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A hydrazide-anchored dendron scaffold for chemoselective ligation strategies†

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Chemoselective ligation, including "click" chemistry, has found wide utility in general synthetic strategies and the specific modification of polymers and biomolecules. This has resulted in a number of applications of such approaches, particularly in the biomedical area, including diagnostic imaging and drug delivery. However, tools to chemoselectively decorate target molecules with multiple copies of a particular drug, ligand or label are lacking. We describe the design and synthesis of a hydrazide-anchored dendron scaffold for chemoselective ligation to carbonyl moieties, and demonstrate its use in the modification of aldehyde-rich surfaces with the RGD integrin-binding ligand.

Introduction

The chemical modification of polymers and biological molecules, including those in whole cells and even tissues, has a wide range of applications, from the generation of molecular tools for basic research to clinical diagnostics and therapy. Early techniques for the modification of biomaterials and biomolecules often relied on the utilization of accessible carboxylic acids to generate activated (usually *N*-hydroxysuccinimide (NHS)) esters, followed by reaction with a species containing a free amine. Alternatively, peptides and proteins can be modified *via* lysine and cysteine residues by reaction with NHS esters or maleimides, respectively. However, because biological molecules are generally rich in these functional groups, non-specific, off-target modification can be a significant issue. To overcome this and ensure selective chemical modification of biomolecules, chemoselective ligation strategies have been developed that employ mutually reactive functional groups that are not normally found within biological systems. Ideally, these reactions occur under conditions that are compatible with biomolecules and living cells, *i.e.* in aqueous media under physiological conditions. To date, there are a number of such bioorthogonal ligation reactions that are commonly used not only to modify biological molecules, but which are also employed in a wide range of chemical syntheses.

Chemoselective ligations include, but are not limited to, those between an alkyne and an azide (now commonly referred to as "click" chemistry),[1–3] the Staudinger ligation between a triarylphosphine and an azide,[4,5] and reactions between carbonyl groups and hydrazides, thiosemicarbazides or aminooxy moieties.[6–8] To employ these ligations for the selective labelling or modification of biological molecules, the biomolecule itself must, of course, possess a suitable bioorthogonal functional group. These can be introduced using standard synthetic strategies or, in the case of living cells, *via* approaches such as the selective periodate oxidation of carbohydrates to yield aldehydes,[9,10] or by metabolic engineering. In the latter approach, the permissiveness of certain metabolic enzymes to structural variations in their substrates has been used to decorate proteins and cell surface glycans, including those within living animals, with a variety of bioorthogonal functional groups.[6,11–16] Chemoselective strategies have, to date, been used for a wide range of applications, including antibody labelling,[14,17] drug delivery strategies,[18,19] nanoparticle functionalization,[20,21] protein immobilization,[22–24] hydrogel formation,[23,25–27] modification of viruses to improve tissue targeting,[28] and the generation of three-dimensional cell aggregates.[10,29–31]

Dendrons and dendrimers have been widely studied in recent decades[32] for their potential application in a number of areas, including drug and gene delivery and clinical diagnostics,[33–36] with their multivalent nature being a key characteristic. While these macromolecules have been assembled using chemoselective ligations[37,38] and have been decorated with bioorthogonal functional groups,[29,39] there is now interest in strategies that enable "dendronization"[40,41] of a target molecule *via* chemoselective decoration with multiple functional moieties. To date, various targets such as polymers,[42–44] proteins,[45] magnetic nanoparticles,[46] carbon nanotubes[47] and nucleotides[48] have been chemoselectively dendronized, but these strategies have been limited to the use of click chemistry.

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The pairing of a carbonyl group with a hydrazide, thiosemicarbazide or aminooxy moiety is perhaps the most versatile chemoselective ligation strategy in terms of their chemical or metabolic installation into co-reacting species and ability to readily ligate in aqueous media. The resultant hydrazone bond is relatively stable, but ultimately undergoes reversible hydrolysis in aqueous environments.[49,50] With these characteristics in mind, we describe the design and development of, to our knowledge, the first dendritic scaffold containing a bioorthogonal hydrazide anchor for the dendronization of carbonyl-containing molecules. Such a hydrazide-anchored dendron scaffold could enable, for example, decoration of biomaterials with multiple biological cues for tissue engineering and regenerative medicine strategies, and enhanced antibody, polymer or nanoparticle labelling for diagnostic, imaging and drug delivery applications.

