# Highly efficient cell adhesion on beads functionalized with clustered peptide ligands†

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**Resin beads were functionalized with either clustered peptide ligands or individual peptide ligands at various ligand densities and then the beads were evaluated in a cell binding assay.**

Multivalent interactions are a ubiquitous strategy that has evolved in biology for a wide range of functions including signal transduction pathways triggering cell adhesion and morphology.[1] These multivalent interactions are more potent than the analogous monovalent interactions.[2] When designing ligands, the most effective strategy is to use chemical entities that display multiple copies of a recognition unit from a central scaffold.[3,4] A variety of scaffolds was exploited to construct multivalent ligands varying in size, shape and physical characteristics.[3,5] We reason that clustered ligands can improve the adhesion of cells to surfaces especially when the concentration of ligands on the surface is decreased. This strategy could be particularly attractive not only for designing surfaces for tissue engineering but also for selectively capturing cells from biological fluids. Previous efforts to study the benefit of multivalent ligands have used clustered RGD (Arg-Gly-Asp) peptides that specifically home to cancer tissue through the $\alpha_v\beta_3$ integrin receptor.[6,7] Herein we evaluated the effects on cancer cell adhesion of a surface functionalized with clustered or individual RGD peptide ligands. Additionally, a fundamental issue regarding the density of ligands on the surface necessary for cell adhesion was addressed.

Our approach basically consists in decorating resin beads with peptide ligands *via* a simple chemical method and in assessing the cell adhesion to the bead using microscopy. For this study, we selected a tetravalent RGD-containing peptide endowed with desirable biological properties (Fig. 1).[7,8] The latter is composed of a cyclic decapeptide scaffold that presents in a spatially controlled manner two independent functional domains: a clustered ligand domain for integrin recognition conferring cell targeting and a domain devoted to a supplementary function (cancer monitoring and/or drug delivery). To study the contribution of the RGD cluster to cell adhesion, we decided to prepare compounds **1** and **2** displaying respectively four and one recognition element by means of fully solid-phase syntheses (Scheme 1). Compound **3** bearing nonsense RβAD (Arg-βAla-Asp) peptides was used as a negative control compound for biological studies. We used the oxime bond formation to construct biomolecular assemblies since this chemoselective reaction has proved to be particularly efficient for preparing artificial protein,[9] chemical microarray,[10] and other bioconjugates.[11] Our chemical approach involves the incorporation of building blocks encompassing a protected aminooxy function within the peptide chain (see ESI†). The 1-ethoxyethylidene (Eei) group was chosen to protect the aminooxy function (dotted frame in Scheme 1) since we recently established that Eei is entirely compatible with the solid-phase peptide synthesis.[12]

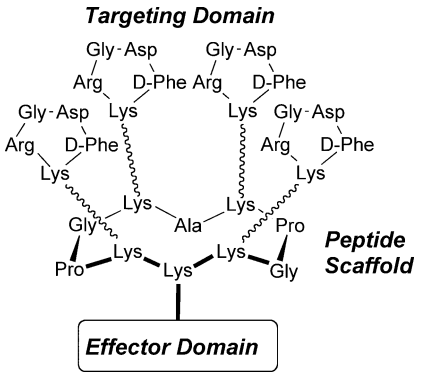


**Fig. 1** General structure of tetrameric RGD-containing peptide.

Briefly, compounds **1–3** were synthesized from PEG-containing resin beads suitable for use in cell adhesion assays. We prepared resins containing different levels of ligand loading (from 0.2 mmol/g to 0.2 pmol/g). This was easily accomplished by adjusting the amount of D-glutamic acid on the resin (see ESI†). The latter was essential to ensure the β-turn directed head-to-tail cyclization within the cyclodecapeptide. To conduct a comparison of resin beads functionalized with compounds **1–3**, it was mandatory to begin the synthesis *via* a common tetrapeptide intermediate **4** (Scheme 1). Afterwards, the one-pot aminooxy deprotection and the chemoselective ligation of the RGD units were carried out under the mild acid conditions that are essential to prevent peptide release from the resin. Compounds **1–3** were finally analyzed and characterized from resin bead samples by means of RP-HPLC and mass spectroscopy (see ESI†).

