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PAPER

Comparing dendritic and self-assembly strategies to multivalency—RGD peptide–integrin interactions†

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This paper compares covalent and non-covalent approaches for the organisation of ligand arrays to bind integrins. In the covalent strategy, linear RGD peptides are conjugated to first and second generation dendrons, and using a fluorescence polarisation competition assay, the first generation compound is demonstrated to show the most effective integrin binding, with an EC_{50} of 125 μM (375 μM per peptide unit). As such, this dendritic compound is significantly more effective than a monovalent ligand, which does not bind integrin, even at concentrations as high as 1 mM. However, the second generation compound is significantly less effective, demonstrating that there is an optimum ligand density for multivalency in this case. In the non-covalent approach to multivalency, the same RGD peptide is functionalised with a hydrophobic C12 chain, giving rise to a lipopeptide which is demonstrated to be capable of self-assembly. This lipopeptide is capable of effective integrin binding at concentrations of 200 μM. These results therefore demonstrate that covalent (dendritic) and non-covalent (micellar self-assembly) approaches have, in this case, comparable efficiency in terms of achieving multivalent organisation of a ligand array.

Introduction

Multivalency, in which moieties with multiple binding ligands are employed, can be a powerful mechanism for enhancing the binding of synthetic ligands to biological targets.[1] Multivalent ligands exhibit enhanced binding through (i) a local concentration effect (in which once a ligand dissociates from its binding partner, there is a high local concentration to favour ligand rebinding) and/or (ii) a primarily entropic effect, which after the initial binding event, favours the binding of a second ligand to a second binding site on the biological binding partner (Fig. 1). As such, the strategy is widely employed for the binding of biomolecules with multiple potential binding sites, such as glycoproteins and DNA.[2]

Integrins are heterodimeric, transmembrane proteins which play important roles in cell adhesion and signalling.[3] They have been of interest in tissue engineering applications,[4] and furthermore, given that some integrins are over-expressed on specific cancer cells, they are an important target for anti-cancer treatments.[5] In key work, the tri-peptide Arg-Gly-Asp (RGD) was shown to bind to integrins.[6] 'Cilengitide', a cyclic RGD peptide with enhanced integrin binding affinity, is currently under development as an anti-angiogenic agent,[7] and the X-ray structure of this molecule bound to the extracellular segment of integrin $\alpha_v\beta_3$, has been published.[8]

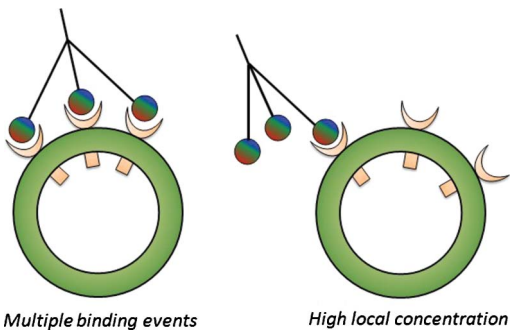

Fig. 1 Origins of multivalency in biomolecular recognition.

As such, understanding the binding of peptides to integrin targets in detail is an important target in studies of molecular recognition. On initial inspection, integrins may appear to be unpromising targets for multivalent binding, as they only possess a single ligand binding site. However, in biological systems, integrins are often clustered in cell membranes. As such, multivalent binding of multiple integrins is a genuine possibility.[9] To enhance the binding of RGD peptides to integrins, there has therefore been interest in using multivalent peptide arrays.[10]

Dendritic molecules,[11] which have an inherently branched architecture offer a versatile approach for organising covalent multivalent ligand arrays.[12] Pre-formed dendrimers have had RGD-containing peptides conjugated to their surfaces, and it has been demonstrated that their biological activities in a number of assays appear to be enhanced, presumably by multivalency.[13] However, it is worth noting that in general, the fundamental

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nature of the interaction between the RGD peptide and the integrin protein has rarely been explored—with a greater focus on biological outcome. Liskamp and co-workers recently reported a dendritic RGD derivative, and carefully assayed its ability to bind integrin using a competition assay,[14] however, the use of cyclic RGDs led to all compounds showing very strong binding, and little evidence of multivalent binding. Dumy and co-workers, have developed template-supported multivalent RGD arrays, and used cell adhesion assays, to show that multivalency is important when integrins are supported in cell membranes.[15]

An alternative approach to multivalency employs self-assembly to spontaneously organise binding units into a multivalent array. RGD-containing lipopeptides have been demonstrated to insert into membranes which gain enhanced affinity for integrins,[16] and show improved cell internalisation,[17] clustering at the integrin binding part of the membrane.[18] RGD-functionalised lipopeptides have also been co-formulated with other active units to generate assemblies capable of enhanced cellular gene delivery.[19] Cyclic RGDs have also been coupled to the surfaces of liposomes, which have then exhibited enhanced cellular uptake.[20] There have also been studies on the ability of RGD functionalised lipids to self-assemble in their own right.[21] For example, Lee and co-workers have demonstrated that lipids functionalised with cyclic RGD self-assemble into nano-ribbons, and can deliver hydrophobic molecules into cells.[22] A number of groups, including those of Stupp and Ulijn, have demonstrated that incorporating RGD peptides into self-assembling soft materials can enhance tissue engineering.[23] It is, however, unclear whether self-assembly enhances the RGD–integrin interaction in thermodynamic terms, or whether it is simply a useful materials formulation tool. There has recently been increasing interest in understanding in fundamental terms how self-assembly can enhance multivalency, for example in interactions with glycoproteins,[24] collagen[25] and DNA,[26] but such effects have not really been probed for integrin–RGD interactions.

