Revue Roumaine de Chimie, **2009**, *54*(10), 807–813

THE INTERACTION OF VANCOMYCIN WITH DNA

Loredana Elena VIJAN*

University of Piteşti, Faculty of Science, Department of physics and chemistry, Tg. Vale Street, No.1, Piteşti, 110040, Roumania

Received July 30, 2008

Vancomycin is the store antibiotic, used only in the grave infections treatments (endocarditis, septicemias, pneumonias, meningitis) with the resistance pathogens of other antibiotics. The interaction of vancomycin with calf thymus DNA was studied by the absorbance measurements. The vancomycin self-association has been investigated in terms of Tipping and Schwarz methods. The binding of vancomycin to DNA has been investigated in terms of Benesi-Hildebrand, Scott and Scatchard methods, supposing a 1:1 binding ratio and do not account explicitly for either the dimerization of the drug or cooperativity effects on the binding.

INTRODUCTION

Glycopeptide antibiotics are an important therapeutic class of compounds used for treating bacterial infections. They are potent antibiotics which have low minimum inhibitory concentrations for Gram-positive strains[1] and are most commonly used in the treatment of virulent gastrointestinal or systemic infections,[2] such as those elicited by staphylococcal and enterococcal organisms. Glycopeptide antibiotics, such as vancomycin, ramoplanin and teicoplanin, are life saving drugs in clinical situations where first-line antibiotics (*e.g.* penicillins, cephalosporins) result in treatment failure.[3,4]

Glycopeptide antibiotics are natural products produced by a diverse group of actinomycetes,[5] the agents with the similarities in structure that account for the biological properties they have in common. The core aglycone portion of these natural products is a heptapeptide that is relatively conserved among members of the class. Glycopeptides differ largely on the basis of number, position and chemical structure of the sugar moieties attached to the heptapeptide core.[6] The majority of these agents contain a monosaccharide or disaccharide attached to the fourth amino acid residue. Vancomycin (Figure 1) has a disaccharide at this position.

Vancomycin is the prototypic glycopeptide antibiotic first described in 1956 and introduced for treatment of serious Gram-positive infections in 1958 by Eli Lilly.[7] For nearly 30 years, vancomycin was used successfully without a significant challenge from acquired resistance development. Transferable, inducible resistance to high concentrations of vancomycin in clinical isolates of *Enterococcus* was not detected until 1986 and was subsequently reported in 1988. The mechanism of this resistance results from a biosynthetic alteration of the molecular target of vancomycin.[8,9] The action' mechanism of vancomycin consists in inhibition of the biosynthesis of bacterial cell wall peptidoglycan[7] by binding carbon-terminal acyl-D-alanyl-D-alanine containing residues in peptidoglycan precursors.

Vancomycin consists of a core heptapeptide with attached saccharide moieties, one of which is the deoxyaminosugar vancosamine. Vancomycin exhibits its antibacterial activity by binding bacterial cell wall mucopeptide precursors terminating in the sequence L-lysyl-D-alanyl-D-alanine.[10] It was found that five hydrogen bonds account for this binding specificity and the disruption of one of these hydrogen bonds by the replacement of the terminal alanine with lactate (D-alanyl-D-lactate) in the mucopeptide precursor is the molecular basis for the resistance to vancomycin. It was also demonstrated that the conformations of vancomycin and its aglycone differ in their alignment of the amide protons, which participate in the hydrogen-binding network with cell- wall precursors.[11] In addition, the alkylation of the 3-amino group on the disaccharide at amino acid residue 4 further enhances the activity, where the alkyl moiety probably serves as a hydrophobic anchor to the cell membrane.[12]