The ability of resin beads to selectively bind cells through RGD–$\alpha_v\beta_3$ integrin recognition was next evaluated using human embryonic kidney cells HEK 293(β3) (high level of $\alpha_v\beta_3$ integrins) and HEK 293(β1) ($\alpha_v\beta_3$ negative but expressing $\alpha_v\beta_1$).[13] We first examined beads displaying a high density of ligand (0.2 mmol/$g_{resin}$). Cells were incubated with resin beads for 30 minutes at 37 °C. As expected, compound **3** did not lead to cell adhesion whereas

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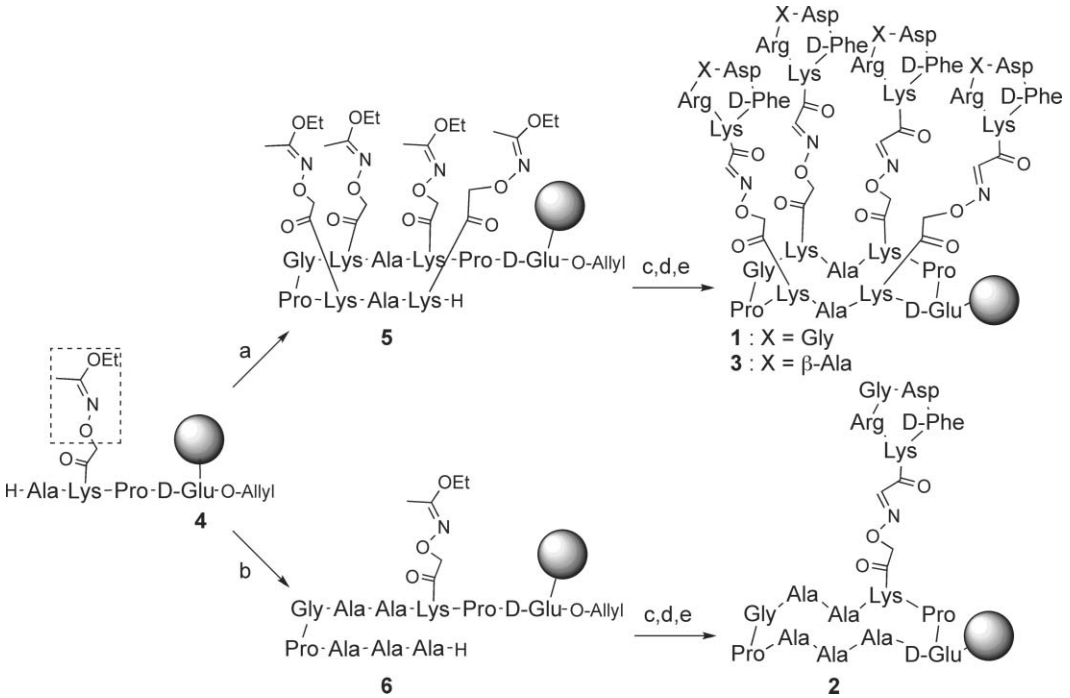
† Electronic supplementary information (ESI) available: Details of the solid phase peptide synthesis, syntheses and spectroscopic characterisation of compounds **1–3**, cell adhesion procedure, optical images of cell adhesion on beads (ligand loading from 0.2 mmol/g to 0.2 pmol/g), and vinculin staining. See DOI: 10.1039/b911440h




**Scheme 1** Solid-phase syntheses of **1–3**. (a–b) standard Fmoc/*t*-Bu solid phase peptide synthesis; (c) $Pd^0(PPh_3)_4$, $PhSiH_3$, $CH_2CH_2$/DMF; (d) PyAOP, DIPEA, DMF; (e) 0.4 mmol/$g_{resin}$ c[-Arg-X-Asp-D Phe-Lys(-CO-CHO)-], $CH_2Cl_2$/TFA/$H_2O$ (94/3/3). Fmoc = 9-fluorenylmethoxy–carbonyl, DMF = *N,N*-dimethylformamide, PyAOP = (7-azabenzo-triazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, DIPEA = diisopropylethylamine, TFA = trifluoroacetic acid.

beads exhibiting compounds **1** and **2** bound strongly positive HEK 293(β3) cells (Fig. 2a and b, see also ESI†). This result indicates that the clustered ligand architecture does not make an important contribution to ligand–integrin interactions. The multivalency effect essentially comes from the high level of resin substitution. Surprisingly, the non specific adhesion of HEK 293(β1) was slightly observed on beads carrying **1** and **2** (Fig. 2c and d) signifying that the density of ligand on the surface is crucial for preserving the selectivity for the target.