The goal of this paper was *not* to develop high-affinity or highly selective integrin binding systems— there are a large number of those already—but rather, to gain insight into different ways in which ligands can be organised for multivalent binding. We therefore chose to use linear RGD peptides for ligand construction. Although they have relatively low affinity binding and poor selectivity these compounds can be easily synthesised in multi-gram quantities using straightforward solution-phase peptide chemistry, and because their integrin binding is less highly optimised and has relatively low affinity (mM), multivalent binding effects may be more significant. In this paper, we therefore report a simple comparison between different RGD peptides. Monomeric **PEG-RGD** is compared with first generation dendritic system **G1-RGD$_3$** having three RGD peptide surface groups, and second generation dendron **G2-RGD$_9$** having nine (Fig. 2). This provides an insight into the effect of covalent multimerisation on integrin binding. We then compared hydrophilic **PEG-RGD** with amphiphilic **C12-RGD**. The latter compound self-assembles and can therefore provide insight into the effect of self-assembly on multivalent ligand organisation. Using this approach, we hoped to develop an enhanced understanding of the way in which ligands can be organised to achieve multivalent binding.

Results and discussion

Synthesis and characterisation

The synthesis and characterisation data for all compounds are provided in the Experimental section and/or ESI.† Dendron **G1-RGD$_3$** was constructed (Scheme 1) from a first generation 'Newkome-type' amide/ether scaffold protected at the focal point

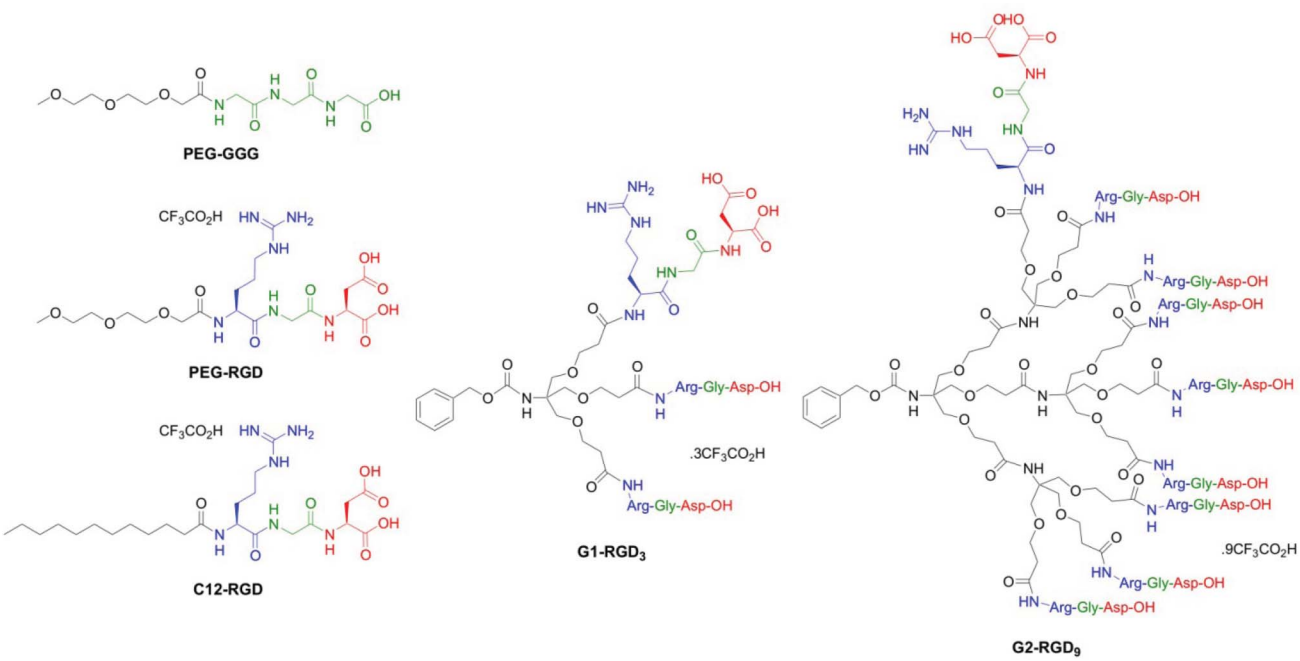
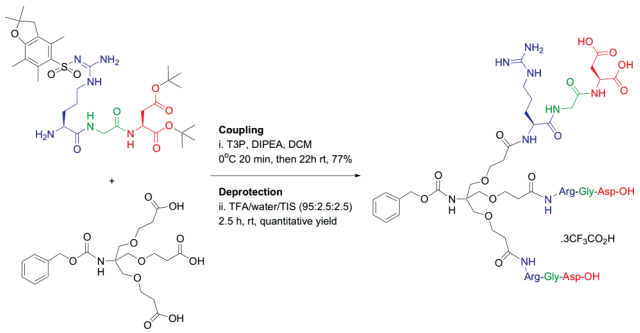


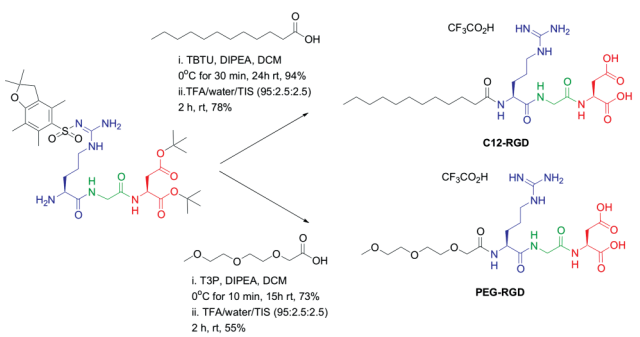
Fig. 2 Compounds investigated in this paper.



