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Enantiomeric and Diastereomeric Self-Assembled Multivalent (SAMul) Nanostructures – Understanding the Effects of Chirality on Binding to Polyanionic Heparin and DNA

Kiri A. Thornalley,[a] Erik Laurini,[b] Sabrina Pricl*[b] and David K. Smith*[a]

This paper is dedicated to the retirement of Prof Francois Diederich in honour of his pioneering work in physical organic supramolecular chemistry and the fields of stereoselective and biological molecular recognition.

Abstract: A family of four self-assembling lipopeptides containing Ala-Lys peptides attached to a C_{16} aliphatic chain was synthesised. These compounds form two enantiomeric pairs that bear a diastereomeric relationship to one another (C_{16}-L-Ala-L-Lys/C_{16}-D-Ala-D-Lys) and (C_{16}-D-Ala-L-Lys/C_{16}-L-Ala-D-Lys). These diastereomeric pairs have very different critical micelle concentrations (CMCs), with LL/DD < DL/LD suggesting more effective assembly of the former. The self-assembled multivalent (SAMul) systems bind biological polyanions as result of the cationic lysine groups on their surfaces. Polyanion binding was investigated using dye displacement assays and isothermal calorimetry (ITC). On heparin binding, there was no significant enantioselectivity, but there was a binding preference for the diastereomeric assemblies with lower CMCs. Conversely, on DNA binding, there was a significant enantioselective preference for systems displaying D-lysine ligands, with a further slight preference for attachment to L-alanine, with the CMC being irrelevant. Binding to adaptive, ill-defined heparin has a large favourable entropic term, suggesting it depends primarily on the cationic SAMul nanostructure maximising surface contact with heparin, which can adapt, displacing solvent and other ions. Conversely, binding to well-defined, shape-persistent DNA has a larger favourable enthalpic term, and combined with the enantioselectivity, this allows us to suggest that its binding is based on optimised individual electrostatic interactions at the molecular level, with a preference for binding to D-lysine.

Polyanions are ubiquitous in biological systems and play vital roles in many processes – both biological and medicinal. [1] Interestingly, given the plethora of anionic species *in vivo*, biology can nonetheless achieve effective control over anion-mediated processes. DNA and heparin are archetypal charge dense polyanions, and given the intense biomedical interest in DNA for gene delivery[2] and heparin for coagulation control,[3] significant attention has focussed on binding them.[2-4] However, only rarely have binding differences between them been probed.[5] This is, in part, because these anions typically reside in different biological compartments, meaning that they rarely come into direct competition. An exception to this is in bacterial biofilms where extracellular DNA is present and has been shown to compete with heparin for binding to the same proteins.[6] Furthermore, in nanoscale therapeutics, a delivery vehicle for (e.g.) genetic material must transit the extracellular medium which is rich in heparin and other glycosaminoglycans – polyanion competition for binding to synthetic systems therefore becomes important.

One key strategy for polyanion binding employs cationic self-assembled colloidal nanosystems,[7] indeed the general importance of colloidal systems in controlled molecular recognition events is increasingly recognised.[8] In cases where the self-assembled systems display specific ligands on the surfaces, and are capable of forming multivalent interactions[9] with the target, this can be classified as a self-assembled multivalent (SAMul) approach.[10] Considerable attention has focussed on the binding of DNA[2,11] or heparin[12] using SAMul ligand arrays. Recently, we have begun to explore similarities and differences in their binding interactions. It is well-known that different polyelectrolytes, such as heparin and DNA, have different persistence lengths, which can impact on their binding.[13] This has led us to introduce the simple descriptions of DNA as 'shape-persistent', and heparin as 'adaptive', to help explain differences in their multivalent interactions.[14]

Given the importance of chirality in biomolecular recognition,[15] there has been some interest in exploring chiral preferences in polyanion binding.[16] We have reported that a chiral SAMul lysine ligand array can control polyanion binding.[17] Interestingly, however, when using lysine ligands, chiral recognition of heparin does not always occur – Wang and Rabenstein reported that there was no impact of chirality in their peptide heparin binders,[18] whilst we found that the precise structure of the self-assembling system determined whether chiral recognition was achieved.[19] In this new study we wanted to gain a more detailed understanding of the chiral preferences of both heparin and DNA, and unambiguously understand, in thermodynamic terms, any inherent differences in the way these important polyanions interact with chiral nanoscale objects.