To discriminate the multivalency effect of clustered RGD **1** from that of the resin bead, we next assessed the cell adhesion under stringent conditions using beads containing very low densities of RGD ligands **1** and **2** (2 nmol/$g_{resin}$). In this context, only beads functionalized with nanoscale clustered RGD peptides **1** generated the adhesion of $\alpha_v\beta_3$-expressing HEK cells (Fig. 2e), while few HEK 293(β3) cells were attached *via* the RGD ligand **2** (Fig. 2f). This trend was also observed in the presence of beads displaying RGD ligands **1** and **2** at intermediate ligand densities (2–20 μmol/$g_{resin}$): we observed an enhanced cell adhesion using **1** until the limit of 2 pmol/$g_{resin}$ where we did not detect any cells (see ESI†). We confirmed our previous finding that $\alpha_v\beta_3$ integrin–ligand interactions can be improved through the clustered ligand domain of the cyclodecapeptide scaffold.[7,14,15] Additionally, beads displaying ligand **1** did not induce HEK 293(β1) cell adhesion when using low resin loading (see Fig. S29 and S30 in the ESI†). This result indicates that control of the ligand density on the surface may be required to ensure the cellular selectivity.

Integrin expression level was determined to be approximately $10^5$ receptors/cell, yielding an integrin density of 100 receptors/μm$^2$ for spread cells.[16] Since the majority of ligands in the interior of the resin bead (~90%) is not reached by cells,[17] we estimate the cell accessibility to be nearly 40–80 ligands/μm$^3$ at the lower level of resin substitution (2 nmol/$g_{resin}$).[18] These data suggest that the minimum density of ligand **1** required for cell adhesion matches with the amount of accessible integrin receptors. For instance, approximately 50–100 fibronectin natural ligands/μm$^2$ are necessary for cell adhesion,[19] while more than 200–5000 molecules/μm$^2$ are required for the linear peptide GRGDSPK.[20]

Finally, it was interesting to exploit the resin beads for selectively capturing $\alpha_v\beta_3$-expressing cells from a biological mixture since most tumour metastases express the $\alpha_v\beta_3$ integrin. Methodologies for selective tumour cell capture from biological samples are of significant importance for cancer monitoring. Their major barriers to use arise from limited capture efficiency and lack of standardization. We therefore applied the resin bead **1** (20 μmol/$g_{resin}$) to capture HEK 293(β3) cells from a mixed cell suspension containing 3LL cells ($\alpha_v\beta_3$ negative Lewis lung carcinoma). As expected, some HEK 293(β3) cells were trapped onto the beads (Fig. 3a). The cells that did not adhere to the beads were readily removed (see ESI†) and the beads subsequently incubated in cell culture medium for 96 hours at 37 °C. As shown in Fig. 3b (see also Fig. S34–S37 in the ESI†), the resin beads were massively coated with cells, signifying a high rate of cell division on beads displaying ligand **1**. Interestingly, the latter mediates morphological differentiation with a highly extended and flattened cellular morphology (see Fig. S38 and S39 in the ESI†) suggesting that an intracellular signalling pathway is activated by ligand **1**.