Scheme 1 Synthesis of **G1-RGD$_3$**.

with a benzyl carbamate group, synthesised using previously published methodology.[27] The three peripheral carboxylic acid groups were then conjugated to the N-terminus of a protected linear RGD peptide (synthesised *via* solution-phase peptide coupling methodology—see ESI†) using propylphosphonic anhydride (T3P) as a coupling agent. Finally, removal of the RGD-peptide protecting groups using trifluoroacetic acid (TFA) and triisopropylsilane (TIS) gave rise to the target compound, **G1-RGD$_3$**. Second generation dendritic compound **G2-RGD$_9$** was synthesised in an analogous manner.

Monovalent control, **PEG-RGD**, and lipopeptide **C12-RGD** were constructed by simple conjugation of the N-terminus of the protected RGD peptide with the appropriate carboxylic acids mediated by propylphosphonic acid (T3P) and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) respectively, followed by deprotection of the RGD-peptide (Scheme 2). Negative control, **PEG-GGG** was synthesised *via* analogous solid-phase peptide and protecting group methodologies (see ESI†). All target compounds were synthesised in good yield, and fully characterised.



Scheme 2 Synthesis of **C12-RGD** and **PEG-RGD**.

Self-assembly of RGD peptides

Initially, we monitored whether the RGD peptides aggregated in aqueous solution, using solubilisation experiments with the hydrophobic dye Nile Red.[28] If self-assembled architectures are present, Nile Red will be solubilised into the hydrophobic domain of the aggregates. Varying the concentration of the self-assembling peptide will lead to increasing levels of dye solubilisation (Fig. 3A) Plotting the fluorescence emission intensity of Nile Red at 635 nm *versus* log[peptide] (Fig. 3B) then allows the determination of critical aggregation concentrations (CACs). As expected, **PEG-RGD**,

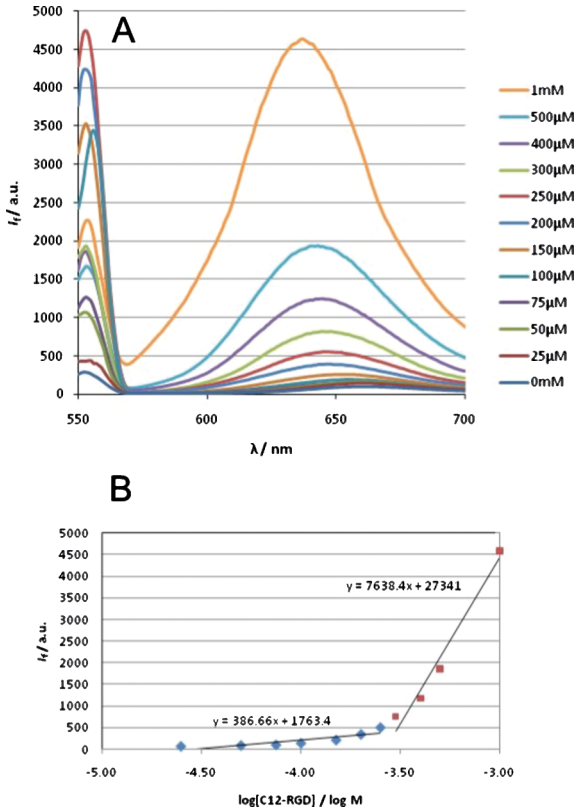


Fig. 3 A) Nile Red fluorescence emission spectra in the presence of increasing concentrations of **C12-RGD**. B) Fluorescence emission intensities of Nile Red at 635 nm plotted against log[**C12-RGD**] in order to determine the Critical Aggregation Concentration (CAC). Excitation wavelength: 550 nm.

G1-RGD$_3$ and **G2-RGD$_9$** showed no evidence of self-assembly at concentrations <1 mM. Conversely, **C12-RGD** showed evidence of self-assembly, with the CAC being calculated as *ca.* 300 μM in aqueous phosphate buffered saline (PBS) (pH 7.4).

Transmission electron microscopy (TEM) was then performed on **C12-RGD** dried from aqueous solution in order to gain some insight into the morphology of the aggregates. This revealed that at high concentration (1 mM) small (10–50 nm diameter) spherical micelles were formed in TRIS (tris(hydroxymethyl)aminomethane) and PBS buffers. On dilution to just above the CAC (400 μM), however, larger lamellar aggregates were observed (see ESI† for TEM images). These experiments clearly demonstrate that self-assembly of **C12-RGD** is taking place.

Integrin binding affinity

We then probed the ability of these peptides to bind integrin $\alpha_v\beta_3$. To monitor the binding, we employed a competitive fluorescence polarisation (FP) assay, first established by Li *et al.*[29] This assay uses the fluorescent probe **5(6)-FL-c[RGDfK]** based on a cyclic RGD peptide[30] (Fig. 4, synthesised according to literature methods—see ESI†). This probe is bound to the integrin, and the ligand of interest is then titrated into the solution. If binding occurs between the ligand and the integrin, the fluorescent probe is displaced, and the FP signal decreases in intensity as the probe mobility increases. Determining the concentration of

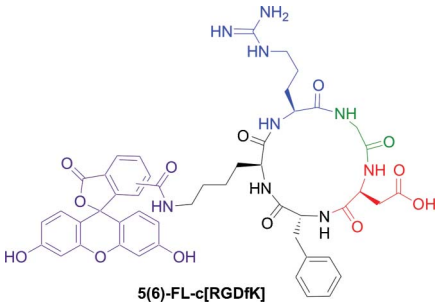


Fig. 4 Structure of cyclic RGD-peptide fluorescent probe **5(6)-FL-c[RGDfK]**.

ligand required for the apparent 50% displacement of the probe from the integrin binding site yields an EC_{50} value (effective concentration) for each ligand of interest. Although there has been some discussion about the advantages and disadvantages of fluorescence polarisation assays,[31] this approach remains a powerful one for comparing related families of ligands and their relative affinities to bind to the same biological target under equivalent conditions, and as such, allows us to gain some insight into the comparative affinities of our family of linear RGD peptides for integrin $\alpha_v\beta_3$.