* Corresponding author: loredana.vijan@upit.ro

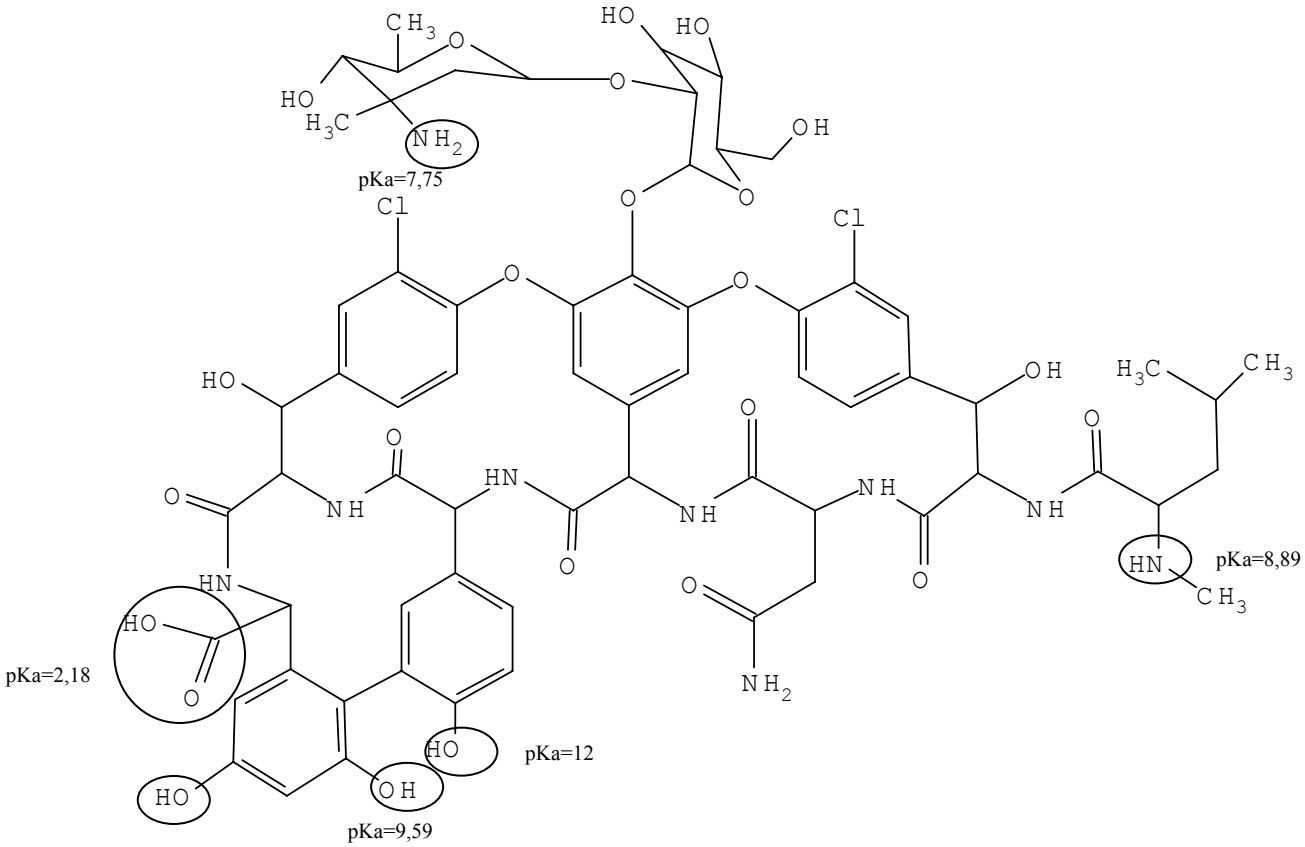


Fig. 1 – The structure of vancomycin.

A target for the modification of vancomycin is the vancosamine moiety. Recently, it was found that N-alkylation of vancosamine with *n*-decyl or 4-chlorobiphenylyl groups results in an antibiotic acting in a different mechanism than vancomycin itself.[13] Quite recently, Kahne's group[14] has developed a general methodology for selective glycosylation of the vancomycine aglycon. It is quite likely that a wide-ranging investigation of different sugars will lead to more significant improvements across a range of bacterial strains.

Vancomycin contains 18 chiral centers surrounding three "pockets" or "cavities" that are bridged by five aromatic rings. Strong polar groups are proximate to the ring structures to offer strong polar interactions with the solutes. It has a number of ionizing groups (two basic and four acidic groups, indicated in Figure 1) and thus it can be used over a range of different pH values and exhibit a wide range of retention characteristics and chiral selectivities.

The acid-base properties and proton-speciation of vancomycin were determined. It is reported to have the following pKa values: 7.75, 8.89 (basic), 2.18, 9.59, 10.4 and 12 (acidic).[15] The net charge of vancomycin across the 0 to 13 pH range were calculated. This is approximately +2.1 to pH = 2. Increasing pH from 3 to 7.4 leads to a decrease in net charge of vancomycin to +0.7. Then, the net charge of the drug grows to +4 at 13 pH.[15]

The present work follows the study of the self-association of vancomycin and their interaction with calf thymus DNA, with a view to determine the binding parameters, supposing that a 1:1 drug – DNA complex is formed. In order to describe the binding processes, it must take into account the cooperative interaction between the binding sites, i.e. the fact that binding at one-site affects the binding at others. For the study of vancomycin aggregation on DNA, a basic model, represented by a linear lattice of equivalent binding sites with nearest-neighbor cooperativity, was used.

