Biomaterials

# Improving Implant Materials by Coating with Nonpeptidic, Highly Specific Integrin Ligands**

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Surface modification for enhanced cell adhesion to improve the properties of the critical interphase between an implant material and the biological tissue is an ongoing issue. Despite considerable improvements there is still a challenge to optimize the following criteria: the efficiency in stimulating cell adhesion, the selectivity for a specific cell type (e.g. osteoblast vs. platelet adhesion), the stability under physiological conditions, the stable covalent attachment to the material, the ease of handling under sterile conditions, and reasonable costs for the coating. Herein we present a new solution to these problems by using anchored nonpeptidic, highly αv-selective integrin ligands to coat titanium, a common implant material.

Osteoblast adhesion can be stimulated by extracelullar matrix (ECM) proteins (e.g. fibronectin, collagen, laminine, and bone sialo protein),[1] their fragments, or by RGD peptides[2] which bind to αvβ3 integrin on osteoblast cells but bind to the platelet integrin αIIbβ3 as well.[3] Selectivity for αvβ3 integrin could be achieved by using optimized cyclic pentapeptides.[4–6] Coating poly(methyl methacrylate) (PMMA) with suitable modified cyclic pentapeptides stimulates osteoblast adhesion in vitro[7] and bone formation in PMMA granulates in vivo (rabbit).[8]

A number of nonpeptidic αv-selective RGD mimics have been developed by us and others[9–12] as potential drugs to treat cancer, osteoporosis, acute renal failure, restenosis, arthritis, and retinopathy.[13–18] Recently the X-ray structure of the αvβ3 head group containing the cyclic peptide cilengitide[5] was reported.[19] Modeling studies on the nonpeptidic αvβ3 ligands elucidated their binding mode.[20] We used this data to identify suitable positions for anchor groups (linkers)

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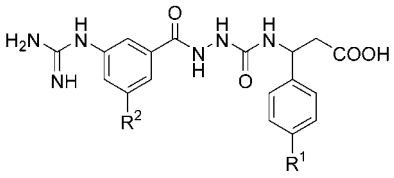
that could be used to attach ligands to the surface without interfering with integrin binding. The guanidine and carboxy groups of the ligand are essential for binding to the integrin subunits α and β, respectively.[21] Therefore we chose the two aromatic rings of our highly αvβ3-selective diacylhydrazine scaffold[10] to position the anchor groups (Scheme 1). By using the AutoDock3 program[22,23] two mimetics with different anchors at $R^1$ and $R^2$ were modeled into the X-ray structure of the αvβ3–cilengitide complex[19] after removal of the peptide ligand. The binding modes were identical to those of the anchor-free mimetic ($R^1 = R^2 = H$; Figure 1),[20] and the linkers showed no disturbing interaction with the integrin, hence we synthesized both variants with different linker groups.



***Scheme 1.*** Substituted nonpeptidic diacylhydrazines with possible linker positions $R^1$ and $R^2$ for anchoring to surfaces.