Results and discussion

For wide utility, we envisaged the synthesis of hydrazide-anchored scaffolds with terminal succinimide esters, enabling one-step functionalization with, primarily, peptides, *via* a primary amine (Fig. 1). As such, it was necessary to construct scaffolds with a protected hydrazide in order to eliminate any possibility of reaction with the activated esters. We also considered the possibility of peptide side chains cross-reacting with the esters, and adopted a general strategy that could be employed for any peptide that contains potentially problematic residues, such as lysine. Using solid phase peptide synthesis followed by cleavage conditions that retain side chain protection, selective reaction of the N-terminus with the dendron scaffold could be realized, following by deprotection of both the side groups and hydrazide anchor (Fig. 1).

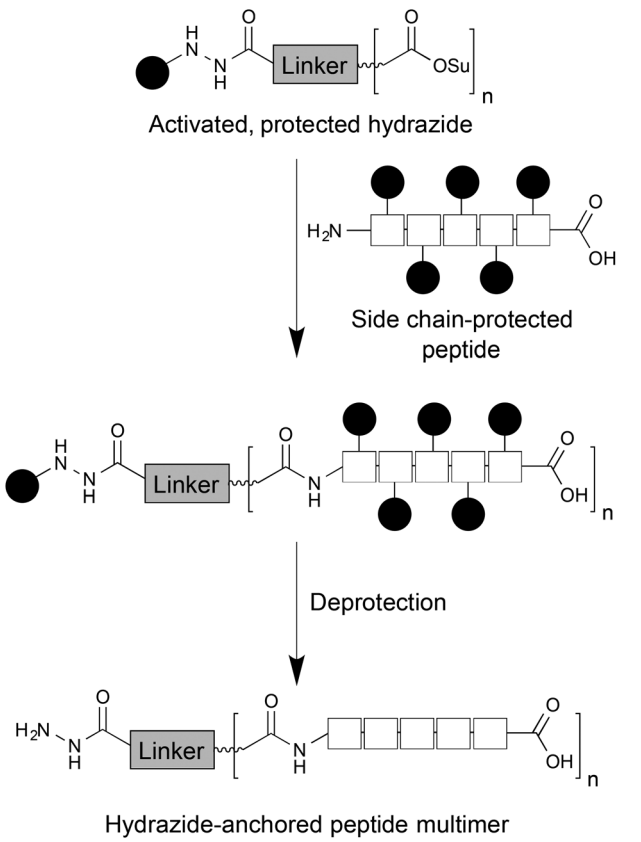
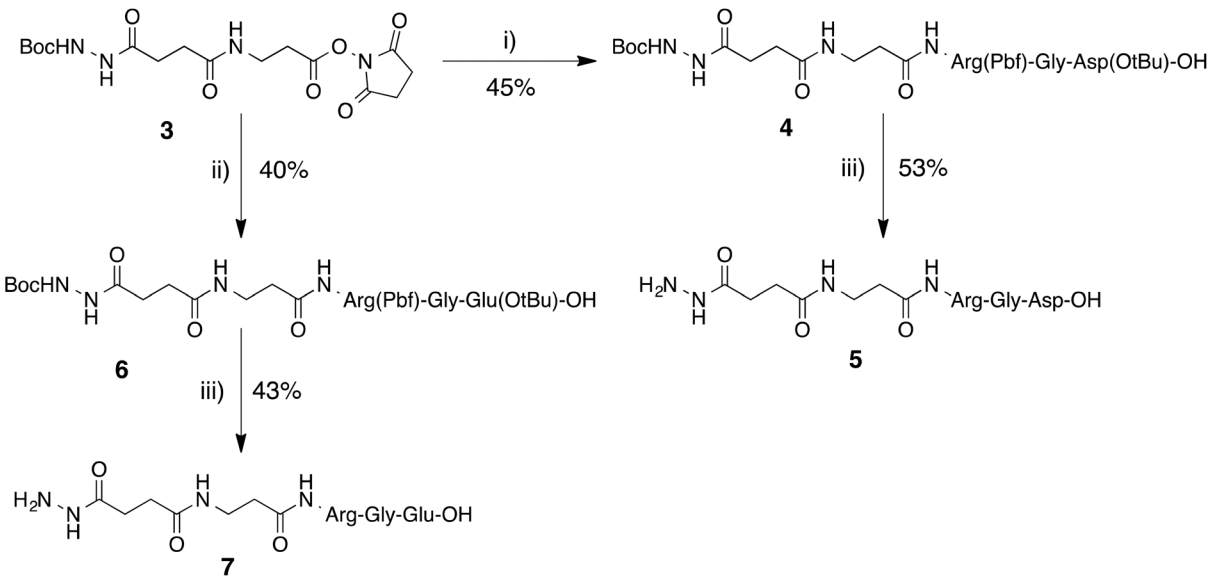


