The quest towards αvβ3- or α5β1-integrin selective peptidomimetics for surface coating - *History, recent advances and future perspectives*

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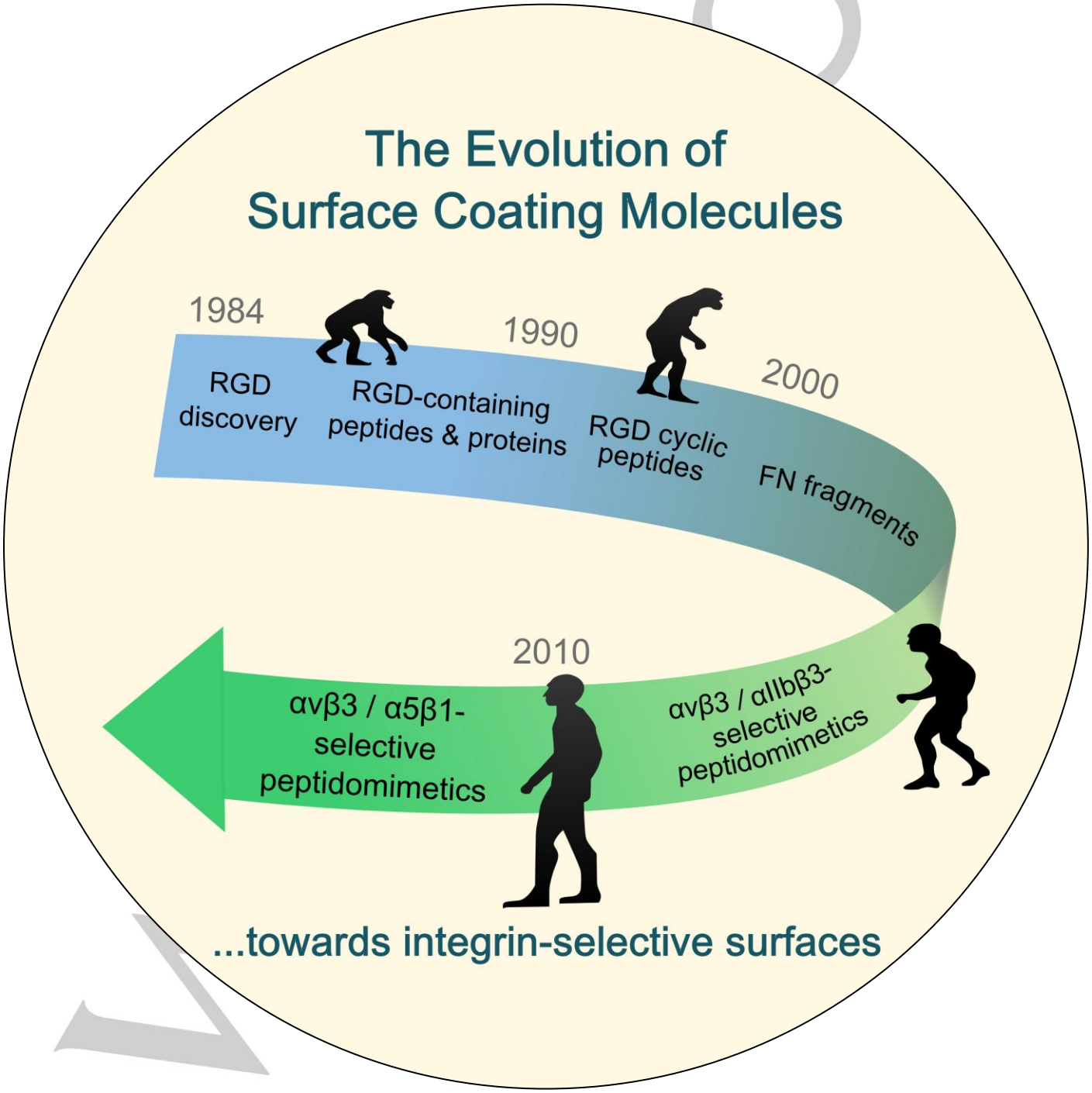

Abstract: Engineering biomaterials with integrin-binding activity is a very powerful approach to promote cell adhesion, modulate cell behavior and induce specific biological responses at the surface level. The aim of this review is to illustrate the evolution of surface coating molecules in this field: from peptides and proteins with relatively low integrin-binding activity and receptor selectivity to highly active and selective peptidomimetic ligands. In particular, we will bring into focus the difficult challenge of achieving selectivity between the two closely related integrin subtypes αvβ3 and α5β1. Functionalization of surfaces with such peptidomimetics opens the way for a new generation of highly specific cell-instructive surfaces to both dissect the biological role of integrin subtypes and implement in tissue engineering and regenerative medicine applications.