For this study, we designed a family of stereoisomeric lipopeptides capable of self-assembly – C_{16}-Ala-Lys (Fig. 1). These molecules each contain two chiral centres (Ala and Lys), and there are four possible stereoisomers overall – two pairs of enantiomers with a diastereomeric relationship to each other: C_{16}-L-Ala-L-Lys and C_{16}-D-Ala-D-Lys (LL and DD), as well as C_{16}-D-Ala-L-Lys and C_{16}-L-Ala-D-Lys (DL and LD). These lipopeptides should bind polyanions as a result of the cationic Lys unit with self-assembly enhancing binding via multivalency. In contrast to

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Supporting information for this article is given via a link at the end of the document previous work,[17,19] the introduction of a second chiral centre (Ala) which does not directly participate at the binding interface allows us to probe both enantio- and diastereo-selectivity and gain much more detailed insight.

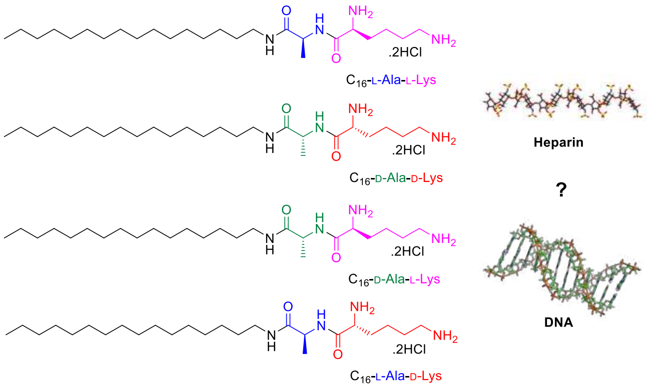
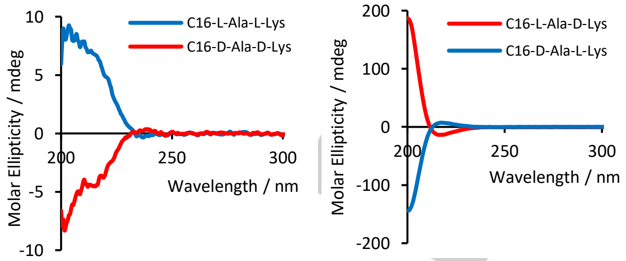


Figure 1. Family of four stereoisomeric self-assembling cationic lipopeptides studied here for their relative abilities to bind heparin and DNA. There are two pairs of enantiomers (LL/DD & DL/LD) with a diastereomeric relationship.

These molecules were synthesised in excellent yield and purity using standard peptide coupling and protecting group methodologies (see ESI). Their NMR spectra were broadly similar to each other – key proton resonances all appeared at equivalent ppm values. However, the coupling patterns, in particular for the CH_2-N protons at ca. 3.1 ppm, were more complex for LL/DD than for DL/LD (see Fig. S24-S27, ESI). This reflects the diastereomeric relationship of these two pairs of compounds, leading to differences in ^1H-^1H coupling. We propose this is induced by differences in conformational preferences between diastereoisomers, leading to changes in the torsion angles between coupled protons. Circular Dichroism (CD) spectroscopy of the four compounds confirmed their existence as two enantiomeric pairs, with mirror image spectra. However, there was a significant difference in peak maxima and ellipticities between diastereomeric LL/DD and DL/LD, supportive of conformational differences between diastereomers.



Fig, 2 CD Spectra of LL/DD (left) and LD/DL (right) measured at a concentration of 2.27 mM in Tris-HCl (10 mM) and NaCl (150 mM), indicating significantly different profiles for each diastereomeric pair of enantiomers.