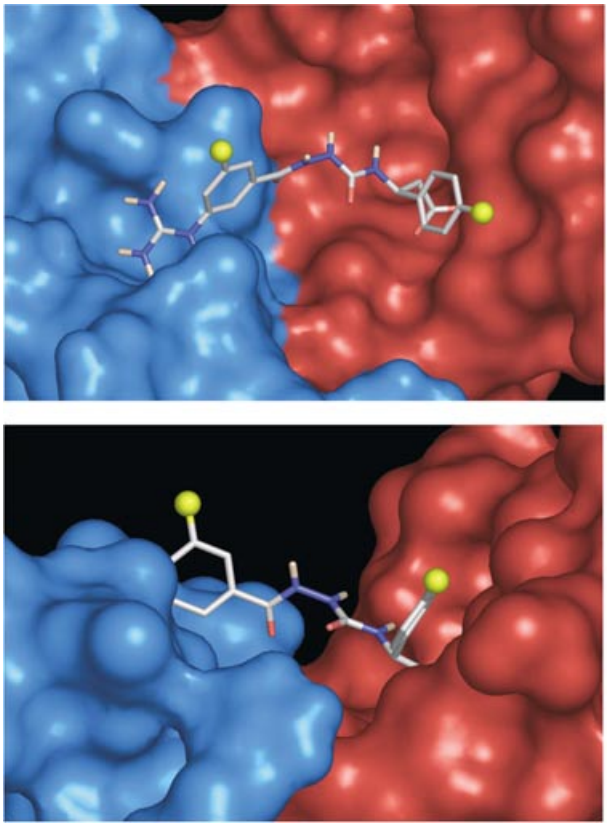
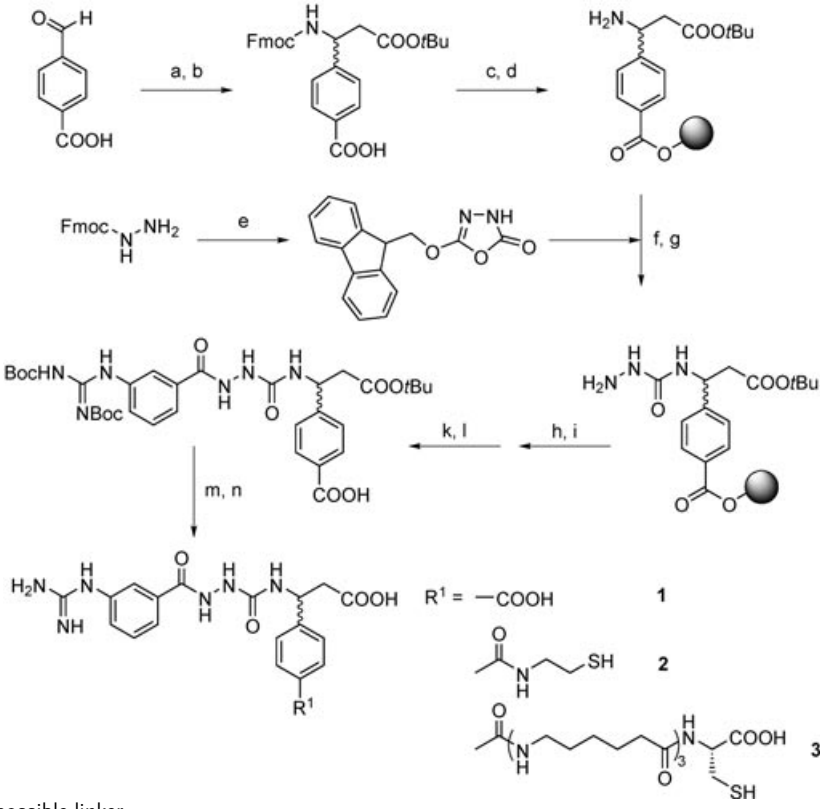


***Figure 1.*** Results of the docking studies: Two diacylhydrazine molecules with anchors on the aromatic rings (stylized as yellow spheres) modeled into the crystal structure of the αvβ3 integrin head group.[19]



***Scheme 2.*** Synthesis of $R^1$ substituted diacylhydrazines on a solid phase. a) $NH_4OAc$ (2 equiv), malonic acid mono-*tert*-butyl ester (1 equiv), EtOH; b) Fmoc-Cl (1.05 equiv), $NaHCO_3$, dioxane; c) TCP-resin, $CH_2Cl_2$, DIEA; d) 20% piperidine in NMP; e) $COCl_2$ (3 equiv; 1.9 M solution in toluene), sat.$NaHCO_3$, $CH_2Cl_2$;[24] f) 5-(9-*H*-fluoren-9-ylmethoxy)-1,3,4-oxadiazol-2-(3 *H*)-one (4 equiv), DMF; g) 20% piperidine in NMP; h) 3-(*N*-Fmoc)-aminobenzoic acid (2 equiv), HATU (1.94 equiv), collidine (22 equiv), NMP; i) 20% piperidine in NMP; k) *N*,*N*′-bis(Boc)guanylpyrazole (10 equiv), $CHCl_3$, 50 °C; l) 20% HFIP in $CH_2Cl_2$; m) linker molecule (1 equiv), HATU (0.97 equiv), HOAt (1.1 equiv), collidine (11 equiv), DMF; n) 50% TFA, 2% triisopropyl silane,[30] 2% water in $CH_2Cl_2$. DIPEA = *N*,*N*-diisopropylethylamine.