Fig. 1 Overview of the strategy for synthesis of a hydrazide-anchored dendron scaffold, allowing multimeric peptide display.

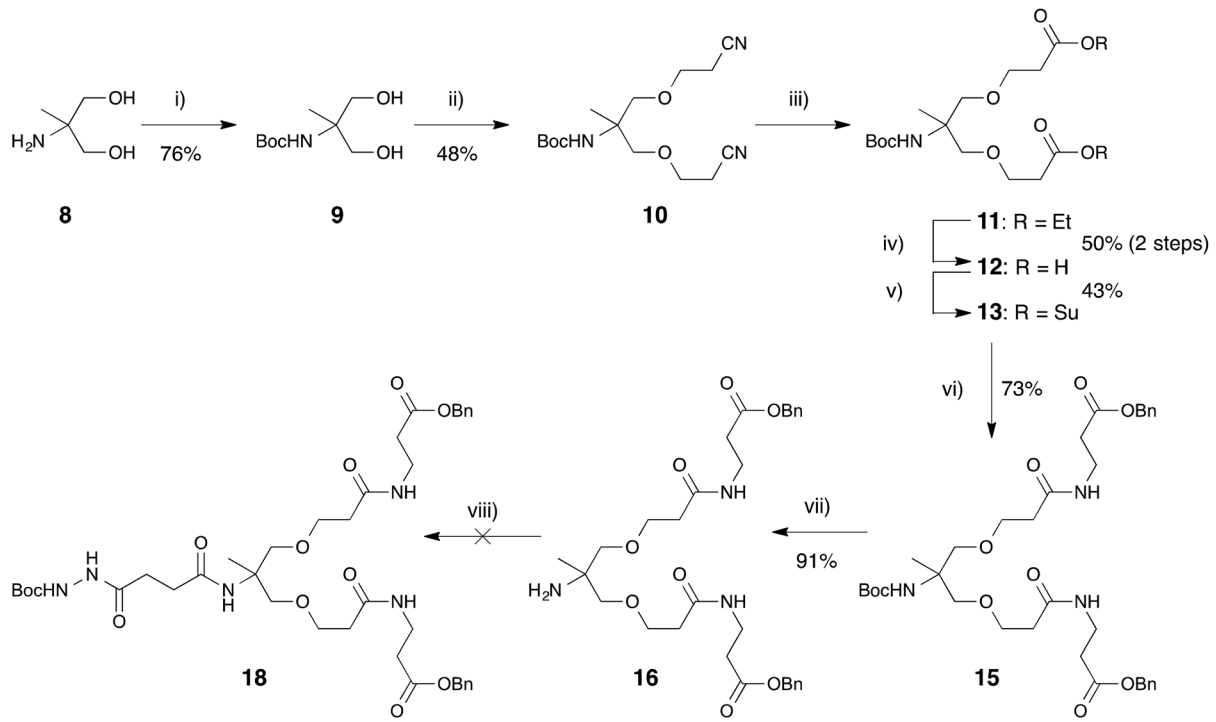
As our interests lie primarily in tissue engineering and regenerative medicine, we chose to demonstrate these scaffolds using the RGD tripeptide. This motif is found in a number of extracellular matrix proteins and is important in cell–matrix adhesion *via* binding to integrin receptors.[51] As a result, RGD and its analogues have been widely studied for improving cell adhesion to poorly adhesive materials.[52] In addition, integrin receptors are overexpressed in some cancers, so there has been extensive interest in this tripeptide for the targeting of drugs or imaging agents to tumours.[53,54] For monomeric peptide hydrazides, partially protected RGD (H-Arg(Pbf)-Gly-Asp(O*t*Bu)-OH **1**), was synthesised by manual Fmoc solid phase peptide synthesis on 2-chlorotrityl resin, followed by cleavage with acetic acid and trifluoroethanol. As a negative control, the partially protected version of the non-adhesive RGE tripeptide (H-Arg(Pbf)-Gly-Glu(O*t*Bu)-OH **2**) was synthesised in an analogous manner. These peptides were reacted with succinimide ester **3** to yield the corresponding protected hydrazides **4** and **6** in yields of 45 and 40%, respectively (Scheme 1). Removal of the remaining, acid-labile protecting groups proceeded smoothly *via* treatment with TFA and triisopropylsilane. However, it was not possible to isolate the resulting peptide hydrazides **5** and **7** using the conventional method of ether precipitation. Instead, excess TFA was removed under vacuum, the crude residue redissolved in 10% acetic acid and extracted with chloroform. Lyophilisation of the aqueous phase yielded the target monosubstituted compounds as crystalline white solids in reasonable yields.

