The Effect of Terminal Group Functionality on the Ability of Dendrimers to Bind Proteins.

Fumiko Chiba,[†] *and Lance. J. Twyman* *

Department of Chemistry, University of Sheffield, Dainton Building, Brook Hill, Sheffield, South Yorkshire, UK. S3 7HF.

* Corresponding author; email address: l.j.twyman@sheffield.ac.uk

† Fumiko Chiba is deceased, having passed away in April 2016. She contributed all of the laboratory work and prepared a first draft of the manuscript.

ABSTRACT

It is known that dendrimers can bind proteins with good selectively. This selectivity comes about from an optimization based on matching the size of the dendrimer with the size of the protein's interfacial binding area. In this paper we report how this selectivity can be moderated by the functionality on the surface of the dendrimer. Specifically, we describe the synthesis of amino acid functionalized dendrimers, and the effect of functionality on the dendrimer's ability to bind and inhibit the enzymatic protein, chymotrypsin. The results show how dendrimer binding can be increased *or* decreased, dependent on the terminal functionality. These results will allow new ligands to be designed and synthesized possessing increased and selective protein binding abilities.

INTRODUCTION

Although not well understood, it is now accepted that most proteins function through cooperative partnerships with other proteins. These protein aggregates have an essential role in many biological processes, including signal transduction, cytoskeletal remodeling, cell regulation, immune response, viral self-assembly and many others.[1-3] However, there is also a possibility for irregular protein interactions, which can result in a range of disease processes. Examples include the homo-dimeric complex HIV-1 protease and the aggregation of misfolded proteins in diseases such as Alzheimer's and several forms of rheumatoid arthritis.[4,5] Additionally a number of cancer therapies rely on the disruption of certain protein-protein complexes. For example, inhibitors of *c-Myc* are important for cancers linked to sustained activation of *c-Myc*.[6]

In general, the size of a protein's binding or interfacial area (known as the hot spot) [7] ranges from 500Å^2 up to 5000Å.[8,9] These interacting surfaces tend to be highly charged, with overall interactions dominated by simple (polyvalent) electrostatics. Preliminary research within our group aimed at mimicking the mechanism of protein-protein binding, investigated the use of a series of dendrimers that could bind proteins *via* a simple size-based mechanism. Specifically, our studies investigated a series of carboxylic acid functionalized dendrimers of varying size and studied their ability to bind proteins with differing interfacial areas (*cytochrome-c* and *chymotrypsin*).[10] The results confirmed that optimum binding occurred when the dendrimer's maximum addressable area matched the dimensions of the target protein's interfacial area. From this study it was clear that matching the size of the dendrimer's addressable area with the interfacial area of the protein was more important than simple charge-charge interactions, which is the case for linear polymers.[11-13] As such, larger dendrimers possessing the most charge do not necessarily result in optimum, or high affinity binding. The best interactions are the result of an optimal synergy between the size and shape of the dendrimer/protein structure and the ensuing enthalpic and entropic properties of the

complex. We were also able to demonstrate (using circular dichroism) that dendrimer/protein binding was not accompanied by changes in protein structure.[14]