Initially, the binding of fluorescent probe **5(6)-FL-c[RGDfK]** to integrin $\alpha_v\beta_3$ was assessed *via* FP titration methods and, in line with the literature,[29] as the integrin was added to the probe (10 nM), the FP signal increased from *ca.* 35 mP (milli polarisation units) in the absence of integrin to over 100 mP when the concentration of integrin was >400 nM. The binding process was kinetically fast and incubating the samples for longer than 5 min did not have any significant effect on the FP signal.

We then performed competition assays using increasing concentrations of our RGD peptides against fixed concentrations of fluorescent probe (10 nM) and integrin (280 nM). In the first instance we compared the binding of **PEG-RGD**, **G1-RGD$_3$** and **G2-RGD$_9$** in order to investigate whether the covalent dendritic strategy to multivalency yielded enhanced integrin binding. Monovalent ligand **PEG-RGD** only reduced the normalised FP signal to *ca.* 70% of its initial value, and was unable to reduce the value below 50% (Fig. 5) even at very high concentrations (*ca.* 1 mM). As expected, monovalent linear RGD only has weak affinity for integrin,[32] and is therefore unable to fully displace the strongly binding cyclic RGD fluorescent probe from the protein. Importantly, **PEG-GGG**, the negative control, did not displace **5(6)-FL-c[RGDfK]** from its integrin binding site at all, with the FP signal remaining unchanged even at the maximum tested concentration. This demonstrates that the RGD peptide in **PEG-RGD** is directly responsible for the decrease in FP signal, which can therefore be attributed to weak integrin binding. Compound **G1-RGD$_3$**, however, showed enhanced integrin binding (Fig. 5), reducing the normalised FP signal to <50% of its initial value at a concentration of *ca.* 125 μM. Compared with cyclic RGD peptides, this is still weak binding, but the enhancement in contrast with the data from **PEG-RGD** clearly indicates the advantage of a multivalent strategy. On a per-RGD-peptide basis, the reduction of FP signal to below 50% of its initial value occurs at 375 μM for **G1-RGD$_3$**—significantly below the concentrations assayed for monovalent **PEG-RGD**.

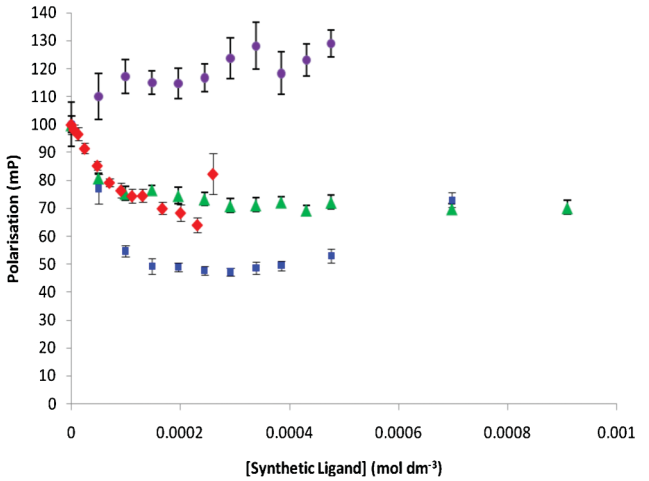


Fig. 5 Normalised titration curves for the displacement of **5(6)-FL-c[RGDfK]** probe (10 nM) from integrin $\alpha_v\beta_3$ (280 nM) on the addition of the synthetic ligands: **G1-RGD$_3$** (blue), **G2-RGD$_9$** (red), **PEG-RGD** (green) and **PEG-GGG** (purple) after incubating at 29 °C for 5 min.‡§

We then investigated **G2-RGD$_9$** binding to integrin $\alpha_v\beta_3$ using the same approach. On adding this second generation dendron to the integrin, there was initially a decrease in the FP signal as the fluorescent probe was displaced from the RGD binding site—however, this did not occur at such low concentrations as for **G1-RGD$_3$** (Fig. 5). This indicates that the first generation system is better able to bind to integrin than the second generation analogue. Similar effects have previously been reported for saccharide binding to glycoproteins, where increased steric hindrance at higher generations can limit the ability of the surface ligands to effectively bind to their biological target.[2a] We can therefore conclude that the first generation system is, in this case, optimal for integrin binding and we suggest that the surface rigidity and steric hindrance of the higher generation dendrimer acts to limit its integrin affinity.

The data above demonstrate the advantage of a dendritic approach to multivalency, and would suggest it is playing a significant role in this binding event. At first sight this is surprising, because integrin only has a single binding site, and the mechanism by which multivalency may operate is not completely clear. However, in this assay, the solution-phase integrin is not strictly 'free' protein. As a membrane-bound protein, integrin $\alpha_v\beta_3$ is supplied stabilised in Triton X-100 surfactant. This was the form of integrin used in the assays here. Fig. 6 shows the TEM image of the Triton X-100-integrin assemblies prior to the addition of **G1-RGD$_3$**. This formulation of integrin protein is somewhat similar to the situation within a cell membrane. As such, we propose that the dispersion of integrin in the surfactant phase allows the significant enhancement of binding on the application of a multivalent, dendritic ligand.

However, it should also be noted that on further addition of all ligands, the FP signal began to rapidly increase in intensity (Fig. 7). This was particularly notable for **G2-RGD$_9$**, where the

‡ Fluorescence polarisation data were normalised to 100 mP units in order to enable comparability between different competition experiments.

§ Data are presented as mean values ± standard deviations from at least 5 independent scans.

