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ORIGINAL PAPER

"Is Macromolecular Crowding Overlooked?"- Effects of Volume Exclusion on DNA-Amino Acids Complexes and Their Reconstitutes

Priyanka D. Pal · Prabhakar M. Dongre · Arunkumar V. Chitre

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Abstract Biological macromolecules evolve and function within intracellular environments that are crowded with other macromolecules. Crowding results in surprisingly large quantitative effects on both the rates and the equilibria of interactions involving macromolecules, but such interactions are commonly studied outside the cell in uncrowded buffers. The addition of high concentrations of natural and synthetic macromolecules to such buffers enables crowding to be mimicked in vitro, and should be encouraged as a routine variable to study. In this study, we propose to understand the changes in DNA character and its modulation in presence of macromolecules such as PEG with reference to binding parameters to amino acids using fluorescence enhancement.

Keywords Macromolecular crowding · Fluorescence spectroscopy · Binding constants · DNA-amino acids

Abbreviations

DNA	Deoxyribose Nucleic Acid
PEG	Poly Ethylene Glycol
EB	Ethidium Bromide

Introduction

The interior of biological cells is a crowded environment, in which the networks of biochemical interactions controlling cellular function may perform very differently compared to the dilute environment of a typical test tube. During recent decades it has gradually become recognized that crowding can considerably alter the reactivity of individual macromolecules, both qualitatively and quantitatively. Crowding can be mimicked experimentally by adding high concentrations of inert synthetic or natural macromolecules, termed crowding agents or crowders, to the system in vitro [1]. Experimental and theoretical work has demonstrated substantial (order-of-magnitude) effects of crowding on a broad range of biochemical, biophysical and physiological processes, including—but not limited to—nucleic acid and protein conformation and stability, protein–protein and protein–DNA association equilibria and kinetics (including protein crystallization, protein fibre formation and bundling), catalytic activity of enzymes and cell volume regulation [2], [1].

The concentration of macromolecules inside a prokaryotic cell is typically 200–400 g/l), implying that macromolecules occupy a significant fraction (up to 20–30 %) of the cellular volume. Biochemical kinetic parameters such as equilibrium constants, association and dissociation rates, however, are usually measured in vitro, at much lower macromolecular concentrations. A plethora of observations, reviewed in, show that the kinetic parameters of many key biochemical processes are different at higher macromolecular concentrations, typical of the in vivo environment [3]. Because the performance of biochemical networks often depends strongly on the interaction of the constituent reactions, we expect crowding to have a significant influence on DNA amino acid binding by direct as well as reconstitution method.

Crowding also has implications for genome structure and function because it influences both the structural organization of DNA and the interactions between DNA and proteins [4], [5]. Crowding effects resulting from changes in the amount of water seem to compensate for the effects of changes in cytoplasmic K^+ ions and contribute to maintaining protein–nucleic-acid equilibria and kinetics in the range required for function in vivo [2].

P. D. Pal · P. M. Dongre · A. V. Chitre (✉)
Department of Biophysics, University Of Mumbai, Vidyanagri Santacruz (E), Mumbai 400098, India
e-mail: chitre_a@hotmail.com

Thus it can be seen that macromolecules like DNA are influenced by the crowded cell environment. It would be therefore interesting and significant to see the influence of crowding on the binding interactions of DNA and amino acids in crowded environment caused by polymers.

Experimental

Materials and Sample Preparation

Deoxyribose Nucleic Acid (DNA), Poly ethylene glycol 300 & 20,000 mol. wt (PEG) and amino acids were obtained from Hi Media Pvt Ltd and Sisco Research laboratories respectively. Fluorescent probes such as Ethidium bromide (EB) was purchased from S.D. Fine Chem Ltd. For salt gradient dialysis, dialysis membrane-110 from Hi Media is used. 1 mg/ml DNA stock solution was prepared in saline. 1 % amino acids, 10^{-3} M Ethidium bromide, 10 % Poly ethylene glycol (PEG) stock was prepared in double distilled water. All the chemicals were of analytical reagent grade.

Methods

Samples of DNA and DNA-amino acid + Dye complex in presence of PEG for direct complex formation method and reconstitution method were prepared by using Peacocke and Skerrette method [6] and changes in the fluorescence intensity of dye was recorded in the wavelength range of 540-700 nm on Varian, Cary Eclipse Fluorescence spectrophotometer at room temperature. Excitation and emission slit widths were set at 5 nm and the PMT voltage was set at 600 V. One cm pathlength rectangular quartz cuvettes were used.

Reconstituted DNA sample was prepared by salt gradient dialysis [7]. Concentration of DNA-amino acid reconstitute was estimated by UV-visible Nanophotometer (Implen). UV-visible absorption spectrum was recorded in the wavelength range of 200–300 nm using one cm path length quartz cuvettes. Volume correction for the changes in the DNA concentration after reconstitution was accounted for the calculation of DNA in the reconstituted samples. The actual concentration of the DNA solution was determined by using equation:(Eq. 1)

$$C_{(DNA)} = (50\ x\ Absorbance\ x\ Dilution\ factor)/1.8 \quad (1)$$

$$C_{(reconstitutedDNA)} = C_{(DNA)} after\ reconstitution / C_{(DNA)} before\ reconstitution \quad (2)$$

The binding parameter ‘n’- number of binding sites for the dye per mg of DNA, ‘1/n’ – mg of DNA per binding sites and ‘K’ - association constant’ were calculated by plotting Scatchard plot [8] using Irvin and Irvin method [9] to obtain the bound C_b and free C_f concentration of dye (Eq. 2).