Although the principle interactions are electrostatic, proteins bind *specifically* and with high affinity because of additional non-covalent interactions provided by the amino acid functionality. Bogan and Thorn studied the role of amino acid functionality and amino acid preferences within the hot spot/interfacial regions, finding that hot spots are enriched by the presence of tryptophan, tyrosine, and arginine.[15] Although the occurrence of these amino acids is relatively rare within a protein's structure, it was determined that when they do occur, they were more likely to appear at the interfacial area.[16] The enhanced binding of these amino acids comes from their ability to contribute additional interactions. For example, tyrosine and tryptophan can contribute a hydrophobic surface, as well as aromatic π-interactions, and in the case of tyrosine, additional hydrogen bonding comes from its 4-hydroxy group.[17] Conversely, there are a number of amino acids known to effect binding negatively, or be of little or no benefit with respect to protein-protein interactions. These include leucine, methionine, serine, threonine and valine, all of which are regarded as "bad" amino acids.[16] The importance of specific amino acids on increasing the binding of synthetic protein ligands was demonstrated by Hamilton using functionalized porphyrin [18] and calixarene [19] scaffolds. Hamilton later showed how a series of functionalized porphyrins with different groups could bind to a series of proteins with high affinity.[20] In addition to our results using dendrimers, Higashi [21] showed how dendrimers terminated with poly-glutamic acid could be used to bind amino acids (as opposed to proteins). The glutamic ends formed α-helices, which preferentially bound D-amino acids with positive cooperativity. Tomalia also reported the use of dendrimers to bind a variety of amino acids and proteins. In this work the dendrimers were functionalized with a spin-label and EPR used to observe and quantify binding.[22]

RESULTS AND DISCUSSION

For the work described in this paper we wanted to construct dendrimers functionalized with "good" and "bad" amino acids and to study their ability to bind a protein. Chymotrypsin was selected as our target protein, as it possess a well-known and defined structure. In addition, chymotrypsin's active site entrance sits within a typical protein hot spot of binding interface. This interface is rich in positive charge and has a binding area around 2400 $Å^2$. Binding to this interface will block substrate access and lead to inhibition, which can be related to binding efficiency/strength.[10] Towards these aims and based on the work of Bogan,[15] three different amino acids were proposed; phenylalanine, tyrosine and valine. We predicted that dendrimers functionalized with phenylalanine or tyrosine would bind better than the equivalent unfunctionalized dendrimer and act as an *enhanced* ligand or inhibitor for *chymotrypsin* (during a hydrolysis experiment). On the other hand, dendrimers functionalized with valine would result in poorer binding and therefore be a worse inhibitor than either the unfunctionalized dendrimer, or the dendrimers modified with the phenylalanine or tyrosine. The idea is shown schematically in Figure 1.

Before stating any synthesis, we first needed to consider how functionalization would affect the dendrimer's size. The interfacial area of *chymotrypsin* is rich in positive charge and ~2400 $Å^2$ in size. Therefore, an optimized dendrimer would need to have a surface rich in negative charge and a binding or addressable area that matched as closely as possible the interfacial area of *chymotrypsin*. Our original experiments [10] determined that the G2.5 dendrimer with 32 terminal carboxylates was the optimum sized dendrimer for *chymotrypsin* binding. However, functionalizing the G 2.5 dendrimer would increase its size above the point where optimum binding had occurred. We therefore selected the smaller G1.5 dendrimer with16 terminal carboxylic groups, as a scaffold to support a layer of amino acids. Although this dendrimer has fewer terminal groups and therefore fewer charges, our previous experiments had clearly shown that matching

dendrimer/protein size was more important than charge density.[11] The unfunctionalized G 1.5 dendrimer possesses a maximum addressable area of just 1400 Å^2. However, when any amino acid is added, the size of the maximum addressable will increase and can be estimated by taking the carboxylic acid, to carboxylic acid diameter. Doing this generates an estimated addressable area ~ 1800 Å^2. However, if we include phenylalanine's and tyrosine's aromatic side chain in our estimate, then the maximum addressable areas increase to around 2000 Å^2 and 2300 Å^2 respectively. The isopropyl group on valine does not significantly affect the dendrimers size. As such we decided to use the G 1.5 dendrimer and functionalize it with tyrosine, phenyl alanine and valine.