RESULTS AND DISCUSSION

Evidence that vancomycin can self-associate and form noncovalent homodimers in aqueous solution was reported as early as 1971.[16] Although the self-association of the drugs is adequately interpreted in

terms of models of the indefinite association, in the domain of concentrations used, the presence of high aggregates may be neglected and only monomer – dimer equilibrium considered. One followed the influence of concentration on the absorption spectra of drug, at constant product of the concentration of drug and the path length.

Starting from the equations Tipping:[17]

$$\sqrt{\frac{C_D^0}{\varepsilon_M-\varepsilon_{app}}}=\frac{1}{\varepsilon_M-\varepsilon_D}\cdot\sqrt{C_D^0\cdot(\varepsilon_M-\varepsilon_{app})}+\sqrt{\frac{1}{2K_d\cdot(\varepsilon_M-\varepsilon_D)}} \quad (1)$$

respectively Schwarz:[18]

$$\sqrt{\frac{\varepsilon_M-\varepsilon_{app}}{C_D^o}}=\sqrt{\frac{2K_d}{\Delta\varepsilon}}\cdot[\Delta\varepsilon-(\varepsilon_M-\varepsilon_{app})] \quad (2)$$

the linear plots, $\sqrt{\frac{C_D^0}{\varepsilon_M-\varepsilon_{app}}}$ against $\sqrt{C_D^0(\varepsilon_M-\varepsilon_{app})}$, respectively $\sqrt{\frac{\varepsilon_M-\varepsilon_{app}}{c_D^o}}$ versus (ε_M-ε_{app}), were obtained and the values for the molar absorption coefficient of dimer (ε_D) and the dimerization constant (K_d) were determined. Both methods lead to a molar absorption coefficient of dimer ε_D=3320(±80)$M^{-1}cm^{-1}$ and a dimerization constant of K_d=460(±10)M^{-1}.

Figure 2 presents a family of absorption spectra in vancomycin – DNA system, at different polymer to drug ratios ($\frac{P}{D}$). One observed the decreasing of bands' intensity with increasing $\frac{P}{D}$ ratios.

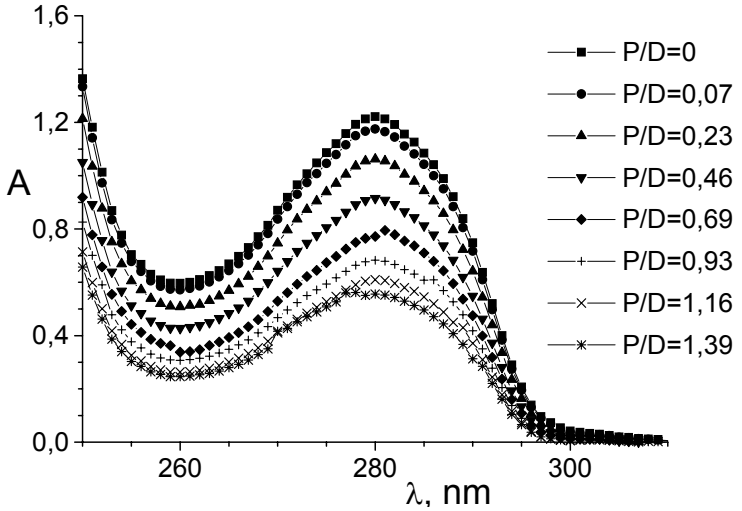


Fig. 2 – Absorption spectra of vancomycin – DNA system.

The equilibrium experiments can be evaluated by a model based on the linear lattice of equivalent binding sites with nearest-neighbor cooperativity. Therefore, we report on the binding interactions of vancomycin with DNA, adopting the basic model of Schwarz[18-20] with just one type of equivalent binding site. This restricts cooperative interactions to those with nearest neighbors.

Schwarz theory implies two binding processes: nucleation – the binding of an isolated drug and aggregation – the binding of the drug in the immediate neighborhood of one that is already bound. The form of binding curves of vancomycin to DNA (ε_{app}=f($\frac{P}{D}$)) presents the features of a cooperative binding caused by stacking interaction of neighboring bound drug molecules. After a rapid decrease in the range of small $\frac{P}{D}$, ε_{app} levels showed a much slower decrease above $\frac{P}{D}\approx 4$, what suggests a cooperative binding of vancomycin to DNA.