1. Introduction

Integrins represent the most important family of cell adhesion receptors. These proteins are bidirectional, heterodimeric cell surface receptors, which are crucial for the interaction of cells with extracellular matrix (ECM) proteins.[1] By interacting with ECM ligands, integrins activate intracellular pathways of signal transduction and mediate cell migration and adhesion. Since the discovery and initial classification of integrins in the late 80s,[2] extensive research has focused on the study of their structure, ligand recognition and biological functions, converting this class of proteins in the most studied adhesion receptors.

The integrin family consists of at least 24 subtypes, built by the non-covalent association of 18 α and 8 β subunits. These subunits are both type-I membrane proteins, each consisting of a large ectodomain and a typically short non-catalytic cytoplasmic domain, linked by a single transmembrane domain.[3] The affinity of integrins to their ligands is regulated by cellular signaling, which can lead to activation, so-called "inside-out" signaling.[4] For instance, intracellular salt bridge formation controls "inside-out" signaling, since abrogation of the salt bridge between the α and β subunit cytoplasmic domains (which stabilizes the resting state of the integrin) strengthens integrin interaction with ECM ligands.[5] Conversely, the binding of ECM ligands induces conformational changes in the structure of integrins, provokes dissociation of the transmembrane helices and contributes to clustering into oligomers, leading to "outside-in" signal transduction (**Figure 1**).[6] Interestingly, ligand binding can occur in the membrane associated (resting) state of integrins; however signal transduction requires the dissociation of the transmembrane helices of the integrins and subsequent oligomerization.[7] Thus, integrins are considered as bidirectional signaling machines, controlling cell polarity, adhesion and survival. In the cell adhesion process, integrins mediate force transmission in focal adhesions (FAs) to ECM proteins, a process known as mechanotransduction.[8]

Embryogenesis, tissue development, angiogenesis and immune system function are therefore highly dependent on integrin activity.[9] Moreover, integrins are also critically involved in pathological processes such as thrombosis, osteoporosis, tumor formation and progression, metastasis and inflammation.[10] On the basis of these biological roles, it is not surprising that integrins have been targeted to develop drugs for treating diverse pathologies.[10b,11] For instance, antagonists of the platelet receptor αIIbβ3 (*abciximab*, *eptifibatide* and *tirofiban*) have been marketed as inhibitors of platelet aggregation to reduce the risk of ischemia in acute coronary syndromes.[12] *Natalizumab*, a drug targeting integrin α4, was prescribed in patients suffering from multiple sclerosis[13] and Crohn's disease;[14] and *efalizumab*, an αLβ2 inhibitor, was approved for the treatment of psoriasis.[15] *Cilengitide*, a highly potent antagonist of integrins αvβ3, αvβ5 and α5β1, reached clinical phase III for the treatment of glioblastomas and is currently in phase II for other cancer types.[16] Although several limitations have been described for these drugs,[17] these examples illustrate the pharmacological potential of targeting integrin receptors.

Understanding the biological role of integrins is paramount to develop novel drugs with high potential and reduced side-effects. Nonetheless, progress in this field has been hampered by the scarcity of integrin-specific ligands. The issue of ligand specificity is clearly illustrated by the canonical integrin binding peptide RGD.[18] Although many integrins bind the ECM via an RGD specific recognition mechanism, these receptors are able to discriminate within distinct natural ligands containing the same RGD recognition motif.[19] The presence of complimentary or synergistic domains, the nature of flanking residues, and the conformation and presentation of the RGD motif to integrins are key determinants of such integrin specificity.