For the binding of dyes to DNA, Scatchard plots were made by plotting: (Eq. 4)

$$r/C_f\ vs\ r\ (where\ r = C_b/[DNA]) \quad (3)$$

$$\Delta G\ values\ were\ calculated\ from\ the\ association\ constants. \Delta G = -RTlnK \quad (4)$$

Results and Discussion

The interactions of a number of dye molecules with DNA and DNA amino acid complexes have been extensively studied; binding parameters have been measured and molecular models suggested. The present studies have explored the influence of molecular crowder on these interactions.

Characteristics of Fluorescence Spectra

On addition of DNA to EB there is an increase in the fluorescence intensity and clear blue shift is observed since EB binds with DNA and stretches the DNA fragment, removing water molecule from the ethidium cation. It is interesting to see that this trend is enhanced on addition of PEG 300 and much higher on addition of PEG 20000. For DNA-amino acids complexes in presence of PEG 300 there is the same trend of blue shift but with hypochromicity and for reconstituted complexes there is no significant shift but very strong hypochromicity. This may be due to the addition of PEG300 and 20000 decreases the activity of water in solution causing a change in the thermodynamics of the system, which one might expect to favour strand pairing in order to decrease the hydrophobic effects present within bases of single stranded DNA. Thus it is clear that molecular crowding has effect on the conformations of the complexes which vary in the different microenvironments [10] (Fig. 1, Fig. 2, Fig. 3).

Binding Parameters

Scatchard plots chart in presence and absence of PEG 300 and 20000 were constructed in Figs. 4 and 5. The low and high molecular weight crowders was used in Scatchard studies in order to test if there was macromolecular crowding effects on the binding between amino acids and DNA using EB as probe. As can be seen