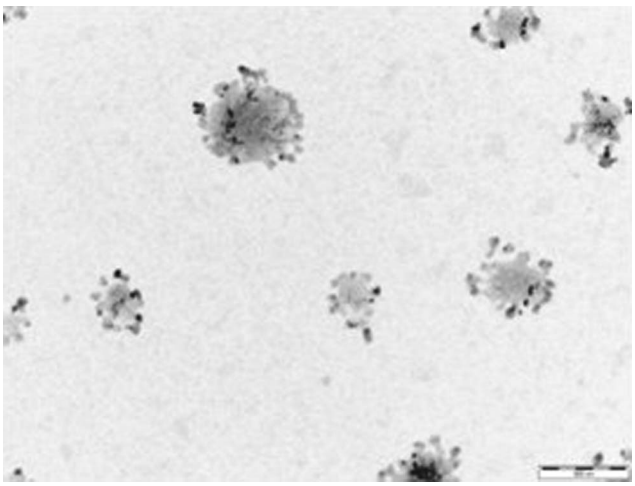


Fig. 6 TEM image of integrin-Triton X-100 assemblies prior to addition of RGD peptide ligands—scale bar = 500 nm.

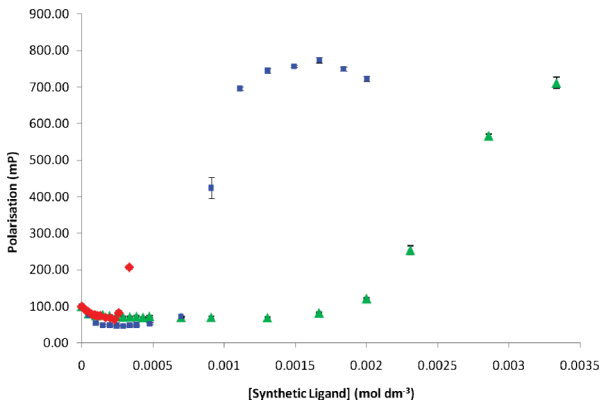


Fig. 7 Normalised titration curves at elevated concentrations for the displacement of **5(6)-FL-c[RGDfK]** probe (10 nM) from integrin $\alpha_v\beta_3$ (280 nM) on the addition of the synthetic ligands: **G1-RGD$_3$** (blue), **G2-RGD$_9$** (red) and **PEG-RGD** (green) after incubating at 29 °C for 5 min.‡§

increase in FP intensity started at 250 μM, but was also the case for **G1-RGD$_3$**, where the increase started at 800 μM and **PEG-RGD**, where the increase started at *ca.* 2 mM. It should be noted that these linear RGD ligands are being applied at relatively high micromolar concentrations in this assay—owing to their low binding affinities for integrins. We therefore propose that this increase in FP signal is caused by a non-specific interaction between the RGD ligands and the integrin target. This could be mediated by terminal carboxyl group on each of the ligands, leading to non-specific binding (*e.g.* integrin surface binding) and subsequent aggregation/precipitation, hence explaining the increase in apparent FP intensity. This argument is supported by the observation that the onset of this process occurs at a concentration of 250 μM for **G2-RGD$_9$**—an effective ligand concentration of 2.25 mM (250 μM × 9), for **G1-RGD$_3$** the effective ligand concentration is 2.4 mM (800 μM × 3) and for **PEG-RGD** the effect is also observed at an effective ligand concentration of *ca.* 2 mM.

We then investigated the potential of **C12-RGD** to bind to integrin, and assess whether self-assembling a multivalent array of RGD ligands is a plausible replacement strategy for the synthetically more demanding construction of a covalent dendritic ligand array, such as **G1-RGD$_3$**. For **C12-RGD**, the normalised FP signal was reduced below 50% of its initial value when the concentration of **C12-RGD** was increased to *ca.* 200 μM (Fig. 8). This makes monovalent **C12-RGD** a significantly more effective ligand in comparative terms than the control monovalent ligand **PEG-RGD**—a potential multivalent effect. It is interesting to note that per RGD unit, **C12-RGD** (200 μM) is a more effective integrin binder than **G1-RGD$_3$** (375 μM). This demonstrates that the self-assembled system uses its peptide units more effectively in binding to the integrin than the covalently structured dendritic array. It is interesting to speculate that the reversible nature of self-assembly generates ligand arrays with a greater degree of flexibility, and potentially a better ability to satisfy the binding requirements of the integrin. This is certainly true when comparing self-assembling **C12-RGD** with second generation **G2-RGD$_9$** where the self-assembly approach yields significantly enhanced integrin binding—perhaps as a result of the better ability of self-assembled ligands to recombine and better adapt to the binding site of integrin.

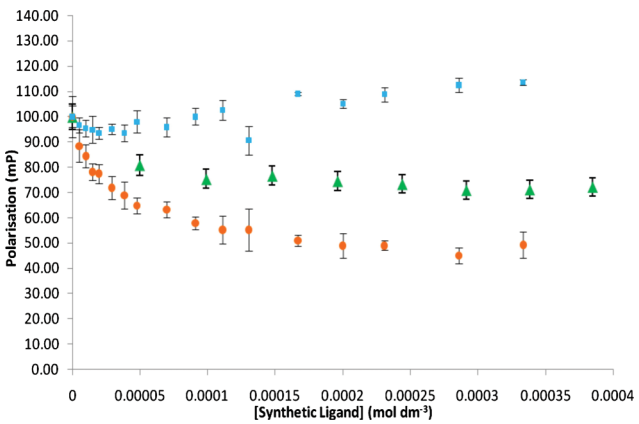


Fig. 8 Normalised titration curves for the displacement of **5(6)-FL-c[RGDfK]** probe (10 nM) from integrin $\alpha_v\beta_3$ (280 nM) on the addition of the synthetic ligands. **C12-RGD** (orange), **PEG-RGD** (green) and **SDS** (light blue) after incubating at 29 °C for 5 min.‡§

We suggest that binding to integrin may actually encourage the surfactant-like peptide to aggregate, even below its CAC. It is also plausible that **C12-RGD** may itself insert into the Triton X-100 assemblies and that the overall combined multivalent aggregate then binds to integrin proteins in adjacent assemblies. TEM imaging indicates that the Triton–integrin assemblies do change morphology somewhat in the presence of **C12-RGD**, in support of this latter hypothesis (see ESI†).