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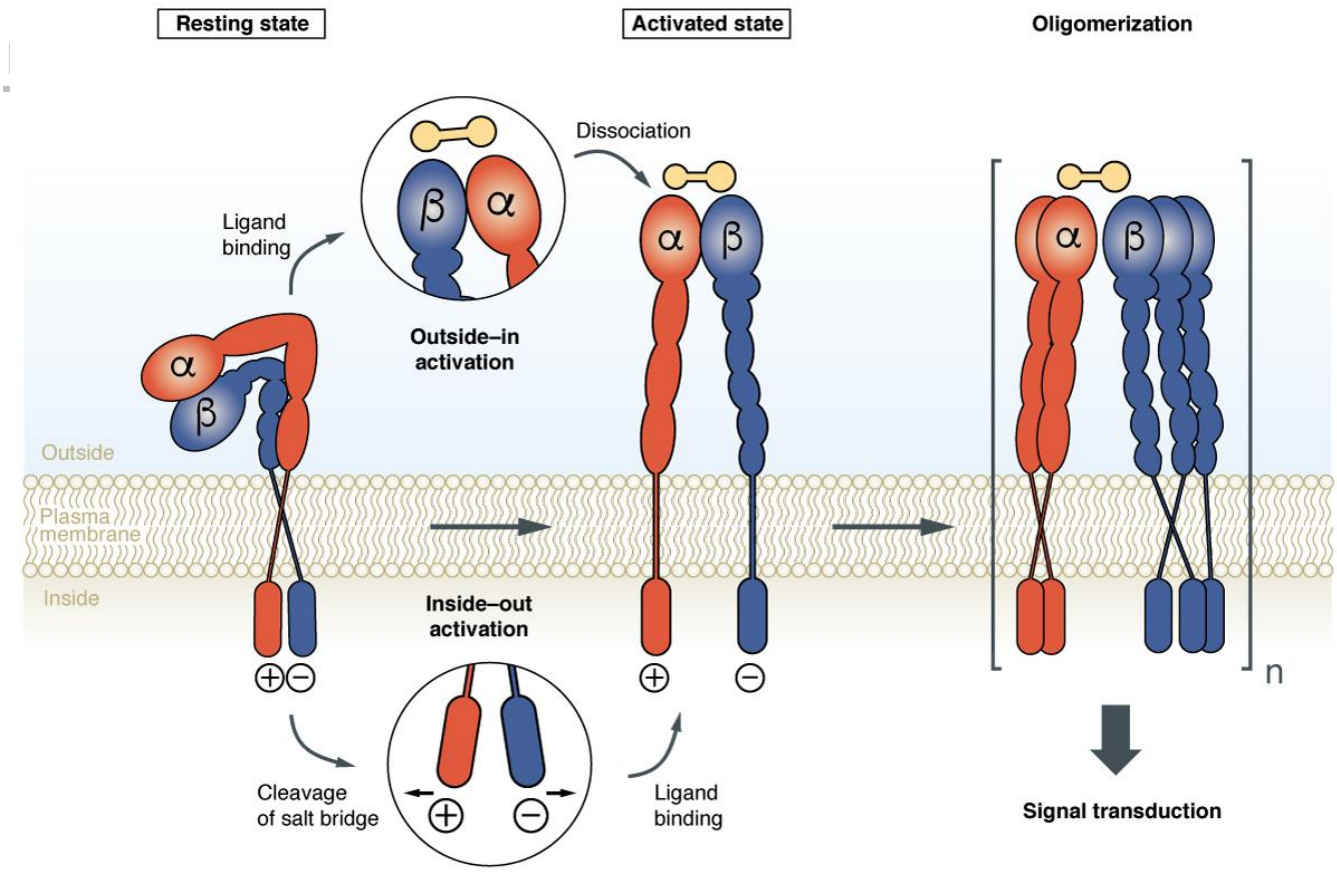


Figure 1. Integrin activation states and "inside-out" and "outside-in" signaling mechanisms. In the bent form integrins have low affinity for their ECM ligands. Inside-out signaling includes cleavage of the intracellular salt bridge established between the cytoplasmic α and β subunits. This induces dissociation of the transmembrane helices and their reorganization and multimerization into a focal adhesion (FA), which binds ligands with high affinity. Conformational changes of the resting integrins and oligomerization are also induced by binding to ECM ligands. This causes stronger binding in the FA. Outside-in signaling requires integrin oligomerization.