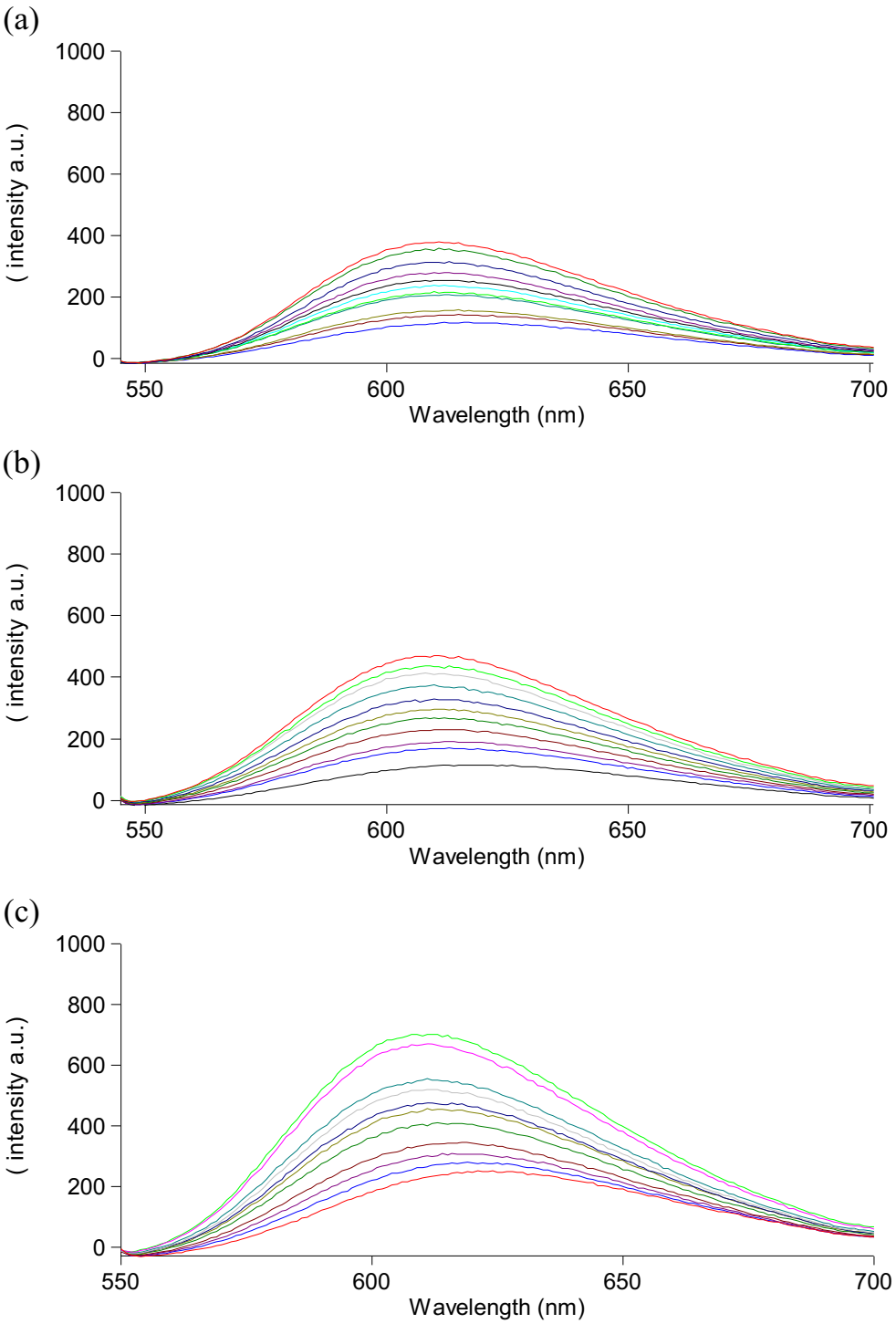

Fig. 1 Fluorescence spectra (*bottom to top*) of (**a**) EB and EB with increasing volume of DNA, (**b**)& (**c**) EB and EB with increasing volume of DNA in presence of PEG 300 and PEG 20000 respectively

from Figs. 4 and 5, there are two straight lines of different slope. This shown that in the presence of complex, there are two stages of EB interaction with DNA (Table 1).

After we use of the formula (3), we can see in the figure that each straight line has two parts i.e. a biphasic plot is obtained: (i) the slope is large (ii) the slope is small. The biphasic Scatchard plot suggests either negative cooperativity or two sites of unequal affinity. Either situation indicates the presence of multiple sites for the interaction of EB and DNA-amino acid complexes in presence of crowders. We have calculated

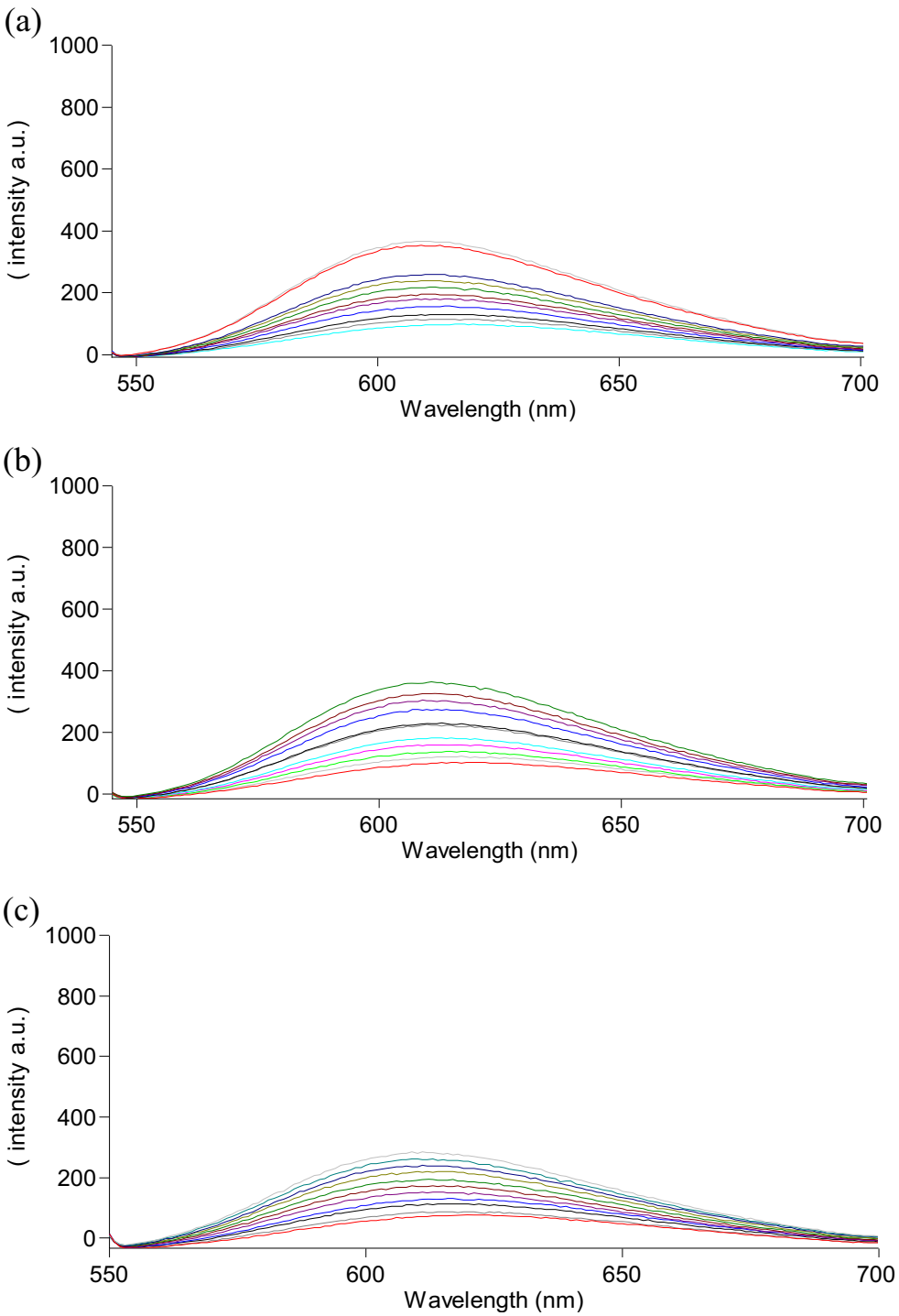

Fig. 2 Fluorescence spectra (*bottom to top*) of (**a**) EB with increasing volume of DNA-arginine complex, (**b**)& (**c**) EB with increasing volume of DNA-arginine complex in presence of PEG 300 and PEG 20000 respectively by direct complex formation method

the values of binding parameters with reference to the primary spontaneous interactions.