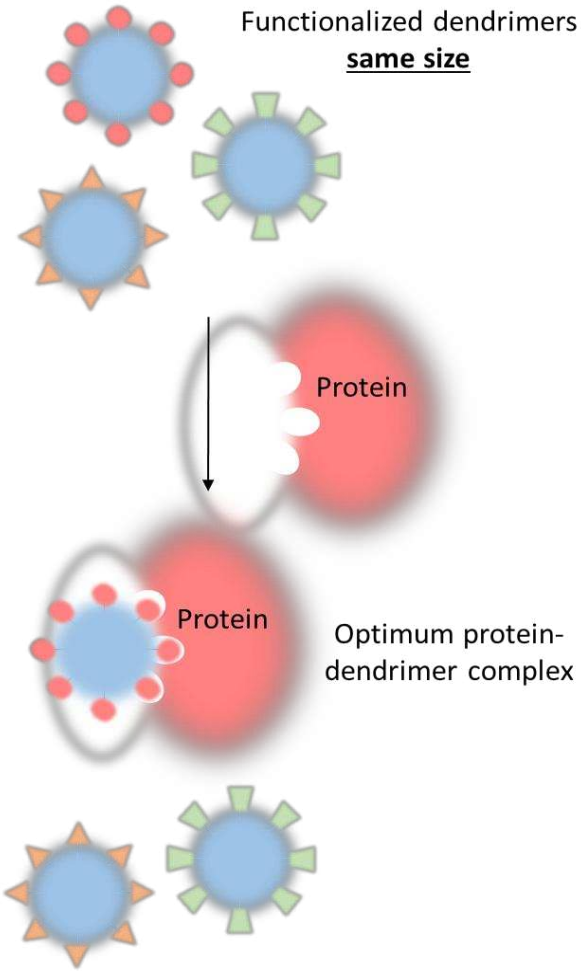


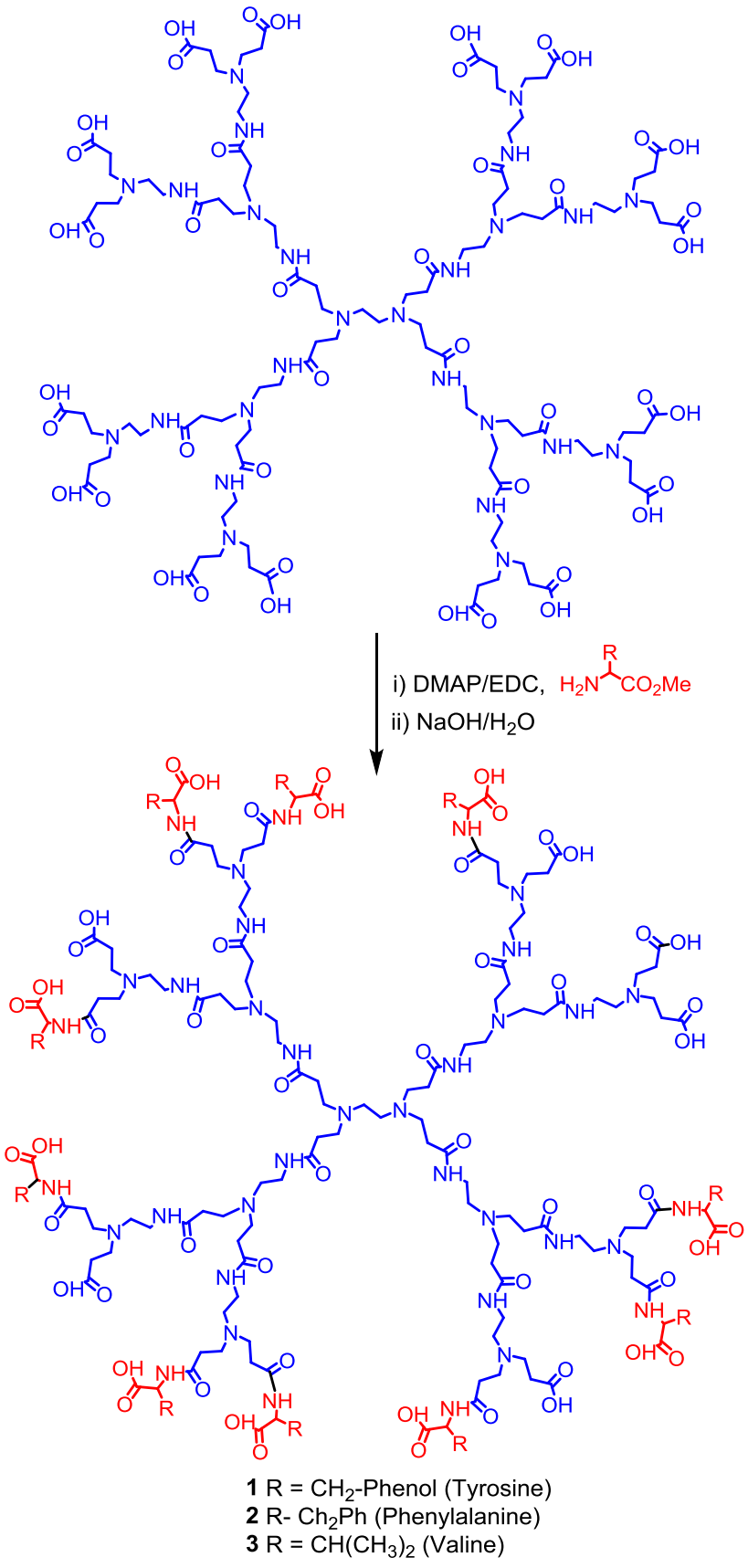
FIGURE 1 – Schematic representation showing how terminal functionality is important with respect to optimum protein binding and selectivity.