Synthesis was performed on solid support (trityl chloride polystyrene resin = TCP-resin) by an Fmoc strategy (Fmoc = 9-fluorenylmethoxycarbonyl) similar to that described elsewhere.[9,10,24] Starting from substituted β-amino acid immobilized on the resin, carbonylated Fmoc-protected hydrazine as the aza-glycine precursor[24] and 3-(*N*-Fmoc)aminobenzoic acid were coupled. Guanidine was successfully incorporated using an excess of *N*,*N*′-bis(Boc)guanylpyrazole (Boc = *tert*-butoxycarbonyl; Scheme 2). After cleavage (hexafluoroisopropanol (HFIP)/$CH_2Cl_2$) the resulting Boc/O*t*Bu-protected compound was coupled in solution on position $R^1$ with two thiol linkers of different length—cysteamine and 6-aminohexanoyl-6′-aminohexanoyl-6″-aminohexanoyl cysteine—and deprotected by trifluoroacetic acid (TFA). Purification was done by reverse-phase (RP) HPLC. The high αvβ3 affinity of all the mimetics, with linkers (**2**, **3**) or not (**1**), was demonstrated in an established $IC_{50}$ assay.[10,25] Whereas the dicarboxy compound **1** is only active for the αvβ3 integrin in the nanomolar range, the linked molecules are biselective

for αvβ3 and αvβ6 integrins (Table 1) as described for other nonpeptides of the diacylhydrazine type.[9]

Steric effects cannot be the reason for the high αvβ3 selectivity of compound **1** as compounds **1**–**3** are equally sterically demanding. Consistent with our theoretical studies on αvβ5 homology models,[26] the residues Lys180 and Asp252 of the SDL (selectivity determining loop) near the MIDAS region could effect an unfavored reorientation of ligand **1** in the binding pocket through electrostatic interactions with the carboxylate. There are uncharged residues at these positions in the β3 subunit and this could explain the strong impact of the carboxylic group in compound **1** for inhibiting the binding in αvβ5.

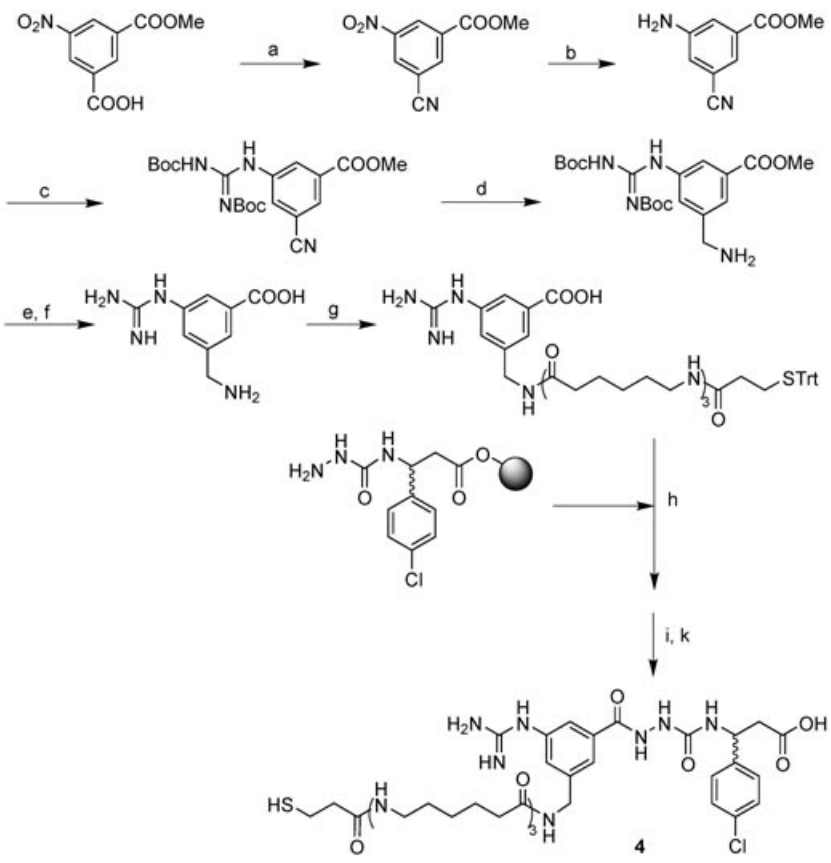
***Table 1:*** Ligand affinities of unanchored (free) nonpeptidic RGD mimetics to different integrins.