The figures are shown for interaction between DNA and arginine under various environments for the purpose of representative illustration. Similar diagrams were obtained for other amino acids.

Direct Complex Formation Method

The results show negligible change for the binding sites of DNA-amino acid complex which indicates that the amino acids do not compete with EB for DNA in presence of both the polymers, PEG 300 and PEG20000. This support

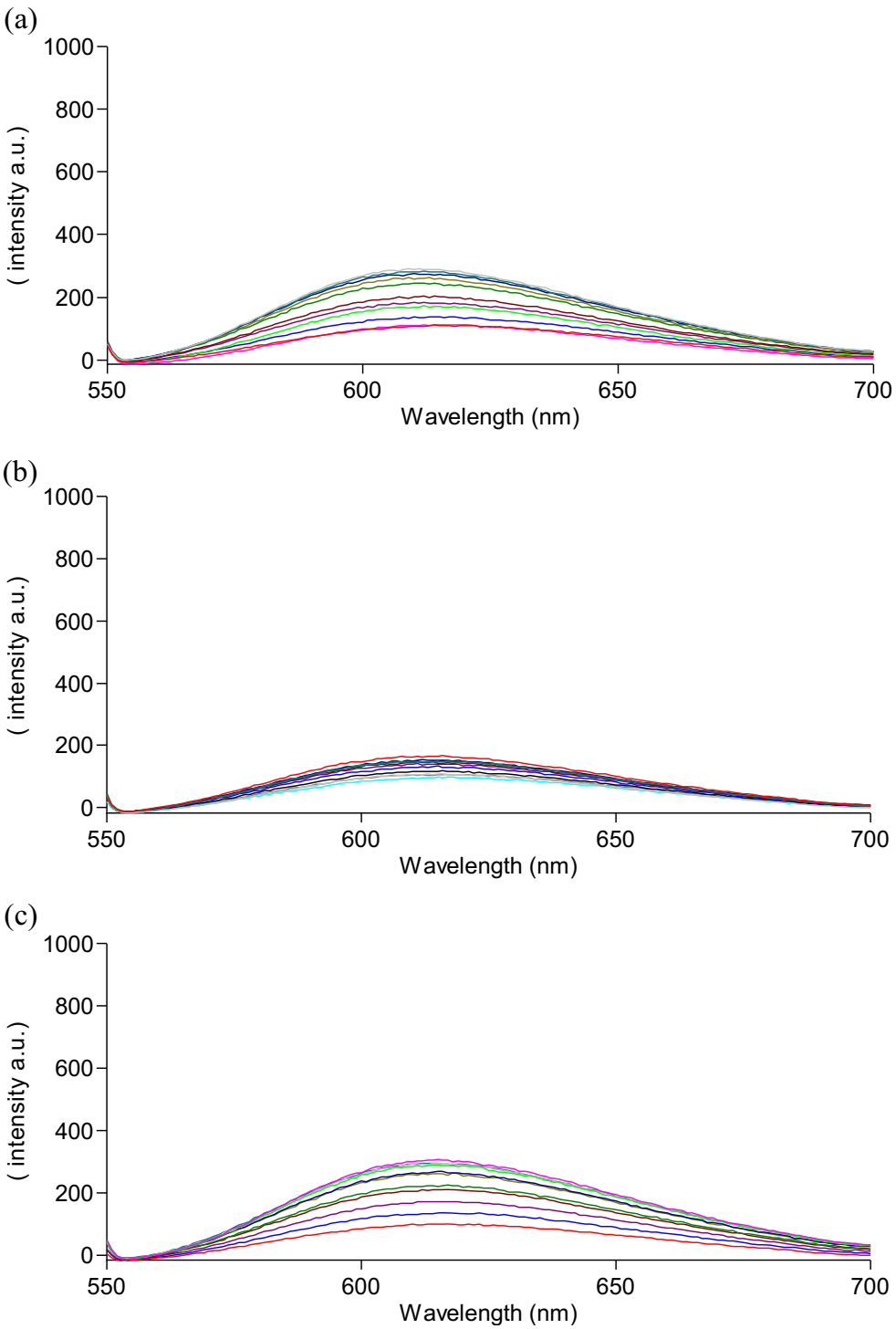

Fig. 3 Fluorescence spectra (*bottom to top*) of (**a**) EB with increasing volume of DNA-arginine complex, (**b**)& (**c**) EB with increasing volume of DNA-arginine complex in presence of PEG 300 and PEG 20000 respectively by reconstitution method

that macromolecular crowding is not involved in the competition. However the binding affinity seems to have increased in presence of PEG 20000 and variability to an extent in the after addition of PEG 300. This indicates that high molecular weight crowder enhance the stability of the DNA-amino acid complex while low molecular weight crowder has variable effect with specific conditions. The destabilizations can be partly attributed to direct interactions with cosolutes, but also explained by a reduced water activity. This is in agreement with the observations of N Sugimoto [10] where he explained that the large PEG generates an area inaccessible to other molecules and increases the solution viscosity. The excluded volume effect that increases the thermodynamic activity of