Initially, we monitored self-assembly using Nile Red assays (Fig. S1-S4) and isothermal titration calorimetry (ITC, Fig. S21). Both methods gave critical micelle concentrations (CMCs) in excellent agreement (Table 1). The enantiomers assembled identically into nanostructures that have equal and opposite chiralities as demonstrated by CD (recorded above the CMC). However, diastereomeric systems had very different self-assembly properties. Specifically, LL and DD have much lower CMCs (ca. 50 μM) than DL and LD (ca. 160 μM). This corresponds to a significant difference in the free energy of micellisation (ΔG_{mic}) – ca. 3 kJmol^{-1}. Interrogating the thermodynamics in more detail indicated that, as is often observed for hydrophobic self-assembly of ionic surfactants,[20] micellisation is entropically driven and slightly enthalpically disfavoured. The self-assembly of LL and DD is slightly enthalpically preferred over DL and LD (1.0-1.5 kJmol^{-1}) and slightly entropically preferred (1.5-2.0 kJmol^{-1}). In this way, the self-assembly of LL and DD is both enthalpically and entropically preferred over diastereomeric DL and LD.

Table 1. Critical Micelle Concentrations (CMCs, μM) determined by Nile Red assay (CMC_{NR}) and isothermal calorimetry (CMC_{ITC}) and thermodynamic data, ΔG_{mic}, ΔH_{mic} and $T\Delta S_{mic}$ (in kJmol^{-1}) extracted from ITC.

	CMC_{NR}	CMC_{ITC}	ΔG_{mic}	ΔH_{mic}	$T\Delta S_{mic}$
LL	50 ±3	52	-24.5	3.2	27.7
DD	43 ±3	48	-24.7	3.3	28.0
DL	155 ±3	159	-21.7	4.3	26.0
LD	166 ±3	172	-21.5	4.8	26.3

Table 2. Data from DLS measurements at a concentration of 1.14 mM to determine the average diameter (nm) from the volume distribution and the zeta potential (mV) of the self-assembled nanostructures. Results are reported as mean ± standard deviation of three experiments.

	Diameter / nm	Zeta Potential /mV
LL	6.49 ± 2.90	35.5 ± 3.3
DD	7.17 ± 2.20	39.2 ± 2.2
DL	9.17 ± 2.75	46.8 ± 0.5
LD	8.60 ± 1.62	43.3 ± 0.6

The self-assembled nanostructures were also characterised using dynamic light scattering (DLS, Fig. S5-S12). In the intensity distribution for each of these self-assembling systems, two types of nanoscale object were observed, one with smaller diameter (<10 nm) and one with a larger diameter (ca. 100-300 nm). For DL/LD the larger assemblies were more significant in the DLS analysis than for LL/DD (see ESI). However, when corrected for the number of assemblies using the volume distribution, it was clear in all cases that the smaller micellar objects dominated the distribution (see ESI). Transmission electron microscopy (TEM) analysis supported this view, with small micellar objects being mainly observed (Fig. S18). Table 2 indicates that the diastereomers form different assemblies, with LL and DD assembling into smaller, better-defined micelles than DL/LD. This would fit with the observation that LL/DD have lower CMCs. The difference in assembly is supported by differences in the Nile Red assay data, which show a smaller fluorescence increase for Nile Red in the case of DL/LD than for LL/DD (Fig. S1-S4), indicative of a less well-organised hydrophobic domain in the former.

Interestingly, however, zeta potentials indicated that DL/LD have somewhat greater surface charge than LL/DD (Table 2) and the larger CD signals would also suggest greater chiral organisation of ligands for DL/LD. We suggest this may be a result of the larger micelles formed by DL/LD having less highly curved surfaces and hence more well-packed charged head groups.

Taking into account these observations, we reasoned there were three possible factors that may influence polyanion binding:

1. LL and DD self-assemble more effectively into SAMul nanostructures than DL and LD, and may therefore be better polyanion binders.
2. DL and LD form SAMul nanostructures with higher surface charge potentials than LL and DD, and may therefore be better polyanion binders.
3. Optimised molecular-scale interactions between the SAMul ligands and biological polyanions may control binding, giving rise to preferences for systems with a specific chirality.