To construct the dimeric scaffold, we chose the commercially available 2-amino-2-methylpropane-1,3-diol **8** as the starting material, enabling the hydrazide functionality to be incorporated *via* the amine group and the arms of the dendron *via* extension of the diol (Scheme 2). The amine was initially Boc protected in high yield to furnish the literature compound **9**,[55,56] which was subsequently converted to the corresponding dinitrile **10** following reaction with acrylonitrile.[57] Attempts to hydrolyse **10** directly to diacid **12** proved unsuccessful, so the dinitrile was converted to diester **11**, which furnished diacid **12** following hydrolysis with 5 M NaOH at 50 °C.

For eventual insertion of the terminal hydrazide functionality, it was necessary at this point to protect the diacid with a suitable protecting group. With this in mind, β-alanine **14**,[58,59] protected as a benzyl ester, was introduced on each arm, following activation of **12** as the corresponding disuccinimide ester **13**, with the effect of adding a spacer group in addition to a masked carboxylic acid to each arm of protected product **15**. To introduce the hydrazide anchor, the Boc group was then cleaved from **15** to expose the free amine of compound **16** for



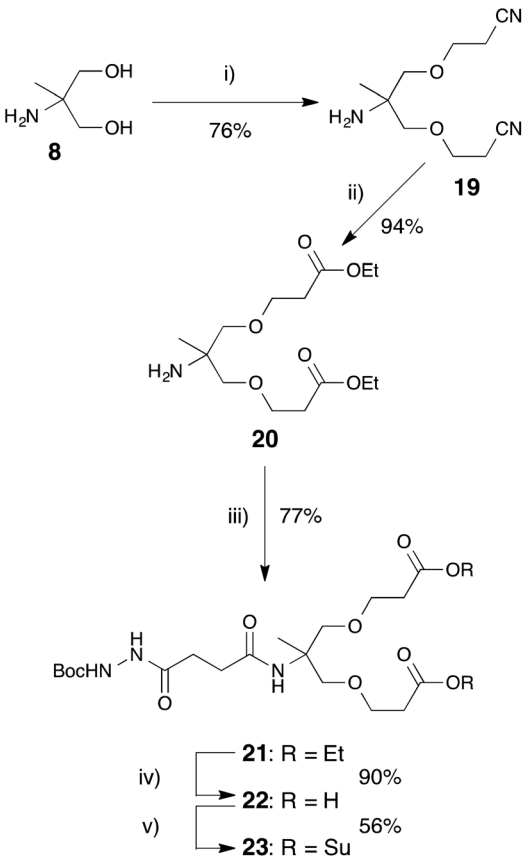
Scheme 1 Synthesis of hydrazide-terminated monomers **5** and **7**. (i) H-Arg(Pbf)-Gly-Asp(O*t*Bu)-OH (**1**), DMAP, DMF, rt; (ii) H-Arg(Pbf)-Gly-Glu(O*t*Bu)-OH (**2**), DMAP, DMF, rt; (iii) TFA–TIS–H_2O, rt.