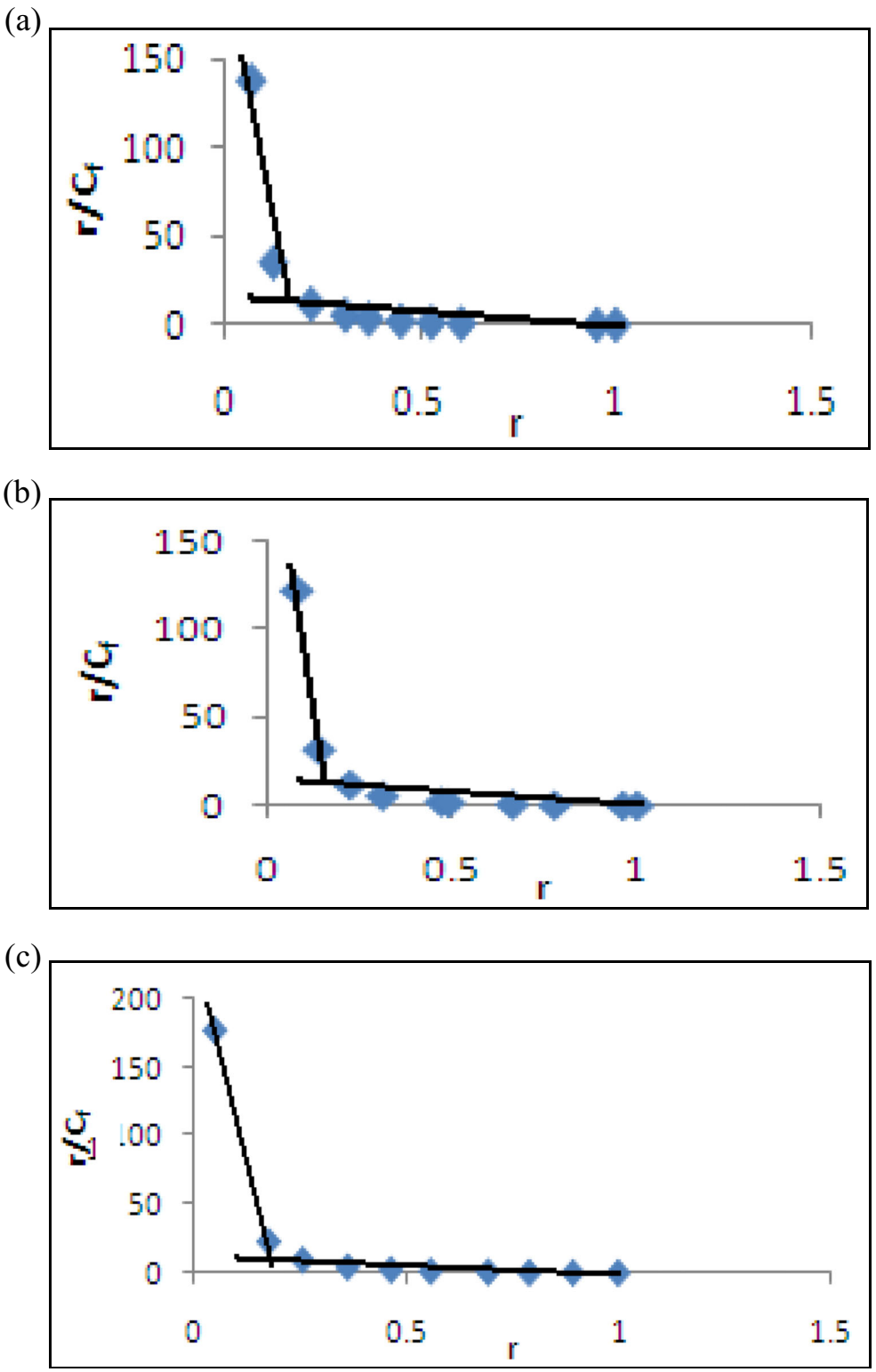

Fig. 4 Scatchard plot of (**a**) EB with increasing volume of DNA-arginine complex, (**b**)& (**c**) EB with increasing volume of DNA-arginine complex in presence of PEG 300 and PEG 20000 respectively by direct complex formation method

biomolecules may increase their association rates; however, the increased viscosity that reduces the diffusion rates overrides the excluded volume effect when the reaction probability is close to unity. In contrast, small cosolute molecules do not act as obstacles, but effectively change the solution property. In particular, ethylene glycol and small PEG molecules decrease the water activity and generate an osmotic pressure (osmotic stress).