| Compound | | | $IC_{50}$ [nM][a]; inhibition at ligand concentration [nM] | | | |
|---|---|---|---|---|---|---|
| | $R^1$ | $R^2$ | αvβ3 | αvβ5 | αvβ6 | αIIbβ3 |
| **1** | COOH | H | 16 | $10^4$ (65%) | $10^3$ (54%) | $10^4$ (22%) |
| **2** | CONH-$(CH_2)_2$-SH | H | 5.7 | 200 | 0.24 | $3.20\times10^3$ |
| **3** | COAhx$_3$Cys[b] | H | 0.72 | 310 | 2.35 | $3.15\times10^3$ |
| **4** | Cl | $CH_2$NHAhx$_3$-CO-$(CH_2)_2$-SH[a] | 0.84 | 245 | 0.089 | $4.15\times10^3$ |
| **5**[10] | H | H | 0.8 | n.m.[c] | n.m.[c] | 8500 |
| **6**[10] | Cl | H | 0.1 | n.m.[c] | n.m.[c] | 5500 |
| *cyclo*(-RGDfK[3-mercaptopropionyl]-)[28] | | | 0.4 | n.m.[c] | n.m.[c] | $>10^4$ |

[a] The data shown represent the mean of at least 2 independent $IC_{50}$ determinations. As previously documented the typical variation in such receptor–ligand inhibition measurements is routinely in the order of the measured value itself.[29] [b] Ahx: 6-Aminohexanoic acid. [c] n.m.: not measured.

$R^2$-linked compound **4** was synthesized by a combined solution and solid-phase strategy (Scheme 3). *p*-Chlorophenyl-substituted β-alanine was chosen as the C-terminus of the molecule because it is found in very potent αvβ3 integrin ligands.[10] A complete solid-phase strategy could not realized, because after coupling of 3-amino-5-(*N*-Fmoc)aminomethylbenzoic acid stabilized with 4-methylbenzenesulfonic acid the coupling of ε-(*N*-Fmoc)-aminohexanoic acid after Fmoc deprotection (only possible with 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)/2% piperidine in *N*-methylpyrrolidone (NMP)) did not work (probably caused by steric hindrance) even though different coupling reagents have been used. Therefore 3-aminomethyl-5-guanidinobenzoic acid was coupled with 3-(*S*-Trt)-mercaptopropionyl-Ahx-Ahx-Ahx-OH (Trt = trityl) in solution. The product was activated with *O*-(7-azabenzoictriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorphosphate (HATU) and coupled on resin-bound 3-(4-chlorophenyl)-3-[(hydrazinocarbonyl)-amino] propionic acid. Cleavage from the resin with HFIP/$CH_2Cl_2$, complete deprotection (TFA/$CH_2Cl_2$), and subsequent purification by RP-HPLC gave in the integrin ligand **4** that is biselective for αvβ3/αvβ6 with an affinity in the subnanomolar range (Table 1).