The binding of the dendrimers would be compared to that obtained suing the unfunctionalized G2.5 dendrimer, which had previously been shown to be optimized with respect to size and chymotrypsin binding.[10] Although functionalizing amine terminated dendrimers is well established,[23] there are far fewer reports describing the successful and total amino acid functionalization of carboxylate dendrimers. Our method used EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) as the coupling agent, as this is known to work in water, which can minimize by-products and simplify purification.[24] The G 1.5 acid terminated PAMAM dendrimer, was subsequently reacted with 16 equivalents of a C-methyl ester-protected amino acid and EDC in water. The methoxy protecting groups were removed by base catalyzed hydrolysis to give the acid terminated dendrimer. The reactions for the functionalization with tyrosine, phenylalanine and valine are shown in Scheme 1. Mass spectrometry revealed dispersity with regards to the level of functionalization, with a number of peaks indicating levels of functionalization between 5 and 16 amino acids. However, the base peaks corresponded to dendrimers with 10 and 11 terminal amino acid groups. This was supported by ^{1}H-NMR, where the average number of amino acids could be determined by comparing integration of the amino acid's aromatic signal(s) or the isopropyl resonance, to those of the dendrimer's CH_2 peaks. This indicated that 11 (of the 16) terminal groups had been functionalized with an amino acid. In this regards, mass spectrometry and ^{1}H NMR were in broad agreement.

Dendrimer	Level of functionalization (out of 16)	Maximum addressable area ($Å^2$)
G1.5	/	1400
G2.5	/	2300
G1.5-Ty	11	2300
G1.5-Phe	11	2000
G1.5-Val	10	1800

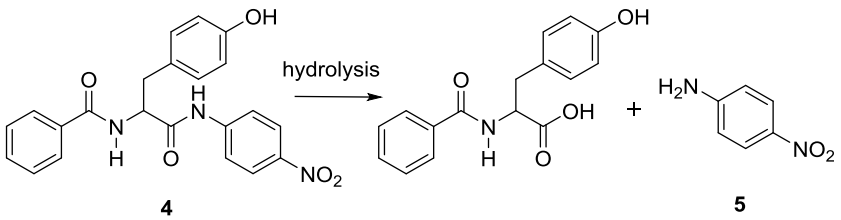
TABLE 1: Functionalization and size data for the dendrimers used in this study.