We were concerned that **C12-RGD** may be affecting the FP signal by interfering with the Triton X-100 surfactant assemblies, rather than forming specific interactions with integrin. We therefore tested the interaction of a control mono-anionic surfactant, which also contained a twelve carbon atom hydrophobic chain, sodiumdodecylsulfate (**SDS**), with the integrin. Detergent **SDS** showed no effect at all on the FP assay (Fig. 8), unambiguously demonstrating that the effect of **C12-RGD** can be attributed to specific interactions between the RGD peptide unit of the lipopeptide and the integrin protein, rather than the disruptive surfactant-like nature of the compound.

Conclusions

In summary, this paper reports the synthesis of dendritic RGD peptides, an RGD lipopeptide and positive and negative control peptides. Interestingly, the dendritic approach appeared to give optimal binding at the first generation, with the more highly branched second generation system having lower affinity recognition as well as a greater degree of non-specific ligand–protein interaction. Endowing the RGD unit with a hydrophobic chain encourages lipopeptide self-assembly and appears to enhance integrin binding. Notably, the self-assembled approach is comparable to, and competitive with the use of a first generation dendritic scaffold to organise a multivalent ligand array (Fig. 9).

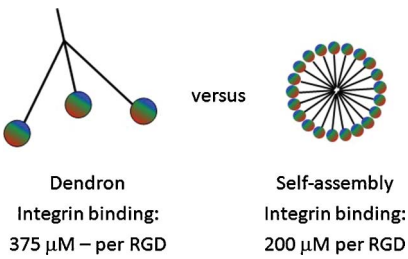


Fig. 9 Comparison of dendritic and self-assembled approaches to multivalency.

As such, the data in this paper indicate that both dendritic (covalent), and self-assembly (non-covalent) strategies to multivalency can be used in similar ways to enhance the binding of RGD peptides to integrins, although in this case, the self-assembled approach appears to give rise to slightly higher affinity integrin binding. In further work, by modifying the peptide (*e.g.* RGDS), or changing the lipophilic group to enhance self-assembly, we believe that significantly enhanced binding affinities will be achieved, and alternative nanostructures with higher integrin affinities will be achieved.

Acknowledgements

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Notes and references

1 (*a*) M. Mammen, S.-K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754–2794; (*b*) A. Mulder, J. Huskens and D. N. Reinhoudt, *Org. Biomol. Chem.*, 2004, **2**, 3409–3424; (*c*) J. D. Badjic, A. Nelson, S. J. Cantrill, W. B. Turnbull and J. F. Stoddart, *Acc. Chem. Res.*, 2005, **38**, 723–732; (*d*) J. Huskens, *Curr. Opin. Chem. Biol.*, 2006, **10**, 537–543; (*e*) V. Martos, P. Castreno, J. Valero and J. de Mendoza, *Curr. Opin. Chem. Biol.*, 2008, **12**, 698–706.

2 (*a*) Y. M. Chabre and R. Roy, *Curr. Top. Med. Chem.*, 2008, **8**, 1237–1285; (*b*) N. Jayaraman, *Chem. Soc. Rev.*, 2009, **38m**, 3463–3483; (*c*) M. A. Kostiainen, J. G. Hardy and D. K. Smith, *Angew. Chem., Int. Ed.*, 2005, **44**, 2556–2559; (*d*) G. M. Pavan, A. Danani, S. Pricl and D. K. Smith, *J. Am. Chem. Soc.*, 2009, **131**, 9686–9694; (*e*) S. Jones, G. M. Pavan, A. Danani, S. Pricl and D. K. Smith, *Chem.–Eur. J.*, 2010, **16**, 4519–4532.

3 (*a*) R. O. Hynes, *Cell*, 1992, **69**, 11–25; (*b*) O. Cleaver and D. A. Melton, *Nat. Med.*, 2003, **9**, 661–668; (*c*) E. F. Plow, T. A. Haas, L. Zhang, J. Loftus and J. W. Smith, *J. Biol. Chem.*, 2000, **275**, 21785–21788; (*d*) R. O. Hynes, *Nat. Med.*, 2002, **8**, 918–921.

4 L. Perlin, S. MacNeil and S. Rimmer, *Soft Matter*, 2008, **4**, 2331–2349.

5 (*a*) P. C. Brooks, R. A. Clark and D. A. Cheresh, *Science*, 1994, **264**, 569–571; (*b*) P. C. Brooks, A. M. P. Montgomery, M. Rosenfeld, R. A. Reisfeld, T. Hu, G. Klier and D. A. Cheresh, *Cell*, 1994, **79**, 1157–1164; (*c*) C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides and D. E. Ingber, *Science*, 1997, **276**, 1425–1428; (*d*) W. Arap, R. Pasqualini and E. Ruoslahti, *Science*, 1998, **279**, 377–380; (*e*) K. E. Gottschalk and H. Kessler, *Angew. Chem., Int. Ed.*, 2002, **41**, 3767–3774; (*f*) K. N. Sugahara, T. Teesalu, P. P. Karmali, V. R. Kotamraju, L. Agemy, D. R. Greenwald and E. Ruoslahti, *Science*, 2010, **328**, 1031–1035.