To evaluate our experimental data, we have adopted the treatment of Schwarz by plotting γ_D^* versus $\frac{P}{D}$, γ_D^* being the total fraction of free drug (monomers and possibly dimers):[19]

$$\gamma_D^* = \gamma_D(1+2K_dC_D^0\gamma_D) \quad (3)$$

where K_d is the previously determined dimerization constant, γ_D - the fraction of free monomeric drug and

To compute:

$$\gamma_D = \frac{C_D}{C_D^0} = \frac{\varepsilon_{app}-\varepsilon_{st}}{\varepsilon_M-\varepsilon_{st}} \quad (4)$$

from the experimental absorbances, it was necessary to investigate the vancomycin – DNA system at a constant $\frac{P}{D}$ ratio, but with variable vancomycin and DNA concentrations. The equation (4) is used to obtain the fraction of free monomeric drug (γ_D) by means of the molar absorption coefficients of the monomer (ε_M) and respectively, of bound and stacked drug (ε_{st}).

At medium $\frac{P}{D}$ ratio and under conditions of cooperativity, the following relationship[19] may be used to determine ε_{st}, valid if the product $KC_D^0 > 1$:

$$\varepsilon_{app} = \varepsilon_{st} + (\varepsilon_M - \varepsilon_{st})\frac{1}{KC_D^0} \quad (5)$$

Plotting the apparent absorption coefficient ε_{app} versus the reciprocal value of the total weighing-in concentration of the drug $\frac{1}{C_D^0}$, at constant $\frac{P}{D}$ ratio, lead to straight lines that converge to ε_{st}. Extrapolation to $\frac{1}{C_D^0} \to 0$ yields the molar absorption coefficient of bound and stacked drug molecules ε_{st} as being the intercept on the ordinate axis. We have found ε_{st}=5000(±120)$M^{-1}cm^{-1}$.

From equation (4), as ε_{st} is already known, we can calculate γ_D values, which are required to compute γ_D^* from equation (3). The plot of γ_D^* versus $\frac{P}{D}$ ratio, at constant concentration of vancomycin, allows the determination of the binding constant K, valid if the binding of the drug to the polymer is stronger than the dimerization tendency of the drug, $K>>K_d$. In our case we find $K>>K_d$ so this is a good approximation.

The bound fraction of the drug is described by the equation:

$$\theta\frac{P}{D}n = 1-\gamma_D^* \quad (6)$$

where θ is the fraction of binding sites occupied by the drug, also called the degree of saturation. At first, when $\frac{P}{D}n << 1$, all the binding sites are occupied. The degree of saturation θ remains equal to unity in this region and γ_D^* will be proportional to $\frac{P}{D}$ ratio. By extrapolating this linear part to the abscissa, one obtains from the intercept for which $\frac{P}{D}n = 1$ (or from its slope) the value of n, the number of binding sites per monomeric segment of the polymer. We have found $n \approx 0.52$, at first sight being a plausible value, because vancomycin has two positive charges and the monomeric segment of DNA, containing a phosphate group, offers only one negative charge.

They were tested several models and found out that it formed a 1:1 vancomycin – DNA complex. In these conditions, the binding constant of drug to DNA, from Benesi-Hildebrand,[21] Scott[22] and Scatchard[23] methods, were determined. In Figure 3 is presented a Benesi-Hildebrand plot for vancomycin – DNA system. The results obtained are summarized in Table 1. As we can notice, the values for the binding constant of vancomycin to DNA obtained by the three methods do not differ too much.

In the absence of the presumption that a 1:1 vancomycin – DNA complex is formed, the experimental data were fitted either to the linear Scatchard plot,

$$\frac{r}{C_F} = (n-r)\cdot K \quad (7)$$

or to a non-linear regression:[24]

$$r = \frac{n\cdot K\cdot C_F}{1+K\cdot C_F} \quad (8)$$

corresponding to a single class of non-interacting binding sites that do not exhibit cooperative behaviour. In these relationships, *r* is the binding

ratio ($r = \frac{C_B}{C_{DNA}}$), C_F and C_B are the concentrations of free, respectively bound drug, n - the number of binding sites, K - the drug - DNA binding constant.