The functionalization data and the maximum addressable area for each dendrimer is shown in Table 1. All attempts to optimize the reaction (for all three amino acids) did not increase the level of functionalization. However, all dendrimers contained a similar level of functionalization. In addition, full functionalization is not required. The key requirement is that *enough* amino acids be added, allowing the dendrimer to present amino acid functionality across all of its surface and over its maximum addressable area. Therefore, functionalizing *all* of the terminal groups is not required. Specifically, and assuming an even distribution of amino acids across the dendrimers surface, a 50% level of functionalization would allow the various functional groups to span the full addressable area. In our case, just under 70% of the dendrimer surface was functionalized. This is more than enough to ensure that the maximum addressable areas have a significant number of amino acids on their surface.



SCHEME 1: C-coupled amino acid functionalization of a G 1,5 dendrimer with 16 terminal groups.

Having synthesized the functionalized dendrimers, the next step was to assess protein binding with respect to terminal group functionality. Binding was assessed using chymotrypsin and the inhibition method previously reported.[10] Chymotrypsin is a member of the serine protease family of enzymes and catalyzes the hydrolysis of peptide bonds, cleaving them at the carboxyl end of the aromatic residue. The active site entrance of chymotrypsin is at the center of its interfacial/binding area. Therefore, when a dendrimer binds to the interfacial area, it blocks the entrance and substrates cannot enter, resulting in enzyme inhibition. Therefore, the dendrimer that binds the best will also inhibit the best and binding can be directly related to inhibition efficiency (i.e. at a particular concentration 50% inhibition equals 50% binding).[10] An initial control experiment was undertaken to determine the activity of the non-inhibited reaction (no dendrimer present). This was carried out by adding a large excess of the amide substrate N-(1-(4-nitrophenylcarbamoyl)-2-(4-hydroxyphenyl)-ethyl) benzamide (BTNA) **4** to an aqueous solution of chymotrypsin, such that the final concentrations were 1.0×10^{-4}M and 5.0×10^{-7}M respectively. As BTNA is hydrolyzed, 4-nitrobenzenamine **5** is generated, which has a strong UV chromophore, absorbing at 410 nm. The process can therefore be monitored by measuring the amount of 4-nitrobenzenamine **5** produced with respect to time, Scheme 2. For the uninhibited reaction (no dendrimer present), a typical reaction profile was observed and an *initial rate* of 1.12×10^{-7} M s^{-1} was obtained, Table 1. We have previously shown that the protein catalyzed hydrolysis of BTNA is unaffected if the reaction is repeated in the presence of a neutral OH terminated dendrimer (i.e. one that is not charged and cannot bind to the protein).[10] Additionally, we have shown that carboxylate terminated dendrimers do not affect or modify the conformation of the protein.[14]



SCHEME 2: The hydrolysis reaction used to follow chymotrypsin inhibition and protein binding.

Having carried out the background/baseline reactions and the control reactions, we then repeated the protein-mediated hydrolysis using equimolar amounts of the functionalized dendrimers and chymotrypsin. The data is shown graphically in Figure 2. In all cases the reaction profiles and initial rates were reduced when compared to the uninhibited reaction, indicating that all dendrimers inhibited the reaction and were therefore bound. However, it is clear from the graph that dendrimers with different terminal functionalities possessed varying abilities to bind and inhibit chymotrypsin. Dendrimers functionalized with tyrosine have the same size as the control/unfunctionalized dendrimer, but showed much stronger binding (shallowest gradient). This increase in binding occurs despite the fact that the tyrosine functionalized dendrimers have fewer charges. Therefore, although electrostatics are important with respect to binding, the increase comes from secondary interactions that add strength cooperatively (aromatic π-π, hydrophobic and H-bonding interactions). The next best inhibitor was the dendrimer functionalized with phenylalanine, whilst the dendrimer that bound worst was the valine functionalized dendrimer (steepest gradient). The initial velocities (v_0) and relative binding are shown in Table 2.