6 M. D. Pierschbacher and E. Ruoslahti, *Nature*, 1984, **309**, 30–33.

7 (*a*) M. A. Dechantsreiter, E. Planker, B. Matha, E. Lohof, G. Holzemann, A. Jonczyk, S. L. Goodman and H. Kessler, *J. Med. Chem.*, 1999, **42**, 3033–3040; (*b*) G. Tabatabai, M. Weller, B. Nabors, M. Picard, D. Reardon, T. Mikkelsen, C. Ruegg and R. Stupp, *Targeted Oncol.*, 2010, **5**, 175–181 .

8 J.-P. Xiong, T. Stehle, R. Zhang, A. Joachimiak, M. Frech, S. L. Goodman and M. A. Arnaout, *Science*, 2002, **296**, 151–155.

9 (*a*) L. J. Kornberg, H. S. Earp, C. E. Turner, C. Prockop and L. Juliano, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 8392–8396; (*b*) S. Miyamoto, S. K. Akiyama and K. M. Yamada, *Science*, 1995, **267**, 883–885; (*c*) G. Maheshwari, G. Brown, D. A. Lauffenburger, A. Wells and L. J. Griffith, *J. Cell Science*, 2000, **113**, 1677–1686; (*d*) D. C. Worth and M. Parsons, *Int. J. Biochem. Cell Biol.*, 2008, **40**, 2397–2409.

10 L. Auzzas, F. Zanardi, L. Battistini, P. Burreddu, P. Carta, G. Rassu, C. Curti and G. Casiraghi, *Curr. Med. Chem.*, 2010, **17**, 1255–1299.

11 (*a*) F. Vögtle, G. Reichardt, N. Werner, and A. J. Rackstraw, *Dendrimer Chemistry -Concepts, Syntheses, Properties, Applications*, Wiley-VCH, Weinheim, 2009; (*b*) G. R. Newkome, and C. N. Moorefield, *Handbook of Dendrimers: Synthesis, Nanoscience and Applications*, Wiley-VCH, Weinheim, 2009; (*c*) D. A. Tomalia, *Prog. Polym. Sci.*, 2005, **30**, 294–324.

12 (*a*) O. Rolland, C.-O. Turrin, A.-M. Caminade and J.-P. Marjoral, *New J. Chem.*, 2009, **33**, 1809–1824; (*b*) U. Boas, J. B. Christensen, and P. M. H. Heegaard, *Dendrimers in Medicine and Biotechnology*, Royal Society of Chemistry, Cambridge, 2006; (*c*) B. Klajnert, and M. Bryszewska, *Dendrimers in Medicine*, Nova Science, UK, 2007; (*d*) I. J. Majoros, and J. R. Baker, *Dendrimer-Based Nanomedicine*, Pan Stanford Publishing,Singapore, 2008.

13 (*a*) S. Monaghan, D. Griffith-Johnson, I. Matthews and M. Bradley, *Arkivoc*, 2001, **2** Part 8; (*b*) R. Shukla, T. P. Thomas, J. Peters, A. Kotlyar, A. Myc and J. R. Baker, *Chem. Commun.*, 2005, 5739–5741; (*c*) H. Yang and W. J. Kao, *Int. J. Med.*, 2007, **2**, 89–99; (*d*) E. Hill, R. Shukla, S. S. Park and J. R. Baker, *Bioconjugate Chem.*, 2007, **18**, 1756–1762; (*e*) C. A. Boswell, P. K. Eck, C. A. S. Regino, M. Bernado, K. J. Wong, D. E. Milenic, P. L. Choyke and M. W. Brechbiel, *Mol. Pharmaceutics*, 2008, **5**, 527–539; (*f*) T. L. Kaneshiro and Z.-R. Lu, *Biomaterials*, 2009, **30**, 5660–5666; (*g*) C. L. Waite and C. M. Roth, *Bioconjugate Chem.*, 2009, **20**, 1908–1916; (*h*) Z. Li, P. Huang, X. Zhang, J. Lin, S. Yang, B. Liu, F. Gao, P. Xi, Q. Ren and D. Cui, *Mol. Pharmaceutics*, 2010, **7**, 94–104; (*i*) D. Q. McNerny, J. F. Kukowska-Latallo, D. G. Mullen, J. M. Wallace, A. M. Desai, R. Shukla, B. Huang, M. M. B. Holl and J. R. Baker, *Bioconjugate Chem.*, 2009, **20**, 1853–1859.

14 (*a*) I. Dijkgraaf, A. Y. Rijnders, A. Soede, A. C. Dechesne, G. W. van Esse, A. J. Brouwer, F. H. M. Corstens, O. C. Boerman, D. T. S. Rijkers and R. M. J. Liskamp, *Org. Biomol. Chem.*, 2007, **5**, 935–944; (*b*) C.-B. Yim, I. Dijkgraaf, R. Merkx, C. Versluis, A. Eek, G. E. Mulder, D. T. S. Rijkers, O. C. Boerman and R. M. J. Liskamp, *J. Med. Chem.*, 2010, **53**, 3944–3953.

15 (*a*) D. Boturyn, J. L. Coll, E. Garanger, M. C. Favrot and P. Dumy, *J. Am. Chem. Soc.*, 2004, **126**, 5730–5739; (*b*) E. Garanger, D. Boturyn, J. L. Coll, M. C. Favrot and P. Dumy, *Org. Biomol. Chem.*, 2006, **4**, 1958–1965; (*c*) Z.-H. Jin, V. Josserand, S. Foillard, D. Boturyn, P. Dumy, M.-C. Favrot and J.-L. Coll, *Mol. Cancer*, 2007, **6**, 41; (*d*) L. Sancey, E. Garanger, S. Foillard, G. Schoehn, A. Hurbin, C. Albiges-Rizo, D. Boturyn, C. Souchier, A. Grichine, P. Dumy and J.-L. Coll, *Mol. Ther.*, 2009, **17**, 837–843.