In this regard, over the last three decades intensive efforts have been devoted to elucidate the structural features that govern integrin-specific interactions. Integrins αvβ3 and α5β1, key mediators of cell adhesion and differentiation, angiogenesis and tumor growth, were considered very promising targets. By restricting the conformational space of RGD peptides via cyclization, introduction of D-amino acids, and through comprehensive structural studies, we developed in the early 90s cyclic RGD peptides with very high affinities for αvβ3 and selective against αIIbβ3.[20] However, discrimination between the closely related αvβ3 and α5β1 integrins could not be achieved by cyclic peptides (excluding some remarkable exceptions, like the recent development of isoDGR peptides).[21] Thus, selectivity between these two receptors was mostly achieved by synthetic RGD-based peptidomimetics. The development of such ligands was only possible after detailed structure-activity relationship studies and the determination of the crystal structures of αvβ3[22] and homology models for α5β1[23] (the crystal structure of this subtype was not be reported until 2012).[24] These compounds have shown potential to be used as integrin antagonists for cancer treatment, tumor imaging, and for biophysical studies to elucidate the exact roles of these very important integrins.

In parallel to these studies, proteins from the ECM and short synthetic peptides have also been used to functionalize a wide range of materials, aiming at improving their bioactivity by instructing cell adhesive processes on the surfaces. An enormous body of research in this direction has shown that integrin activation and signaling on the surface of a bioinert material efficiently promotes cell attachment, proliferation and differentiation, and thus this strategy has been used to develop a new generation of biomaterials for applications in tissue engineering and regenerative medicine. Surprisingly, the use of peptidomimetics to coat surfaces has been scarce and most of the strategies in this field have focused on using RGD-containing peptides and proteins with poor integrin receptor selectivity.[25]

The aim of this review is thus to introduce the use of RGD-based peptidomimetics with αvβ3/α5β1 integrin selectivity to install integrin-specific activity on the surface of biomaterials. In this work, we will present a historical perspective on the development of integrin-subtype peptidomimetics based on the RGD motif, illustrating some representative examples from our research group. The application of these types of molecules for surface coating, both for medical applications and biophysical

studies, will be examined, and future prospects for this strategy will be outlined.

2. Biological role of integrin subtypes αvβ3 and α5β1

Integrin subtypes αvβ3 and α5β1 were identified by Ruoslahti and coworkers in 1985 and originally named after their natural ECM ligands as the vitronectin (VN) and fibronectin (FN) receptors, respectively.[26] Both subtypes recognized the RGD sequence, which had been described as the minimal adhesive binding motif in 1984.[18] To date, almost half of the 24 known integrin subtypes are reported to bind the RGD motif, including all αv integrins, the integrin α5β1 and the blood platelet integrin αIIbβ3.[27] Whereas αvβ3 can bind to several ECM protein including VN, FN, osteopontin and bone sialoprotein, the α5β1 integrin primarily recognizes FN due to the presence of the synergistic amino acid sequence PHSRN in the cell attachment site of the protein.[28] Nonetheless, both integrins have been described to bind to other ECM ligands with varying degrees of affinity.[27b,27c]

Focal contact formation and development is also integrin-dependent. The geometric localization, shape and dimension of these points of anchorage to the ECM deeply vary with the integrin expression profile, the ligands available in the microenvironment, and the culture time. In this context, Geiger and co-workers found that nascent focal complexes are rich in αvβ3, while α5β1 is present in mature fibrillar FAs.[29] Each subtype is associated to diverse organizations of the actin cytoskeleton and, therefore, of cell shape: cells overexpressing αvβ3 are characterized by broad lamellipodia and low RhoA activity (a small GTPase protein); on the contrary, well-defined actin fibers and high RhoA activity are observed in α5β1-rich cells.[30] These observations on cell shape and actin organization are well reflected in the force sensing ability of each integrin subtype. Roca-Cusachs *et al*. showed that clusters of α5β1 support high matrix forces, while αvβ3 initiates mechanotransduction and is responsible for reinforcement in response to an applied force on FN-coated beads.[31] In agreement with this finding, it has also recently been shown that cells binding to substrates via α5β1 exert higher forces than if they bind via αvβ3.[32] In another study, Giannone and coworkers have reported distinct dynamic nanoscale organizations of β1 and β3 integrins, which can control local forces and signaling during cellular functions such as migration and ECM remodeling.[33] Recently, the group of Fässler has shown that α5β1 integrins accomplish force generation, whereas αv integrins mediate structural adaption to forces on FN-based microenvironments.[34] This study identified diverse functions for the integrins αvβ3 and α5β1, which cooperate to regulate cell contractility and rigidity sensing of cells.