We initially probed heparin binding for these systems using both Mallard Blue (MalB) dye displacement assays[21] and ITC (see ESI for details). From the MalB displacement assay (Fig. S13-S14), LL and DD were better able to displace the dye, with lower EC_{50} values, indicating they are better heparin binders than DL or LD (Table 3). There was no significant difference between enantiomers, with LL≈DD and DL≈LD. This is in agreement with some of the previous results from Wang and Rabenstein,[18] and ourselves[19] suggesting a lack of enantioselectivity in heparin binding with lysine ligands. Interestingly, the observed EC_{50} values suggest that in this assay, these compounds only bind heparin once they have achieved the CMC value (i.e., $EC_{50} \geq$ CMC). As such, we suggest that in this assay, heparin binding depends primarily on the ability of the compounds to self-assemble into a multivalent array. This assay was repeated in human serum and binding preferences were maintained with LL/DD > DL/LD, although the EC_{50} values were somewhat higher (Table S1, Fig. S15). TEM imaging in the presence of heparin proves that the micellar objects remain stable on binding (Fig. S19).[22]

Table 3. Heparin binding data. EC_{50} values (μM) from MalB displacement assay report the concentration of SAMul nanosystem required to displace 50% of MalB from its complex with heparin. Thermodynamic data for SAMul nanosystems binding to heparin, ΔG_{bind}, ΔH_{bind} and $T\Delta S_{bind}$ (in $kJmol^{-1}$), are extracted from isothermal titration calorimetry. Results are expressed as mean ± standard deviation of three experiments.

Heparin	EC_{50} (MalB)	ΔG_{bind}	ΔH_{bind}	$T\Delta S_{bind}$
LL	125.5 ± 4.5	-31.0 ± 0.1	-13.4 ± 0.1	17.6 ± 0.2
DD	110.0 ± 2.2	-30.8 ± 0.1	-13.6 ± 0.2	17.2 ± 0.1
DL	145.7 ±12.0	-28.4 ± 0.2	-12.2 ± 0.1	16.2 ± 0.1
LD	135.0 ± 6.5	-29.1 ± 0.3	-12.5 ± 0.1	16.6 ± 0.2

ITC measurements confirmed these results (Table 3, Fig. S22). ITC was performed above the CMC value to avoid complications from the energetics of demicellisation. There were small differences between the binding free energies of LL/DD (average ΔG_{bind} -30.9 $kJmol^{-1}$) and DL/LD (average ΔG_{bind} = -28.8 $kJmol^{-1}$) – an enhancement of ca. 2 $kJmol^{-1}$ for LL/DD. ITC therefore also suggests that LL/DD, which more effectively form smaller micelles, are better heparin binders. In all cases, binding is enthalpically favoured, as a result of high-affinity electrostatic interactions between the SAMul cation and polyanionic heparin, and entropically favoured, as a result of the release of water and ions from the charged surfaces. The difference in binding free energy between LL/DD and DL/LD has both an enthalpic component (ca. 1 $kJmol^{-1}$), and an entropic component (ca. 1 $kJmol^{-1}$).

We then studied DNA binding to these SAMul systems, using Ethidium Bromide (EthBr) dye displacement assays,[23] and ITC – the results were remarkably different to those observed for heparin (Table 4). In the EthBr displacement assay (Fig. S16-S17), the EC_{50} values were much lower than those observed for heparin binding. However, these are competition assays, performed against dye complexes of different affinities, and at different concentrations, so conclusions comparing DNA and heparin binding using this approach must be drawn with caution – the ITC results discussed below provide the best assessment of binding strength. Notably, however, the EC_{50} values for DNA binding are below the CMC values, which would suggest that binding can even occur in the absence of self-assembly. Indeed, it is well-known that binding to polyanions can occur below the CMC, encouraging self-assembly in the process.[24] Furthermore, the different stereoisomers behave very differently to the heparin binding experiments. Compounds DD and LD are better DNA binders than LL or DL. This would suggest that compounds displaying D-lysine ligands are more effective DNA binders than those with L-lysine ligands – an enantioselective effect. This appears to be largely independent of the chirality of the alanine unit, although there is a smaller effect, which suggests those compounds with L-alanine units perform slightly better than those with D-alanine. As such, compound LD (C_{16}-L-Ala-D-Lys) is the best DNA binder in this assay. TEM imaging in the presence of DNA demonstrates that the self-assembled micelles remain stable on binding (Fig. S20)