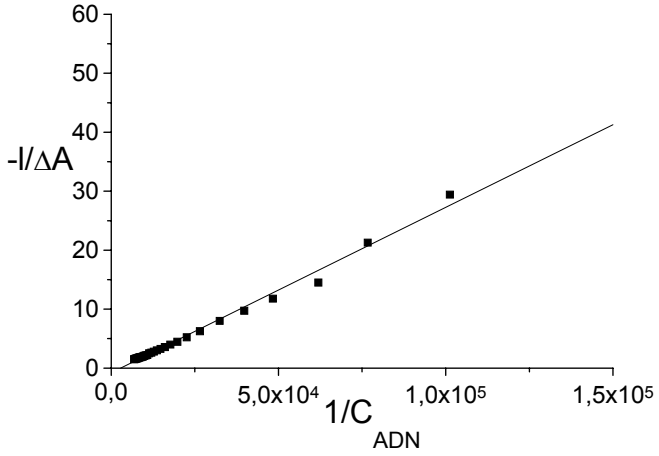


Fig. 3 – Benesi-Hildebrand plot.

Table 1

Results of the vancomycin – DNA interaction

Method	Equation	K, M^{-1}	n
Schwarz	$K = (\gamma_D^o c_D^o)^{-1} + 2K_d$	$3.18(\pm 0.26)\cdot 10^4$	0.52
Benesi-Hildebrand	$\frac{l}{\Delta A} = \frac{1}{C_D^0 \cdot K \cdot \Delta\varepsilon} \cdot \frac{1}{C_{ADN}} + \frac{1}{C_D^0 \cdot \Delta\varepsilon}$	$0.28(\pm 0.06)\cdot 10^4$	-
Scott	$\frac{l \cdot C_{ADN}}{\Delta A} = \frac{1}{C_D^0 \cdot \Delta\varepsilon} \cdot C_{ADN} + \frac{1}{C_D^0 \cdot K \cdot \Delta\varepsilon}$	$0.12(\pm 0.04)\cdot 10^4$	-
Scatchard	$\frac{\Delta A}{l \cdot C_{ADN}} = -\frac{K}{l} \cdot \Delta A + C_D^0 \cdot K \cdot \Delta\varepsilon$	$0.11(\pm 0.03)\cdot 10^4$	-
	$\frac{r}{C_F} = (n - r) \cdot K$	$3.87(\pm 0.18)\cdot 10^4$	0.48
	$r = \frac{n \cdot K \cdot C_F}{1 + K \cdot C_F}$	$2.61(\pm 0.14)\cdot 10^4$	0.45

where $\Delta\varepsilon$ is the difference of molar absorptivity ($\Delta\varepsilon=\varepsilon_B-\varepsilon_F$), ε_F, ε_B are the molar absorption coefficients of free, respectively bound drug, l - path length, ΔA - the observed absorbance change, C_D^0 - the total concentration of drug, C_{DNA} – the concentration of DNA.

On the assumption of the absorption is due only to the free form of drug ($f_B = 0$), the concentrations of free and bound drug are given by:

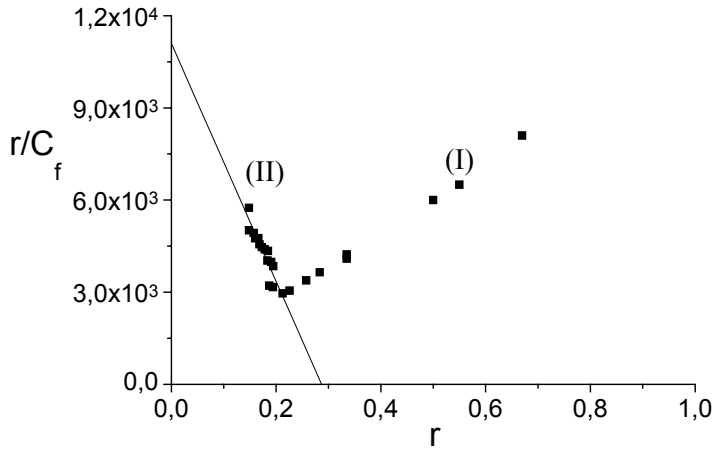
$$C_B = C_D^0 \cdot \frac{A_0 - A}{A_0} \quad (9)$$

$$C_F = C_D^0 - C_B \quad (10)$$

The Scatchard plot presented in Figure 4 attest the presence of two binding processes: the process (I), at small values of $\frac{P}{D}$ ratio and the process (II), at medium values of $\frac{P}{D}$ ratio. The solid line represents the best fit of the linear portion of plot and is characteristic for non-cooperative binding to one class with N equivalent sites. Considering only this linear part, the binding constant, $K=3.87(\pm 0.18)\cdot 10^4 M^{-1}$ and the number of sites, n=0.48 were obtained.