Apart from FAs and actin fiber organization, the engagement of a specific integrin subtype has been shown to influence cell proliferation and differentiation. However, investigations on the role of the αvβ3 and α5β1 subunits on cell growth are often contradictory. The α5β1 receptor has been demonstrated to support cell adhesion and proliferation in several studies.[35] For example, blocking of α5β1 significantly reduced the expression of the transcription factor c-Fos, which is associated with cell proliferation.[35c] Nonetheless, others authors observed no effect of this receptor on cell growth, nor *in vitro*, nor *in vivo*.[36] A study by Martino *et al*.[37] on FN fragments presenting different affinity for α5β1 pointed out that blocking this receptor only affects proliferation on highly affine substrates (containing both RGD and PHSRN sequences), while full-length FN and fragments only containing the RGD motif are still capable of fostering cell growth due to the numerous unspecific signals mediated by other cell receptors. Fewer studies focused on the αvβ3 subtype. Murine cells overexpressing this integrin showed increased proliferation rate compared to non-transfected cells, and this effect was abolished by incubating transfected cells with an αvβ3 blocking antibody.[38] On the other hand, García and coworkers observed no effect on proliferation after blocking this receptor.[35d]

The discrepancies observed in the literature may respond to different reasons. ECM ligands are often characterized for their affinity towards only one integrin subtype (e.g. αvβ3 or α5β1), but the determination of binding affinity for other subtypes is often neglected. Thus, a biological effect may be associated to one specific integrin receptor, underestimating the role of other integrins. Moreover, the pattern of integrin expression on each cell strongly varies depending on cell type, culturing conditions, substrate used, etc. thus not allowing a direct comparison between different studies. On top of that, it should be taken into consideration that integrins have overlapping roles, and the suppressed function of one blocked integrin may be substituted by another.

The role of these two integrins on the differentiation of mesenchymal stem cells (MSCs) is a very hot topic of research, given the rising interest in stem cell therapies and in the development of cell instructive biomaterials. An increasing need to control the plasticity of stem cells is emerging, either for keeping them undifferentiated in culture[39] or for inducing a specific phenotype.[40] Apart from classical molecular mediators of differentiation, such as growth factors, the microenvironment has proved a promising tool for guiding stem cell fate.[41] In this regard, the role of integrins in the progression of the undifferentiated cell toward a specific lineage is not fully established yet. Several studies have detected a positive role of the α5β1 subtype in the induction of osteogenesis. This receptor has been shown to upregulate the expression of osteogenic markers and alkaline phosphatase (ALP) activity *in vitro*[36,37,42] and to induce implant osseointegration[42b,43] and ectopic bone formation *in vivo*.[42a] Decreased levels of α5β1 were also associated to bone loss in an animal model of skeletal unloading.[44]

On the contrary, the role of the αvβ3 subtype remains controversial, with studies claiming a suppression of osteoblastic

differentiation caused by this receptor,[38,42b] while others ascribe increased matrix mineralization to the binding of the αvβ3.[45] In a recent study, Kilian and Mrksich[46] demonstrated that a cyclic RGD peptide with high affinity for αvβ3 directed MSCs toward the osteoblastic lineage. They observed increased expression of several osteogenic markers, such as high ALP activity, high level of runt-related transcription factor-2 (Runx2) and greater cell spreading on surfaces coated with the cyclic peptide. Interestingly, a linear RGD peptide with lower affinity for αvβ3 induced myogenic differentiation instead. Nonetheless, not many studies have focused on the osteogenic potential of αvβ3, since this receptor has been traditionally investigated for its role in bone resorption. In fact, osteoclasts are the cell type with highest *in vivo* expression of the αvβ3 integrin.[47] Osteoclast binding to the ECM is mediated by this integrin subtype and interference with this receptor has been demonstrated to inhibit bone resorption.[47] This effect, which has been corroborated with blocking antibodies *in vitro*[48] and β3-lacking mice *in vivo*,[49] is attributed to the αvβ3-dependent migration of osteoclasts.[48] Noteworthy, the role of αvβ3 in cell migration has been observed in many other cell types, from smooth muscle cells[50] to endothelial cells,[51] and various tumor cell lines.[9b]