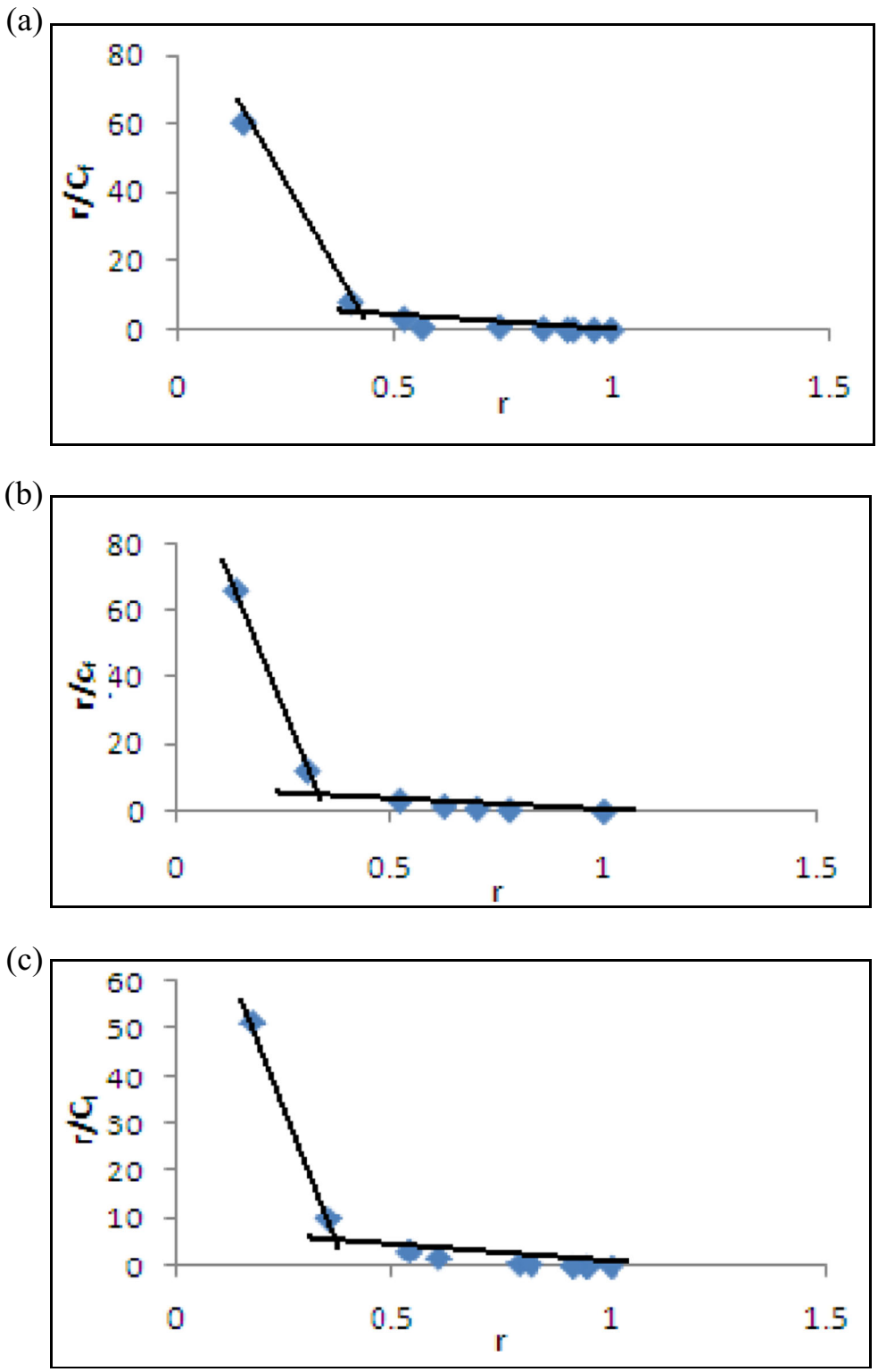

Fig. 5 Scatchard plot of (**a**) EB with increasing volume of DNA-arginine complex, (**b**)& (**c**) EB with increasing volume of DNA-arginine complex in presence of PEG 300 and PEG 20000 respectively by reconstitution method

Reconstitution Method

When EB was allowed to interact with DNA-amino acid complex in presence of PEG 300 and 20000 by salt gradient dialysis method, following observations are obtained. In presence of PEG20000 the number of binding sites for ethidium bromide decreases in the DNA-amino acid complex for aliphatic, basic and sulphur side chain amino acids. This indicate that kinetic process is affected by PEG 20000 whereas an increases