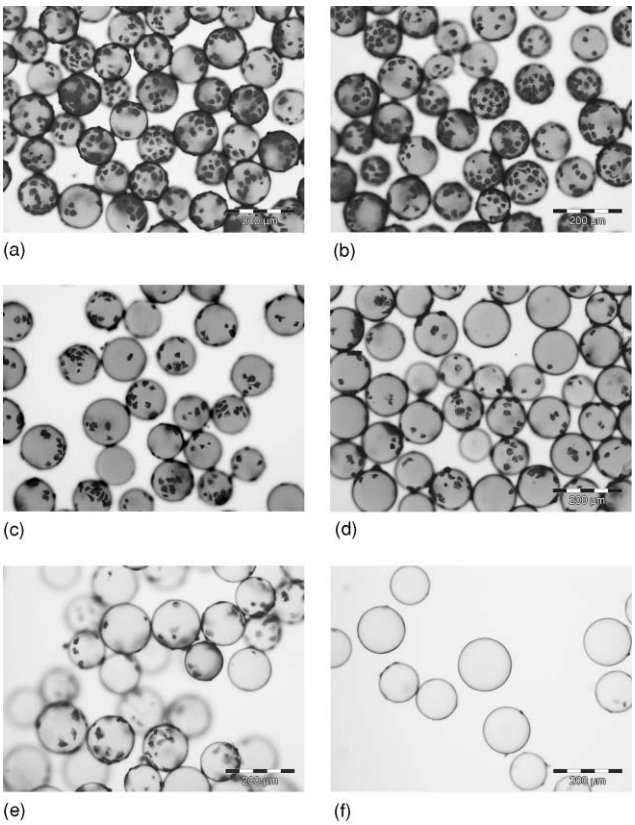



**Fig. 2** Optical microscopy images of cell adhesion to 120 µm resin beads. Cells (1 million) were treated with beads for 30 min at 37 °C then fixed and stained with methylene blue. HEK 293 (β3) were incubated with (a) **1** (0.2 mmol/$g_{resin}$), (b) **2** (0.2 mmol/$g_{resin}$), (e) **1** (0.2 nmol/$g_{resin}$), (f) **2** (0.2 nmol/$g_{resin}$). HEK 293 (β1) were incubated with (c) **1** (0.2 mmol/$g_{resin}$), (d) **2** (0.2 mmol/$g_{resin}$).

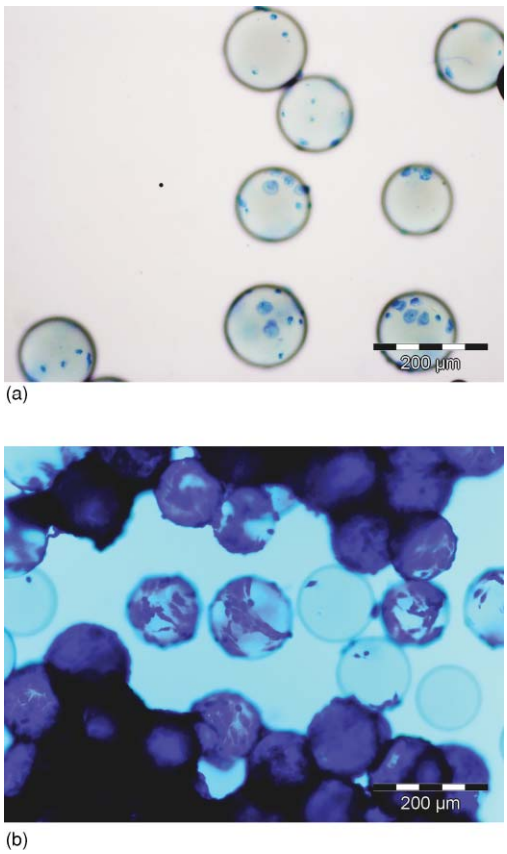



**Fig. 3** Optical microscopy images of cell adhesion to 120 µm resin beads. A mixed HEK 293(β3)/3LL cell suspension (1/9; 1 million) was treated with beads **1** (20 µmol/$g_{resin}$) for 30 min at 37 °C, (a) then fixed and stained with methylene blue; (b) beads were incubated in cell culture medium for 96 hours at 37 °C in a humidified 5% $CO_2$ atmosphere, then fixed and stained with methylene blue.

These results indicate that beads displaying a nanoscale distribution of compound **1** mimic an extracellular matrix protein such as vitronectin, the natural ligand of the $\alpha_v\beta_3$ integrin. They extend our previous observations, obtained with multivalent RGD peptides (Fig. 1),[7,8,13,14] that the clustered architecture improves integrin binding. We hypothesize that the observed multivalent effect arises from a statistical rebinding of ligand **1** due to the high local concentration of RGD elements.

In addition, our approach is not limited to $\alpha_v\beta_3$ integrin-expressing cells: by adapting the corresponding domain on the scaffold (*i.e.* intermediate **5** in Scheme 1), resin beads may be easily exploited to adhere to various cells due to the increasing number of selective ligands selected *in vivo*. At the present time, we are extending our study to selectively target CD20-expressing cells such as lymphoma.