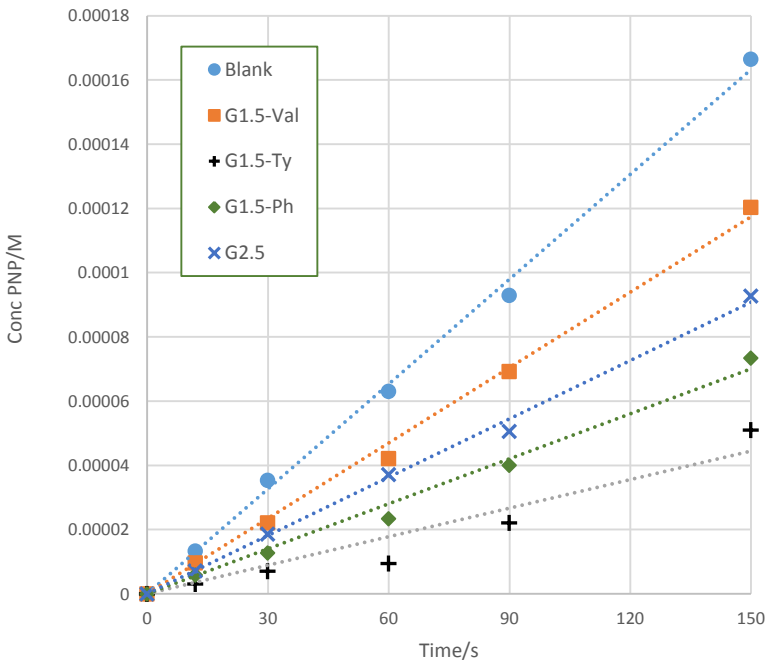


FIGURE 2: Initial rate plot for chymotrypsin/BTNA hydrolysis in the presence and absence of various dendrimers.

Overall the data confirms our predications, which were based on the work of Bogan and Thorn [15] regarding the general population of amino acids throughout a protein and their availability at binding/interfacial areas. Specifically, dendrimers functionalized with tyrosine and phenylalanine demonstrated a high binding affinity for chymotrypsin. Whereas, dendrimers functionalized with valine, which is relatively common in protein structures, but has a low availability at the binding/interfacial area, showed the lowest inhibition and therefore weakest binding. To quantify binding on a relative scale, initial rates were calculated and compared with the initial rate obtained from the uninhibited control reaction (no dendrimer present). Inhibition percentages were calculated by taking the v_0 ratio for the control to dendrimer reactions, and multiplying by 100. The extent of reaction (as a percentage) was easily obtained by subtracting the inhibition percentage from 100. The results were then normalized by setting the best inhibitor to 100% and quoting all other values relative to this. Since inhibition is directly related to binding, the relative inhibitions obtained

translate directly to a relative binding efficiency. The data is shown in Table 2. The dendrimer with tyrosine bound best with a relative binding value of 68%, whilst the valine dendrimer bound worst with an affinity of just 47%. When compared to previous results using the non-functionalized G2.5 dendrimer,[10] this equated to a 16% *increase* and a 19% *decrease* in the binding affinity for the tyrosine and valine dendrimers respectively. These results confirm the importance of the secondary interactions provided by the amino acid functionality, which may result in improved selectivity for protein binding.

Dendrimer	v_0 (x10^{-8} Ms^{-1})	Extent of reaction (%)	Relative binding (%)
None	11.8	100	0
G 2.5	4.89	42	58
G1.5-Ty	3.73	31	69
G1.5-Ph	4.01	35	65
G1.5-Val	6.24	53	47

TABLE 2: Initial rates and binding data for the functionalized and unfunctionalized dendrimers.