Table 1 (**a**) Association constant, (**b**) number of binding sites for the dye per mg of DNA and (**c**) mg of DNA per binding sites (**d**) free energy of various amino acid-DNA complexes in presence of PEG 300 and PEG 20000

a

Association Constant of Amino acids	Direct Complex formation- K'			Reconstitution Method- K'		
	DNA-Amino Acid	DNA-Amino Acid + Peg 300	DNA-Amino Acid + Peg 20000	DNA-Amino Acid	DNA-Amino Acid + Peg 300	DNA-Amino Acid + Peg 20000
Aliphatic Side Chain Amino Acids						
Glycine	0.00813	0.011	0.063	0.13	0.3	0.6
Alanine	0.0075	0.012	0.046	0.067	0.08	0.052
Valine	0.04	0.06	0.074	0.006	0.007	0.017
Leucine	0.0096	0.008	0.026	0.03	0.039	0.022
Isoleucine	0.036	0.015	0.066	0.03	0.015	0.051
Proline	0.016	0.0098	0.051	0.12	0.11	0.13
Basic Side Chain Amino Acids						
Lysine	0.07	0.058	0.068	0.064	0.029	0.051
Arginine	0.0075	0.011	0.065	0.1	0.059	0.057
Histidine	0.011	0.013	0.039	0.004	0.01	0.015
Aromatic Side Chain Amino Acids						
Phenylalanine	0.009	0.012	0.084	0.043	0.049	0.073
Tryptophan	0.012	0.016	0.077	0.118	0.02	0.11
Amide Derivatives Side Chain Amino Acids						
Glutamine	0.03	0.02	0.045	0.89	0.034	0.045
Asparagine	0.062	0.067	0.06	0.1	0.15	0.31
Aliphatic Hydroxyl Side Chain Amino Acids						
Serine	0.019	0.011	0.052	0.056	0.018	0.059
Threonine	0.011	0.008	0.07	0.029	0.017	0.082
Sulphur-Containing Side Chain Amino Acid						
Methionine	0.0063	0.011	0.064	0.07	0.046	0.052

b

Binding Sites of Amino Acids	Direct Complex Formation- N			Reconstitution Method- N		
	DNA-Amino Acid	DNA-Amino Acid + Peg 300	DNA-Amino Acid + Peg 20000	DNA-Amino Acid	DNA-Amino Acid + Peg 300	DNA-Amino Acid + Peg 20000
Aliphatic Side Chain Amino Acids						
Glycine	0.2	0.2	0.18	0.33	0.31	0.4
Alanine	0.19	0.2	0.19	0.33	0.32	0.32
Valine	0.22	0.22	0.22	0.53	0.3	0.4
Leucine	0.21	0.22	0.2	0.48	0.4	0.39
Isoleucine	0.21	0.21	0.19	0.51	0.31	0.33
Proline	0.17	0.21	0.2	0.55	0.3	0.46
Basic Side Chain Amino Acids						
Lysine	0.2	0.2	0.2	0.38	0.26	0.4
Arginine	0.18	0.18	0.2	0.44	0.35	0.4
Histidine	0.21	0.22	0.2	0.55	0.35	0.34
Aromatic Side Chain Amino Acids						
Phenylalanine	0.18	0.19	0.21	0.39	0.22	0.44
Tryptophan	0.21	0.21	0.21	0.28	0.26	0.48
Amide Derivatives Side Chain Amino Acids						
Glutamine	0.18	0.16	0.18	0.38	0.32	0.39
Asparagine	0.28	0.21	0.15	0.4	0.59	0.58
Aliphatic Hydroxyl Side Chain Amino Acids						

Table 1 (continued)

Serine	0.29	0.2	0.14	0.32	0.48	0.5
Threonine	0.23	0.18	0.18	0.15	0.15	0.42
Sulphur-Containing Side Chain Amino Acid						
Methionine	0.14	0.24	0.15	0.48	0.25	0.34

c

mg of DNA per Binding Sites of Amino Acids	Direct Complex Formation- 1/n			Reconstitution Method- 1/n		
	DNA-Amino Acid	DNA-Amino Acid + Peg 300	DNA-Amino Acid + Peg 20000	DNA-Amino Acid	DNA-Amino Acid + Peg 300	DNA-Amino Acid + Peg 20000
Aliphatic Side Chain Amino Acids						
Glycine	5	5	5.555556	3.030303	3.225806	2.5
Alanine	5.263158	5	5.263158	3.030303	3.125	3.125
Valine	4.545455	4.545455	4.545455	1.886792	3.333333	2.5
Leucine	4.761905	4.545455	5	2.083333	2.5	2.564103
Isoleucine	4.761905	4.761905	5.263158	1.960784	3.225806	3.030303
Proline	5.882353	4.761905	5	1.818182	3.333333	2.173913
Basic Side Chain Amino Acids						
Lysine	5	5	5	2.631579	3.846154	2.5
Arginine	5.555556	5.555556	5	2.272727	2.857143	2.5
Histidine	4.761905	4.545455	5	1.818182	2.857143	2.941176
Aromatic Side Chain Amino Acids						
Phenylalanine	5.555556	5.263158	4.761905	2.564103	4.545455	2.272727
Tryptophan	4.761905	4.761905	4.761905	3.571429	3.846154	2.083333
Amide Derivatives Side Chain Amino Acids						
Glutamine	5.555556	6.25	5.555556	2.631579	3.125	2.564103
Asparagine	3.571429	4.761905	6.666667	2.5	1.694915	1.724138
Aliphatic Hydroxyl Side Chain Amino Acids						
Serine	3.448276	5	7.142857	3.125	2.083333	2
Threonine	4.347826	5.555556	5.555556	6.666667	6.666667	2.380952
Sulphur-Containing Side Chain Amino Acid						
Methionine	7.142857	4.166667	6.666667	2.083333	4	2.941176