Table 4. DNA binding data. EC_{50} values (μM) from EthBr displacement assay report the concentration of SAMul nanosystem required to displace 50% of EthBr from its complex with DNA. Thermodynamic data for SAMul nanosystems binding to heparin, ΔG_{bind}, ΔH_{bind} and $T\Delta S_{bind}$ (in $kJmol^{-1}$), are extracted from isothermal titration calorimetry. Results are expressed as mean ± standard deviation of three experiments.

DNA	EC_{50} (EthBr)	ΔG_{bind}	ΔH_{bind}	$T\Delta S_{bind}$
LL	15.6 ± 2.1	-22.7 ± 0.2	-11.3 ± 0.2	11.4 ± 0.3
DD	9.5 ±1.3	-26.7 ± 0.3	-15.2 ± 0.1	11.5 ± 0.2
DL	18.8 ±1.2	-21.4 ± 0.2	-10.9 ± 0.2	10.5 ± 0.3
LD	6.1 ±1.0	-27.1 ± 0.1	-15.4 ± 0.3	11.7 ± 0.1

The results from ITC replicate these observations (Table 4, Fig. S23), with LD and DD being the best DNA binders (LD slightly better than DD), and LL/DL being worse DNA binders (LL slightly better than DL). The difference in binding free energy (ΔG_{bind}) between the best binder (LD) and the worst binder (DL) is large: 5.7 kJmol^{-1}. This is significantly larger than, the 2.6 kJmol^{-1} difference in ΔG_{bind} we observed previously for related systems with glycine instead of alanine units,[19] suggesting that chiral alanine units help preorganise the lysine ligands for enantioselective recognition. More detailed thermodynamic analysis indicates that the difference between DNA binders is primarily a result of different enthalpies of binding (ΔH_{bind} differs by up to 4.5 kJmol^{-1}).

Comparing differences between heparin and DNA (Fig. 3), it is evident that heparin binds these SAMul systems with higher affinity (ΔG_{bind} = -28.4 to -31.0 kJmol^{-1}) than DNA (ΔG_{bind} = -21.4 to -27.1 kJmol^{-1}). This primarily arises from the larger entropic benefits of heparin binding (see discussion below).

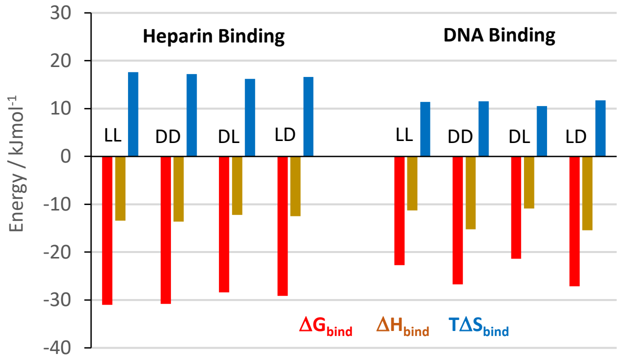


Figure 3. Summary of thermodynamic data extracted from ITC for binding of stereoisomeric SAMul nanosystems to heparin and DNA, indicating that binding is both enthalpically and entropically favoured. The minor preference in heparin binding of LL/DD can be observed, as can the significant, enthalpically driven, preference in DNA binding of DD and LD. ΔG_{bind} is shown in red, ΔH_{bind} in gold and $T\Delta S_{\mathrm{bind}}$ in blue.