CONCLUSIONS

In conclusion, this work has shown that dendrimer-protein binding can be moderated by the dendrimer's terminal functionality. Binding can be affected in a negative of positive way, suggesting that increased selectivity are possible with respect to dendrimer-protein binding. When the results are compared with the data obtained using unfunctionalized dendrimers (of similar size and therefore similar addressable areas), it was apparent that dendrimers modified with aromatic amino acids resulted in increased binding; the strongest binding occurring with dendrimers possessing terminal tyrosine units at their periphery. This binding was slightly stronger than that observed for phenyl alanine and the additional strength can be attributed to the additional hydrogen

bond provided by tyrosine's OH group (in addition to the hydrophobic and π–π interactions common for both of the aromatic amino acids). Overall, these secondary interactions work cooperatively, resulting in the strongest binding. On the other hand, the valine-functionalized dendrimers are unable to contribute secondary interactions, resulting in weaker binding. Overall the results correlate well with theoretical and experimental studies into the population and distribution of particular amino acids within protein structures and their relative population on hot spot/protein binding surfaces.[9] As such, these results have increased our understanding of the importance of size and terminal functionality with respect to the application of dendrimers to protein binding. These results will allow us to develop "next generation" macromolecules for application as selective or specific protein ligands and as inhibitors to protein-protein interactions. Towards these aims, we are currently constructing new multivalent dendrimers that possess a number of different amino acids on their surface. Whilst preparing to submit this manuscript, a paper detailing how a selective binding can be increased using a multivalent/macromolecular approach was published.[25] In addition, we also plan on developing dendrimer based combinatorial approach for the synthesis, selection and identification of optimum ligands for specific proteins.

SUPPORTING INFORMATION

The supporting information provides details of the synthesis, characterization of the amino acid functionalized dendrimers. The experimental procedure for protein binding is also described. This information is available free of charge via the internet at http://pubs.acs.org

AUTHOR INFORMATION

Corresponding Author

Email: l.j.twyman@sheffield.ac.uk

ORCID

Lance J. Twyman: 0000-0002-6396-8225

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by a research scholarship funded by the British Council (ORS) and awarded to Fumiko Chiba.

NOTES AND REFERENCES

1. J. D., Jordan, E. M., Landau, and Iyengar, R. (2000) Signaling networks: the origins of cellular multitasking. *Cell, 103(2),* 193-200.

2. D. R., Hipfner, and Cohen, S. M. (2004) Connecting proliferation and apoptosis in development and disease. *Nat. Rev. Mol. Cell Biol., 5(10),* 805-815.

3. M., Pellegrini, D., Haynor, and Johnson, J. M. (2004) Protein interaction networks. *Expert Rev. Proteomics, 1(2),* 239-49.

4. Stites, W.E. (1997) Protein-Protein interactions: interface structure, binding thermodynamics, and mutational analysis. *Chem. Rev*., *97(5),* 1233-1250.

5. H., Yin, and Hamilton, A. D. (2005) Strategies for targeting protein-protein interactions with synthetic agents. *Angew. Chem. Int. Ed*. *44(27),* 4130-4163.

6. C. M., Koh,_S., Arianna and Guccione G. (2016) Targeting MYC in cancer therapy: RNA processing offers new opportunities. *Bioessays 38*: 266–275.

7. S., Jones, and Thornton, J. M. (1996) Principles of protein-protein interactions. *Proc. Nat. Acad. Sci.*, *93*, 13-20

8. L. L., Conte, C. Chothia, and Janin, J. (1999) The atomic structure of protein-protein recognition sites. *J. Mol. Bio., 285(5),* 2177-2198.

9. D., Reichmann, O., Rahat, M., Cohen, H., Neuvirth, and Schreiber, G. (2007) The molecular architecture of protein-protein binding sites. *Curr. Opin. Struct. Biol. 17*, 67-76.

10. F., Chiba, T. C., Hu, L. J., Twyman, and Wagstaff, M. (2008) Dendrimers as size selective inhibitors to protein-protein binding. *Chem. Comm., 36*, 4351-4353.

11. N., Welsch, J., Dzubiella, A., Graeberta. and Ballauff, M. (2012) Protein binding to soft polymeric layers: a quantitative study by fluorescence spectroscopy. *Soft Matter*, *8,* 12043-12052.