Substances **2**–**4** were tested for their cell adhesion properties on surfaces, the thiol linkers enable irreversible immobilization on titanium (an implant material). MC3T3E1 mouse osteoblasts, expressing the αvβ3 integrin,[8] were seeded onto the modified titanium discs (Ti6Al4V, ∅1 cm), after 1 h the number of adherent cells was measured by detecting the hexaminidase activity.[27] Our study indicates that mouse osteoblasts bind to surfaces coated with compounds **2** or **3** (Figure 2). The plating efficiency was increased up to 42.9% (100 μM compound **3** in coating solution) compared to 9.4% on the unmodified titanium. Compound **3** stimulates cell adhesion as efficiently as the *cyclo*-(-RGDfK[3-mercaptopropionyl]-) peptide.[28] Compound **2** is slightly less potent, probably caused by the significant shorter linker, which makes the integrin ligand less accessible to the integrin. Compound **4**, although having comparable activity in the binding assay of isolated αvβ3




***Scheme 3.*** Synthesis of $R^2$ substituted diacylhydrazine **4**. a) $SO_2(NH_2)_2$ (1.2 equiv), $SOCl_2$ (3.6 equiv), sulfalone, 42 h reflux; b) Pd/C, $H_2$, MeOH; c) $SC(NHBoc)_2$ (1 equiv), $NEt_3$ (4 equiv), $HgCl_2$ (1.3 equiv), MeOH; d) Pd/C, $H_2$ 20 bar, 2M $NH_3$/EtOH, 50°C; e) LiOH (3 equiv), MeOH/$H_2O$; f) 40% aqua.TFA (v/v); g) linker molecule (1 equiv), HATU (1 equiv), HOAt (1 equiv), collidine (10 equiv), DMF; h) HATU (1 equiv); i) 20% HFIP in $CH_2Cl_2$; k) 40% TFA, 2% triisopropyl silane,[30] 2% water in $CH_2Cl_2$.

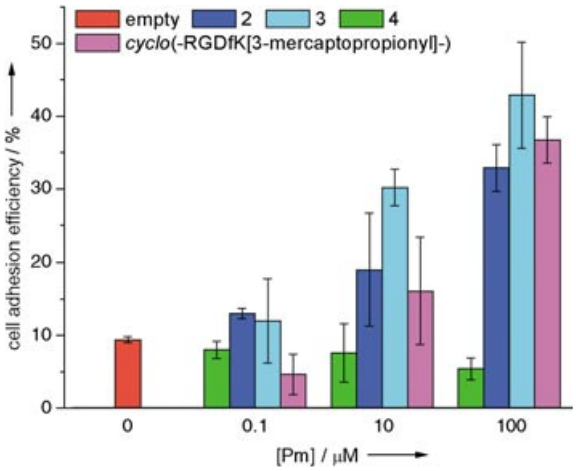



***Figure 2.*** Adhesion of MC3T3 E1 mouse osteoblasts on uncoated and coated titanium surfaces. The mean values of each point given is the result of triplicate determinations, the error bars represent standard deviations. [Pm] = mimic concentration in the coating solution.

integrin (Table 1), yielded no stimulation of osteoblast adhesion when bound to the titanium surface in repeated testing. An explanation could be that in spite of its huge linker the immobilized ligand has an unfavored orientation for integrin binding; the possibility that compound **4** does not immobilize on titanium is unlikely since we have investigated many peptidic derivatives of cyclo(-RGDfK[3-mercaptopropionyl]-) in the past (unpublished data) and all of them coated well on the titanium surfaces (checked by ELISA and cell adhesion assays, data not shown). In the case of the RGD mimic, immobilization could not be directly measured by ELISA because the antibody used recognizes only the cyclic RGD peptide and not the mimetics.

In conclusion, compounds **2** and **3** are the first nonpeptidic αv-selective integrin ligands for surface coating which exhibit a potency for stimulated osteoblast adhesion similar to that of *cyclo*(-RGDfK[3-mercaptopropionyl]-) when immobilized on titanium. Compounds **2** and **3** are more stable to enzymatic degeneration, pH variations, and heat and their synthesis is much cheaper than that of the cyclic peptide.

Spectroscopic and analytical data for compounds **1**–**4** are included in the Supporting Information.

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