For DNA binding, there are larger binding enthalpies (ΔH_{bind} up to -15.4 kJmol^{-1} for DNA but just -13.6 kJmol^{-1} for heparin). Shape-persistent DNA is better able to optimise individual interactions for enthalpic gain as a result of its well-defined repetitive structure, but heparin has relatively poorly organised electrostatic interactions. This optimisation of enthalpic interactions gives rise to the stereochemical preferences expressed by DNA, which appears to have a clear primary preference for D-lysine and a small secondary preference for L-alanine. DNA has a well-defined double helical structure, with regularly repeating phosphate groups down the rigid backbone of the structure. Indeed, it is well-known that DNA can achieve highly selective interactions with cationic ligands.[25] This is in sharp contrast to heparin, which has a more disperse structure with relatively randomly organised anionic saccharides along its more flexible backbone.

Heparin has much larger binding entropies ($T\Delta S_{\mathrm{bind}}$ values up to 17.6 kJmol^{-1} for heparin but just 11.5 kJmol^{-1} for DNA). Adaptive heparin can reorganise to achieve greater surface contact with the SAMul nanosystem leading to desolvation, but shape-persistent DNA cannot wrap round the SAMul nanostructure as effectively to maximise surface contact. We suggest that this adaptability explains why heparin prefers to bind to the SAMul systems based on LL/DD that are better able to assemble into well-defined small micelles.

In conclusion, the structural chiral information programmed into these self-assembling systems therefore plays a key role in controlling their performance – charge density alone is insufficient to explain biological polyanion binding.[26] Firstly, chirality controls self-assembly, with LL/DD being thermodynamically preferred over DL/LD. The binding of adaptive, ill-defined heparin is driven primarily by the ability of the SAMul systems to self-assemble, with heparin wrapping round the nanosystem but not forming highly optimised electrostatic interactions with it. Conversely, the binding of well-defined, shape-persistent DNA is controlled by the molecular-scale information programmed into the SAMul ligand systems, with D-lysine ligands being strongly preferred for effective binding. There is also a minor preference for L-alanine over D-alanine, suggesting this second amino acid can play a role in helping pre-organise the lysine ligands for optimal binding. Thermodynamic data support this hypothesis, and indicate that these SAMul systems bind heparin more strongly than DNA, primarily as a result of the entropic gain associated with adaptive binding and surface desolvation, while DNA binding is more enthalpically favoured, as a result of the ability of the shape-persistent well-defined polyanion to optimise individual electrostatic interactions. These differences in polyanion binding can be quite large – for example the difference in free energy of binding between heparin and DNA ($\Delta\Delta G_{\mathrm{bind}}$) is 8.3 kJmol^{-1} for LL (the best heparin binder) but only 2.0 kJmol^{-1} for LD (the best DNA binder). Changing just one chiral centre in the ligand therefore has a significant effect on this type of polyanion selectivity (6.3 kJmol^{-1}). As such, a SAMul-based DNA delivery system based on LD would be much less adversely affected by the presence of heparin than one based on LL. Given the key biological and medicinal roles of these polyanions, we suggest that understanding structural effects of chirality on their nanoscale binding is of general significance.

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Keywords: DNA • heparin • multivalent • self-assembly • supramolecular

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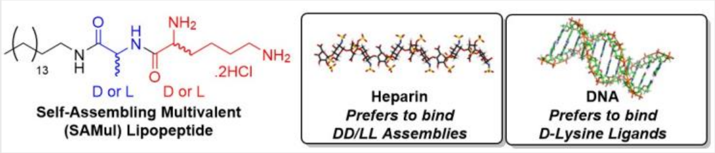
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Self-Assembling Multivalent (SAMul) Lipopeptide
Heparin
Prefers to bind DD/LL Assemblies
DNA
Prefers to bind D-Lysine Ligands

Not all polyanions are the same: Heparin and DNA, both charge dense biological polyanions, behave very differently when binding to stereoisomeric self-assembled nanostructures – their structural differences control their chiral binding preferences.

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