12. J. Y., Gao, and Dubin, P. I. (1999) Binding of proteins to copolymers of varying hydrophobicity. *Biopolymers*, *49.* 185-193.

13. Q., Wei, T., Becherer, S. A., Angioletti-Uberti, J., Dzubiella, C., Wischke, A.T., Neffe, A., Lendlein, and Ballauff, M. (2014) Protein Interactions with Polymer Coatings and Biomaterials. *Angew. Chem. Int. Ed*., *53,* 8004-8031.

14. F., Chiba, G., Mann, and Twyman, L. J. (2010) Investigating possible changes in protein structure during dendrimer–protein binding. *Org Biomol Chem*, *8(22),* 5056-5058.

15. A. A., Bogan, and Thorn, K. S. (1998) Anatomy of hot spots in protein interfaces. *J. Mol. Bio.,* 280*(1),* 1-9.

16. N. H., Jha, S., Vishveshwara, and Banavar, J. R. (2010) Amino acid interaction preferences in proteins. *Protein Sci*. *19(3),* 603-616.

17. C. N., Pace, G., Horn, E. J., Hebert, J., Bechert, K., Shaw, L., Urbanikova, J. M., Scholtz, and Sevcik, J. (2001) Tyrosine hydrogen bonds make a large contribution to protein stability. *J. Mol. Bio., 312(2),* 393-404.

18. H., Zhou, L., Baldini, J., Hong, A. J., Wilson, and Hamilton, A. D. (2006) Pattern Recognition of Proteins Based on an Array of Functionalized Porphyrins. *J. Am. Chem. Soc. 128(7),* 2421-2425.

19. Y., Hamuro, M. C., Calama, H. S., Park, and Hamilton, A. D. (1997) Calixarene with Four Peptide Loops: An Antibody Mimic for Recognition of Protein Surfaces. *Angew. Chem. Int. Ed. Eng., 36(23),* 2680-2683.

20. L., Baldini, A. J., Wilson, J., Hong, and Hamilton, A. D. (2004) Pattern-Based Detection of Different Proteins Using an Array of Protein Surface Receptors. *J. Am. Chem. Soc. 126(18),* 5656-5657.

21. N., Higashi, T., Koga, and Niwa, M. (2002) Enantioselective Binding and Stable Encapsulation of a-Amino Acids in a Helical Poly (L-glutamic acid)-Shelled Dendrimer in Aqueous Solutions. *ChemBioChem*. *3*, 448-454.

22. M. F., Ottaviani, S., Jockusch, N. J., Turro, D. A., Tomalia, and Barbon, A. (2004) Interactions of Dendrimers with Selected Amino Acids and Proteins Studied by Continuous Wave EPR and Fourier Transform EPR. *Langmuir*, *20*, 10238-10245.

23. P., Ballester, R. M., Gomila, C. A., Hunter, A. S. H., King, and Twyman, L. J. (2003) Dendrimers as scaffolds for the synthesis of spherical porphyrin arrays. *Chem. Commun. 1,* 38-39.

24. N. S., Cameron, A., Eisenberg, and Brown, G. R. (2002) Amphiphilic Block Copolymers as Bile Acid Sorbents: 1. Polystyrene-*b*-poly (*N*,*N*,*N*-trimethylammoniumethylene acrylamide chloride). *Biomacromolecules*, 3*(1),* 124-132.

25. P., Gilles, K., Wenck, I., Stratmann, M., Kirsch, D. A., Smolin, T., Schaller, H., de Groot, A., Kraft, and Schrader, T. (2017) High-Affinity Copolymers Inhibit Digestive Enzymes by Surface Recognition. *Biomacromolecules*, *18*, 1772−1784.

TOC-GRAPHIC

The Effect of Terminal Group Functionality on the Ability of Dendrimers to Bind Proteins.