d

$\Delta G *10^2$ of Amino Acids	Direct Complex Formation			Reconstitution Method		
	DNA-Amino Acid	DNA-Amino Acid+Peg 300	DNA-Amino Acid+ Peg 20000	DNA-Amino Acid	DNA-Amino Acid+Peg 300	DNA-Amino Acid+ Peg 20000
Aliphatic Side Chain Amino Acids						
Glycine	−0.0481	−0.06508	−0.37271	−0.76908	−1.7748	−3.5496
Alanine	−0.04437	−0.07099	−0.27214	−0.39637	−0.47328	−0.30763
Valine	−0.23664	−0.35496	−0.43778	−0.0355	−0.04141	−0.10057
Leucine	−0.05679	−0.04733	−0.15382	−0.17748	−0.23072	−0.13015
Isoleucine	−0.21298	−0.08874	−0.39046	−0.17748	−0.08874	−0.30172
Proline	−0.09466	−0.05798	−0.30172	−0.70992	−0.65076	−0.76908
Basic Side Chain Amino Acids						
Lysine	−0.41412	−0.34313	−0.40229	−0.37862	−0.17156	−0.30172
Arginine	−0.04437	−0.06508	−0.38454	−0.5916	−0.34904	−0.33721
Histidine	−0.06508	−0.07691	−0.23072	−0.02366	−0.05916	−0.08874
Aromatic Side Chain Amino Acids						
Phenylalanine	−0.05324	−0.07099	−0.49694	−0.25439	−0.28988	−0.43187
Tryptophan	−0.07099	−0.09466	−0.45553	−0.69809	−0.11832	−0.65076
Amide Derivatives Side Chain Amino Acids						
Glutamine	−0.17748	−0.11832	−0.26622	−5.26524	−0.20114	−0.26622
Asparagine	−0.36679	−0.39637	−0.35496	−0.5916	−0.8874	−1.83396
Aliphatic Hydroxyl Side Chain Amino Acids						
Serine	−0.1124	−0.06508	−0.30763	−0.3313	−0.10649	−0.34904
Threonine	−0.06508	−0.04733	−0.41412	−0.17156	−0.10057	−0.48511
Sulphur-Containing Side Chain Amino Acid						
Methionine	−0.03727	−0.06508	−0.37862	−0.41412	−0.27214	−0.30763

is observed for aromatic, alcoholic and amine side chain amino acids suggesting that slow process helps EB to occupy more sites. A clear reduction in the binding sites for all the DNA-amino acid complex was seen in presence of PEG 300. The stability of the complexes is not significantly affected by both the

polymers except for arginine, lysine and methionine DNA complexes.

There is an overall lowering of ΔG values as compared to DNA suggesting increase in the spontaneity of dye DNA interaction.

Conclusion

The effect of molecular crowding was observed through the decrease in the fluorescence intensity which we believe is a result of compaction of the DNA conformation with increasing crowding. Decrease in binding sites and slight effect upon binding constant suggest that addition of PEG might prevent intercalation by blocking the sites of insertion, by altering the flexibility of DNA or by preventing electrostatic interaction between the ethidium cation and DNA. These studies show the significance of volume exclusion effects of the macromolecular crowders PEG 300 and 20000 on the interaction between DNA and various amino acids complexes prepared by direct addition and reconstitution. It is seen that high molecular weight polymer, PEG 20000, has considerable effect on these interaction and help in stabilization of DNA whereas low molecular weight PEG 300 does not show much crowding effect but has other effects. Addition of polymers favors thermodynamic spontaneity.

References

1. Ellis RJ (2001) Macromolecular crowding: obvious but underappreciated. Trends Biochem Sci 26:597–604
2. Zimmerman SB, Minton AP (1993) Macromolecular crowding: biophysical, biochemical, and physiological consequences. Annu Rev Biophys Biomol Struct 22:27–65
3. Marco J. Morelli, Rosalind J. Allen, and Pieter Rein ten Wolde; Effects of Macromolecular Crowding on Genetic Networks Biophys J. 2011 December 21; 101 (12): 2882–2891.
4. Zimmerman SB (1993) Macromolecular crowding effects on macromolecular interactions: some implications for genome structure and function. Biochim Biophys Acta 1216:175–185
5. Zimmerman SB, Murphy LD (1996) Macromolecular crowding and the mandatory condensation of DNA in bacteria. FEBS Lett 390: 245–248
6. Peacocke AR, Skerrett JNH (1956) The interaction of aminoacridines with nucleic acids. Trans, Faraday Soc 52:261–79
7. Olins DE, Olws AL, And PH, Hippel V (1967) Model Nucleoprotein Complexes: Studies on the Interaction of Cationic Homopolypeptides with DNA; *J.* Mol Biol 24:157–176
8. Scatchard, G. *(*1949*)* The attractions of proteins for small molecules and ions. Ann. New York Acad. Sci. 51: 660–672
9. Irvin JL, Irvin EM (1954) The interaction of quinacrine withadenine nucleotides. J Biol Chem 210:45–56
10. Nakano S-i, Miyoshi D, Sugimoto N (2014) Effects of Molecular Crowding on the Structures, Interactions, and Functions of Nucleic Acids. Chem Rev 114:2733–2758