Indeed, αvβ3 integrin is a critical regulator of physiological as well as pathological angiogenesis, which represents a critical step in tumor progression and metastasis.[9b,10b,52] At the very beginning of tumor progression, hypoxia can induce the so-called "angiogenic switch"[53] in dormant tumors, thus inducing secretion of growth factors, for example, vascular endothelial growth factor (VEGF), and as a consequence leading to upregulation of integrins. By interaction of αvβ3 with its natural ECM ligands, the migrating endothelial cells participate in the formation of new blood vessels, thus providing the tumor with oxygen and nutrients.[54] Since the first studies revealing that αvβ3 is involved in pathological angiogenesis,[55] many studies have shown upregulation of this subtype on tumor cells, pointing towards a proangiogenic role of αvβ3. However, observations that mice lacking all αv integrins show extensive angiogenesis[56] and mice that lack β3 and β5 integrins show pathological angiogenesis and increased tumor growth[57] point to an important, but not essential role of αvβ3 in the regulation of angiogenesis.[9a,58]

The biological function of α5β1 in angiogenesis is not fully established either. Its ability to co-traffic with the epidermal growth factor receptor (EGFR)[59] as well as its upregulation during angiogenesis and on blood vessels in tumors[60] suggests a tumor-promoting role. Other reports point to a context-dependent function with a promoting role in certain tumors and an inhibitory function in others. Recently, Hynes and coworkers have shown that αv and α5 may cooperate and even substitute each other during vascular remodeling.[61]

3. The development of subtype-specific αvβ3 and α5β1 ligands

3.1. RGD Peptides and beyond

Seminal work by Pierschbacher and Ruoslahti described in 1984 the tetrapeptide Arg-Gly-Asp-Ser (RGDS) as the minimal cell binding motif in FN.[18] In these studies, synthetic peptides displaying this sequence inhibited fibroblast attachment to surfaces coated with FN. Remarkably, coating of agarose beads with this sequence also promoted fibroblast adhesion. Further investigations on the role of each amino acid of the tetrapeptide revealed that Arg, Gly and Asp were essential for the activity but not Ser, which accepted a number of substitutions without loss of the biological activity.[18,62] Interestingly, the RGD motif was also found in fibrinogen and type I collagen, and short peptides derived from these proteins containing this sequence supported cell attachment as well.[18] These remarkable findings suggested that cells expressed a common receptor to bind the ECM via the RGD recognition motif (i.e. integrins), and subsequent studies identified the RGD motif in many other ECM proteins, including VN,[63] von Willebrand factor,[64] osteopontin,[65] and laminin.[66]

Even if many integrins recognize ECM proteins via the RGD motif, the specificity governing this interaction is not trivial. This was soon illustrated by integrins α5β1 and αvβ3, which showed mutually exclusive specific interactions with the ECM. In detail, liposomes containing α5β1 were able to bind to FN-coated surfaces but not to VN-coated substrates. In contrast, when αvβ3 was inserted into liposomes the opposite behavior was observed.[26b] Nonetheless, in both cases the same RGD peptide inhibited protein binding. Nowadays, it is well established that ligand specificity for integrins depends on multiple factors. Although there are notable examples on the influence of synergistic domains that confer integrin specificity (i.e. the PHSRN sequence, which synergizes in FN the binding of RGD to α5β1),[28] the conformation and spatial presentation of the RGD motif within ECM proteins is one of the major determinants.[19,67]