16 (*a*) B. Hu, D. Finsinger, K. Peter, Z. Guttenberg, M. Barmann, H. Kessler, A. Escherich, L. Moroder, J. Bohm, W. Baumeister, S. Sui and E. Sackmann, *Biochemistry*, 2000, **39**, 12284–12294; (*b*) V. Marchi-Artzner, B. Lorz, U. Hellerer, M. Kantlehner, H. Kessler and E. Sackmann, *Chem.–Eur. J.*, 2001, **7**, 1095–1101.

17 S. Cressman, I. Dobson, J. B. Lee, Y. Y. C. Tam and P. R. Cullis, *Bioconjugate Chem.*, 2009, **20**, 1404–1411.

18 V. Marchi-Artzner, B. Lorz, C. Gosse, L. Jullien, R. Merkel, H. Kessler and E. Sackmann, *Langmuir*, 2003, **19**, 835–841.

19 (*a*) M. Colin, R. P. Harbottle, A. Knight, M. Kornprobst, R. G. Cooper, A. D. Miller, G. Trugnan, J. Capeau, G. Coutelle and M. C. Brahimi-Horn, *Gene Ther.*, 1998, **5**, 1488–1498; (*b*) M. F. M. Mustapha, S. M. Grosse, L. Kudsiova, M. Elbs, E. A. Raiber, J. B. Wong, A. P. R. Brain, H. E. J. Armer, A. Warley, M. Keppler, T. Ng, M. J. Lawrence, S. L. Hart, H. C. Hailes and A. B. Tabor, *Bioconjugate Chem.*, 2009, **20**, 518–532.
20 A. P. C. A. Janssen, R. M. Schiffelers, T. L. M. ten Hagen, G. A. Koning, A. J. Schraa, R. J. Kok, G. Storm and G. Molema, *Int. J. Pharm.*, 2003, **254**, 55–58.
21 J. A. Zupancich, F. S. Bates and M. A. Hillmyer, *Biomacromolecules*, 2009, **10**, 1554–1563.
22 (*a*) Y.-B. Lim, E. Lee and M. Lee, *Angew. Chem., Int. Ed.*, 2007, **46**, 3475–3478; (*b*) Y.-B. Lim, O.-J. Kwon, E. Lee, P.-H. Kim, C.-O. Yun and M. Lee, *Org. Biomol. Chem.*, 2008, **6**, 1944–1948.
23 (*a*) M. O. Guler, L. Hsu, S. Soukasene, D. A. Harrington, J. F. Hulvat and S. I. Stupp, *Biomacromolecules*, 2006, **7**, 1855–1863; (*b*) Z. Huang, T. D. Sargeant, J. F. Hulvat, A. Mata, P. Bringas, C.-Y. Koh, S. I. Stupp and M. L. Snead, *J. Bone Miner. Res.*, 2008, **23**, 1995–2006; (*c*) M. Zhou, A. M. Smith, A. K. Das, N. W. Hodgson, R. F. Collins, R. V. Ulijn and J. E. Gough, *Biomaterials*, 2009, **30**, 2523–2530.
24 (*a*) B. S. Kim, D. J. Hong, J. Bae and M. Lee, *J. Am. Chem. Soc.*, 2005, **127**, 16333–16337; (*b*) Y. Lim and M. Lee, *Org. Biomol. Chem.*, 2007, **5**, 401–405; (*c*) M. K. Müller and L. Brunsveld, *Angew. Chem., Int. Ed.*, 2009, **48**, 2921–2924.
25 S. W. A. Reulen, P. Y. W. Dankers, P. H. H. Bomans, E. W. Meijer and M. Merkx, *J. Am. Chem. Soc.*, 2009, **131**, 7304–7312.
26 (*a*) P. P. Karmali and A. Chaudhuri, *Med. Res. Rev.*, 2007, **27**, 696–722; (*b*) S. Bhattacharya and A. Balaj, *Chem. Commun.*, 2009, 4632–4656; (*c*) N. M. Rao, *Chem. Phys. Lipids*, 2010, **163**, 245–252; (*d*) P. Posocco, S. Pricl, S. Jones, A. Barnard and D. K. Smith, *Chem. Sci.*, 2010, **1**, 393–404; (*e*) S. P. Jones, N. P. Gabrielson, C.-H. Wong, H.-F. Chow, D. W. Pack, P. Posocco, M. Fermeglia, S. Pricl and D. K. Smith, *Mol. Pharmaceutics*, 2011, **8**, 416–429.
27 (*a*) G. R. Newkome and X. Lin, *Macromolecules*, 1991, **24**, 1443–1444; (*b*) G. R. Newkome, X. Lin and C. D. Weis, *Tetrahedron: Asymmetry*, 1991, **2**, 957–960; (*c*) J. K. Young, G. R. Baker, G. R. Newkome, K. F. Morris and C. S. Johnson Jr., *Macromolecules*, 1994, **27**, 3464–3471; (*d*) C. M. Cordona, R. E. Gawley and C. Gawley, *J. Org. Chem.*, 2002, **67**, 1411–1413.
28 M. C. A. Stuart, J. C. van de Pas and J. B. F. N. Engberts, *J. Phys. Org. Chem.*, 2005, **18**, 929–934.
29 W. Wang, Q. Wu, M. Pasuelo, J. S. McMurray and C. Li, *Bioconjugate Chem.*, 2005, **16**, 729–734.
30 For original reports of **c**[**RGDfK**] see: R. Haubner, R. Gratias, B. Diefenbach, S. L. Goodman, A. Jonczyk and H. Kessler, *J. Am. Chem. Soc.*, 1996, **118**, 7461–7472.
31 D. M. Jameson and J. A. Ross, *Chem. Rev.*, 2010, **110**, 2685–2708.
32 X. Montet, M. Funovics, K. Montet-Abou, R. Weissleder and L. Josephson, *J. Med. Chem.*, 2006, **49**, 6087–6093.