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## Notes and references

1 M. Mammen, S. K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2754.
2 T. K. Dam and C. F. Brewer, *Biochemistry*, 2008, **47**, 8470.
3 (*a*) L. L. Kiessling, J. E. Gestwicki and L. E. Strong, *Angew. Chem., Int. Ed.*, 2006, **45**, 2348; (*b*) R. Haag and F. Kratz, *Angew. Chem., Int. Ed.*, 2006, **45**, 1198.
4 For elegant examples, see: (*a*) P. I. Kitov, G. L. Mulvey, T. P. Griener, T. Lipinski, D. Solomon, E. Paszkiewicz, J. M. Jacobson, J. M. Sadowska, M. Suzuki, K. Yamamura, G. D. Armstrong and D. R. Bundle, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 16837; (*b*) V. M. Hernández-Rocamora, B. Maestro, B. de Waal, M. Morales, P. Garcia, E. W. Meijer, M. Merkx and J. M. Sanz, *Angew. Chem., Int. Ed.*, 2009, **48**, 948.
5 C. W. Cairo, *ACS Chem. Biol.*, 2007, **2**, 652.
6 (*a*) H. D. Maynard, S. Y. Okada and R. H. Grubbs, *J. Am. Chem. Soc.*, 2001, **123**, 1275; (*b*) R. J. Kok, A. J. Schraa, E. J. Bos, H. E. Moorlag, S. A. Ásgeirsdóttir, M. Everts, D. K. F. Meijer and G. Molema, *Bioconjugate Chem.*, 2002, **13**, 128; (*c*) G. Thumshirn, U. Hersel, S. L. Goodman and H. Kessler, *Chem.–Eur. J.*, 2003, **9**, 2717.
7 D. Boturyn, J.-L. Coll, E. Garanger, M.-C. Favrot and P. Dumy, *J. Am. Chem. Soc.*, 2004, **126**, 5730.
8 (*a*) J. Razkin, V. Josserand, D. Boturyn, Z. Jin, P. Dumy, M. Favrot, J.-L. Coll and I. Texier, *ChemMedChem*, 2006, **1**, 1069; (*b*) S. Foillard, Z. Jin, E. Garanger, D. Boturyn, M.-C. Favrot, J.-L. Coll and P. Dumy,

*ChemBioChem*, 2008, **9**, 2326; (*c*) S. Foillard, L. Sancey, J.-L. Coll, D. Boturyn and P. Dumy, *Org. Biomol. Chem.*, 2009, **7**, 221.
9 K. Rose, *J. Am. Chem. Soc.*, 1994, **116**, 30.
10 J. R. Falsey, M. Renil, S. Park, S. Li and K. S. Lam, *Bioconjugate Chem.*, 2001, **12**, 346.
11 (*a*) X. Chen, G. S. Lee, A. Zettl and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2004, **43**, 6112; (*b*) G.-A. Cremer, N. Bureaud, D. Lelièvre, V. Piller, F. Piller and A. Delmas, *Chem.–Eur. J.*, 2004, **10**, 6353; (*c*) A. Dirksen and P. E. Dawson, *Bioconjugate Chem.*, 2008, **19**, 2543; (*d*) D. Forget, D. Boturyn, E. Defrancq, J. Lhomme and P. Dumy, *Chem.–Eur. J.*, 2001, **7**, 3976; (*e*) S. Grigalevicius, S. Chierici, O. Renaudet, R. Lo-Man, E. Deriaud, C. Leclerc and P. Dumy, *Bioconjugate Chem.*, 2005, **16**, 1149.
12 S. Foillard, M. Ohsten Rasmussen, J. Razkin, D. Boturyn and P. Dumy, *J. Org. Chem.*, 2008, **73**, 983.
13 E. Garanger, D. Boturyn, Z. Jin, P. Dumy, M.-C. Favrot and J.-L. Coll, *Mol. Ther.*, 2005, **12**, 1168.
14 E. Garanger, D. Boturyn, J.-L. Coll, M.-C. Favrot and P. Dumy, *Org. Biomol. Chem.*, 2006, **4**, 1958.
15 We measured the constant of affinity of analogous compounds of **1** and **2** for $\alpha_v\beta_3$ integrins: KD = 4 nM and 42 nM respectively. 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.
16 S. K. Akiyama and K. M. Yamada, *J. Biol. Chem.*, 1985, **260**, 4492.
17 J. Vágner, G. Barany, K. S. Lam, V. Krchňák, N. F. Sepetov, J. A. Ostrem, P. Strop and M. Lebl, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 8194.
18 To calculate the ligand density within the resin bead, we considered that beads are perfect spheres with a uniform distribution of functional sites. The size of beads varies between 100 and 120 mm, and 1 g of resin contains 2.9 × 106 beads (information provided by the furnisher). We then estimated the density of ligands to be 600 ± 200 molecules/mm$^3$ within a 0.2 nmol/g-bead.
19 A. R. Asthagiri, C. M. Nelson, A. F. Horwitz and D. A. Lauffenburger, *J. Biol. Chem.*, 1999, **274**, 27119.
20 L. Y. Koo, D. J. Irvine, A. M. Mayes, D. A. Lauffenburger and L. G. Griffith, *J. Cell Sci.*, 2002, **115**, 1423.

 