Scheme 2 (i) Boc anhydride, NEt_3, CH_2Cl_2; (ii) acrylonitrile, KOH, MeCN, rt; (iii) (a) HCl, reflux, (b) EtOH, reflux; (iv) 5 M NaOH, THF, EtOH, 50 °C; (v) NHS, DIC, DMAP, CH_2Cl_2, rt; (vi) 3-(benzyloxy)-3-oxopropan-1-aminium 4-methylbenzene-1-sulfonate (**14**), pyridine, CH_2Cl_2, rt; (vii) TFA, CH_2Cl_2, rt; (viii) BocNHNHCO$(CH_2)_2CO_2H$ (**17**), various conditions.

coupling with Boc-protected hydrazide **17**.[60] However, despite investigating a number of different, standard coupling conditions, the reaction between compounds **16** and **17** did not proceed as expected, possibly due to steric hindrance of the primary amine by the mobile arms of dimer **16**. As a result, it was decided to introduce the hydrazide terminus earlier in the synthesis.

This modified approach (Scheme 3) also utilised diol **8** as the starting material but, rather than employing Boc protection at the start of the synthesis, the amine group was left unmodified for earlier introduction of the hydrazide. Analogous to Scheme 2, diol **8** was treated with acrylonitrile to yield dinitrile **19** in good yield of 76% following purification *via* column chromatography. Conversion of **19** to the corresponding



Scheme 3 Synthesis of hydrazide-terminated dimer scaffold. (i) Acrylonitrile, 1,4-dioxane, KOH, rt; (ii) HCl, EtOH, reflux; (iii) BocNHNHCO-$(CH_2)_2CO_2H$ (**17**), DIC, HOBt, DMF, rt; (iv) NaOH, EtOH, THF, 50 °C; (v) *N*-hydroxysuccinimide, DIC, CH_2Cl_2, rt.

diester **20** proceeded in almost quantitative yield of 94%. In order to introduce the terminal hydrazide, diester **20** was treated with acid **17**[60] in the presence of a variety of coupling agents (DIC, EDC, HATU, PyBOP), all of which proved unsuccessful. However, when two activating agents (DIC and HOBt) were employed, the reaction proceeded smoothly to furnish compound **21** in good yield. Base hydrolysis of diester **21** led to isolation of the desired diacid **22**. As a result of the earlier introduction of the protected hydrazide, further orthogonal protection of the acid dimer was not necessary and conversion to disuccinimide ester **23** yielded our target, activated dimer, capable of further functionalization with suitable nucleophilic species.

To investigate the application of this dimeric scaffold in chemoselective modification of biomaterials, activated ester **23** was treated with partially protected tripeptides RGD **1** or RGE **2** in DMF. Following reaction for 72 hours at room temperature, the corresponding protected, hydrazide-terminated peptide dimers **24** and **26** were isolated in yields of 11% and 21%, respectively. These low yields can be largely attributed to poor chromatographic separation of the starting materials and products. However, following treatment of **24** and **26** with TFA and TIS, the target deprotected hydrazide dimers **25** and **27**