Fitting from non-linear regression the values of r corresponding the linear part of Scatchard plot, with equation (8) (Figure 5), the binding parameters: $K=2.61(\pm 0.14)\cdot 10^4 M^{-1}$ and n=0.45 were obtained. At small values of polymer to drug ratio, high deviation from estimated linearity were observed. These deviations attest the existence of the cooperative interactions, the different classes of the binding sites or the multiple contacts.[18,23]

Fig. 4 – Scatchard plot for vancomycin – DNA system.



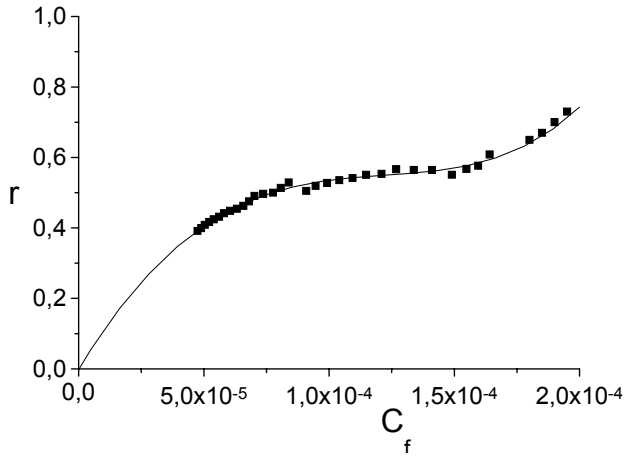


Fig. 5 – Fitting of the binding' data from non-linear regression.

EXPERIMENTAL

Vancomycin (vancomycin hydrochloride, edicin) was obtained from Lek Pharmaceutical and Chemical Company, Ljubljana, Slovenia. Calf thymus DNA was obtained from Sigma Aldrich, Germania. The stock solutions of vancomycin and DNA were prepared in aqueous medium. The concentrations of the stock solutions of reagents were determined by the molar absorption coefficients: ε_{280nm}=6690$M^{-1}cm^{-1}$ for vancomycin and ε_{260nm}=6600$M^{-1}cm^{-1}$ for DNA. The absorption spectra were recorded on a Perkin-Elmer Lambda 25 UV-VIS spectrophotometer, at room temperature, using the quartz cell.

CONCLUSIONS

The interaction of vancomycin with calf thymus DNA was investigated using UV-Vis absorption spectroscopy. A linear lattice of equivalent binding sites with nearest neighbor cooperativity was used as a basic model for a linear biopolymer displaying cooperative binding of small ligands. Complicating effects due to the dimerization of free ligand were taken into account.

The results have outlined that the vancomycin – DNA interaction competes with the self-association of the vancomycin. In addition, the analysis of the vancomycin – DNA interaction, using Benesi-Hildebrand, Scott and Scatchard methods, points out two types of the binding: a non-electrostatic (internal) binding, consisting of the intercalation of the drug between the base-pairs of the nucleic acid and an external binding, cooperative, where the electrostatic interactions with the phosphate groups of DNA are predominant.

It is evident that the binding of vancomycin to calf thymus DNA is more complicated than being represented by the simple model on which the Schwarz theory is based. This model accounts for only one kind of neighbor interaction, independent of the length of a stack of interacting molecules. Nevertheless, the analysis of our experiments with this model allows characterization of the nature of the cooperativity in the interactions between rather large, multiplycharged molecules with a hydrophobic chromophore. Without a neutralizing polymer present, the tendency of these molecules to form dimers is dependent on the presence of a shielding atmosphere of counterions. The presence of an oppositely-charged polymer stabilizes the interactions of dimers or of a multimeric stack of

molecules. When the long-range electrostatic repulsion forces are sufficiently weakened by the attraction to the oppositely-charged polymer, the short-range van der Waals attraction forces provide the required stability for the multimeric structure. In the case of vancomycin, the multimeric structure competes with the formation of bond dimers, which are the most stable form when a large excess of binding sites is available on the polymer.

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