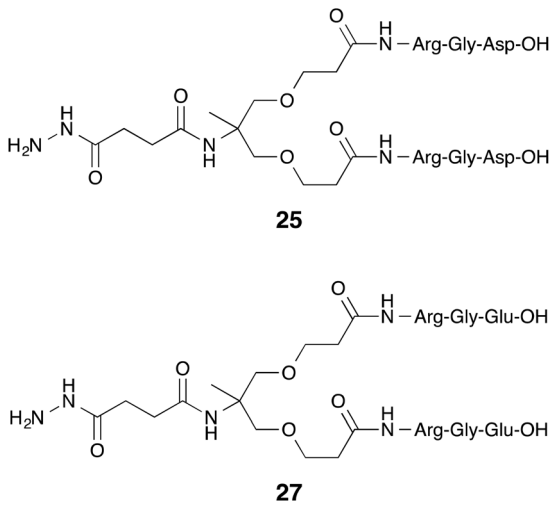


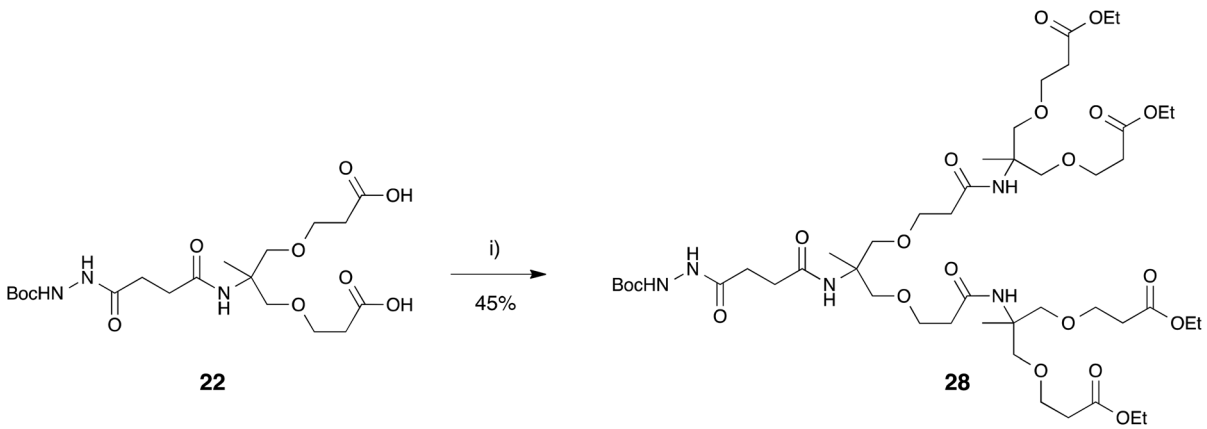
Fig. 2 Structures of target peptide hydrazide dimers **25** and **27**.

(2 × RGD hydrazide and 2 × RGE hydrazide; Fig. 2) were isolated in good yield as described for their monomer analogues.

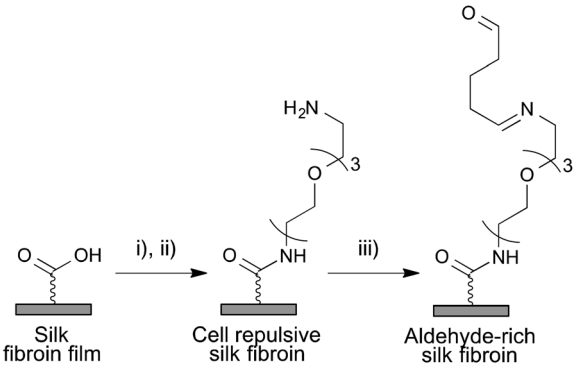
We also wished to investigate whether the methodology we developed could be extended to more complicated dendrimeric species. Hence, we set about preparing a tetramer analogue of ester **21** (Scheme 4). Diacid **22** was treated with EDC and NHS to generate activated ester **23** *in situ*. Reaction with amine **20** under ambient conditions led to target compound **28**, which, despite the very similar R_f values of product and starting material, was isolated in reasonable yield of 45% following flash column chromatography. These results suggest that this approach to hydrazide-anchored dendrons has the potential for expansion of the number of branches, enabling the chemoselective decoration of suitable target molecules with multiple bioactive moieties.

In order to evaluate the ability of our RGD terminated hydrazides to control cell adhesion to a surface in a chemoselective manner, it was necessary to prepare an aldehyde-rich substrate, which would repel cell adhesion until the introduction of the hydrazide-anchored RGD tripeptides. Films of silk fibroin protein, an extensively studied biomaterial,[61] from *Bombyx mori* silk cocoons were utilized for this purpose. The available carboxylic acid groups present on the surface of the silk fibroin film were activated using carbodiimide chemistry and subsequently reacted with aminated tetraethylene glycol **29**[62,63] to yield a cell-repulsive surface (Scheme 5).[63–65] Aldehyde functionality was then incorporated *via* reaction of these surfaces with glutaraldehyde. The presence of aldehyde groups on these modified silk films was confirmed by their chemoselective reaction with fluorescein thiosemicarbazide (FTSC) at pH 5.5. Only films that had been treated with glutaraldehyde were found to fluoresce following subsequent incubation with FTSC (Fig. S1†).

Following the confirmation of the presence of aldehydes on the silk films, we then investigated the monomeric and dimeric peptide hydrazides for their ability to chemoselectively



Scheme 4 Synthesis of tetramer ester **28**. (i) **20**, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide, *N*-hydroxysuccinimide, DMF, rt.



Scheme 5 Chemical modification of *Bombyx mori* silk fibroin films (i) EDC, NHS, PBS, rt; (ii) 50 μM $NH_2(CH_2CH_2O)_3CH_2CH_2NH_2$ **29**, MeOH–H_2O (1 : 1 v/v), rt; (iii) 0.01 M glutaraldehyde, PBS, rt.

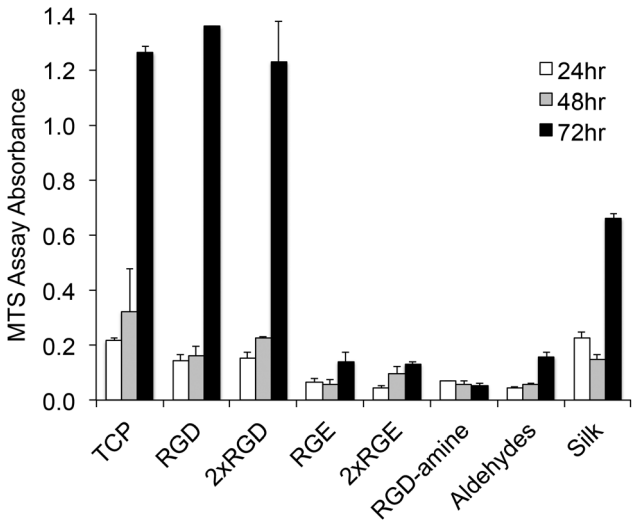


Fig. 3 Relative cell number over time following seeding of C2C12 myoblasts on functionalized silk fibroin surfaces in comparison to tissue culture plastic (TCP). Data represent the mean ± standard error of MTS absorbance values (n = 3).

reintroduce cell-adhesivity to these non-adhesive surfaces. Modified films were incubated with both the RGD and RGE peptide hydrazides for 3 hours at 37 °C (pH 5.5), alongside commercially available RGD as an amine-terminated control. C2C12 myoblasts, as a model cell type, were then seeded on the films and cultured for a period of 72 hours. At 24 hour intervals, relative cell number on the surfaces was assessed using the MTS assay, which detects metabolically active cells (see ESI† for detailed description).

Comparison of the various modified surfaces to tissue culture plastic (TCP) revealed that cells grew selectively on surfaces treated with hydrazides containing RGD peptides, with little or no growth on RGE-containing surfaces, confirming the cell adhesivity of RGD in comparison to RGE (Fig. 3). The chemoselectivity of these hydrazide-anchored peptides towards aldehydes was demonstrated by the lack of significant cell growth on surfaces incubated with native RGD, *i.e.* the primary amine did not react with the aldehyde-rich surface under physiological conditions. While cells exhibited a reasonable degree of proliferation on unmodified silk, the extent of cell growth at 72 h was significantly less than the growth on TCP, RGD hydrazide and 2 × RGD hydrazide, while the introduction of aldehyde functionality all but eliminated proliferation (Fig. 3). Interestingly, there was no significant difference between cell growth on hydrazide-anchored RGD and 2 × RGD surfaces, despite the increased number of RGD motifs present in the latter. RGD clustering and spacing has been shown to be involved in cell adhesion and spreading.[66,67] In this case, the similarity in cell growth on RGD and 2 × RGD surfaces could simply be due to there being sufficient RGD ligands on the monomeric surfaces meaning that doubling the number of ligands had no effect. Alternatively, on the 2 × RGD surfaces, the peptides may have been too close to be distinguished by cell surface integrins and could require additional spacer groups to be added to the scaffold. Nonetheless, both of these species were successfully and selectively ligated to aldehyde-rich surfaces and elicited the expected cellular response confirming the utility of this approach.

Conclusions

In summary, we have designed and developed a novel, hydrazide-anchored dendron scaffold for the chemoselective functionalization of carbonyl-containing molecules. The terminal NHS ester groups enable this scaffold to be readily decorated with a wide range of biomedically useful molecules. In particular, we have demonstrated that peptides can be coupled to this scaffold following solid phase synthesis and mild cleavage from the resin, leaving side chain protecting groups intact and, thus, preventing non-specific reactions. This approach has potential applications in a number of synthetic and biomedical areas where chemoselective decoration of a target molecule or cell with an increased density of drugs, ligands or labels is required.

Acknowledgements

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