The integration of an amino acid sequence into a cyclic peptide represents a feasible way to restrict its conformational space and increase its bioactivity and receptor selectivity.[68] Early studies with a disulfide-bridged RGD-cyclopeptide, showed an improved inhibition of VN-mediated fibroblast adhesion, but not inhibitory activity of cell adhesion to FN, compared to the unselective stem linear peptide.[69] In another study, reduction of disulfide bonds in an RGD-containing venom peptide resulted in suppression of the peptide's inhibitory activity, probably due to a reduced integrin affinity by loss of its bioactive conformation.[70]

In this context, our group pioneered in the 90s a series of studies to determine conformation-dependent integrin subtype selectivity.[20] The study of the effect of a single D-amino acid substitution of a cyclic peptide on its conformation and biological activity, a process named "spatial screening",[71] resulted in the development of the pentapeptide ***c*(RGDfV) (Table 1)**, which showed a 100-fold increased inhibition of A375 cell adhesion to VN compared to the linear control peptide and selectivity against the platelet integrin αIIbβ3.[20a] This peptide showed disruption of

tumor-induced angiogenesis in a chick chorioallantoic membrane (CAM) model[55b] and served as lead structure for the development of many other αvβ3-selective integrin ligands.[72] As such, ***c*(RGDfV)** was subjected to a number of modifications like the reduction of peptide bonds,[73] the incorporation of turn mimetics,[74] the use of sugar amino acids[75] and the synthesis of retro-inverso analogues.[76] While good selectivity was attained between αvβ3 and αIIbβ3, none of these studies reported selectivity against α5β1.

N-Methylation of ***c*(RGDfV)** led to the drug candidate *cilengitide* ***c*(RGDf(*N*Me)V) (Table 1)**,[16] which has antagonistic activity for αvβ3 in the subnanomolar range (IC_{50} = 0.58 nM) and for αvβ5 and α5β1 in the nanomolar range (IC_{50} values of 11.7 nM and 13.2 nM, respectively).[77] Although peptidic compounds often show very poor enzymatic stability, cyclization and *N*-methylation[78] led to high metabolic and enzymatic stability for this peptide. Because of its high activity against proangiogenic integrins, but also for its selectivity against integrin αIIbβ3, *cilengitide* is currently undergoing clinical phase II studies for the treatment of several tumor types.[16b] It reached a phase III trial for the treatment of glioblastomas,[16b,79] unfortunately, patients treated with *cilengitide* and chemoradiotherapy did not live significantly longer and its use for these aggressive tumors was suspended.[80] A cross-talk between α5β1 and the tumor suppressor protein p53 was recently reported to mediate the induction of apoptosis in glioma cells. Such biological effect has not been described for αvβ3. Thus, the reduced affinity of *cilengitide* towards α5β1 could explain the lack of efficacy of the peptide in the treatment of glioblastomas.[81] As *cilengitide* was administered intravenously twice a week or daily (2 g per patient) due to its short half-life in man (the drug is excreted after 4 h without being metabolized), its low concentration in the blood after a few hours may be enough to activate resting integrins but not to block binding and prevent signal transduction.[82] Up to now, no integrin ligand targeting αvβ3 or α5β1 was able to get approval by the FDA.[11b]

Targeting the α5β1 subtype has also been a hot topic of research, and for more than 20 years we and others have also focused on the development of peptides with affinity for this receptor. In this regard, cyclic peptides[72a] as well as linear peptides derived from phage display[83] have been reported to be active for α5β1, however, with no remarkable selectivity against the αvβ3 subtype. A few years later, a non-RGD linear peptide derived from FN targeting the synergistic domain of α5β1 was discovered, and its acetylated analogue, Ac-PHSCN-NH_2, later dubbed *ATN-161*, showed anti-invasive, anti-tumorigenic and anti-metastatic activities in prostate cancer cell lines.[84] *ATN-161* is currently undergoing clinical phase II for the treatment of cancer, however, also activity for αvβ3[85] as well as for αvβ5[11b] is reported.

The group of Sewald also synthesized several cyclic RGD peptides incorporating β-amino acids to investigate their influence on the peptides secondary structure.[86] In this way, the cyclic tetrapeptide **c(RGD-β-H-Phe)** was identified as αvβ3-active ligand (63 nM in an isolated integrin assay)[87] with very low affinity for α5β1 (> 1000 μM in a cellular adhesion assay with K562 cells) **(Table 1)**.[88] In these studies, other highly active peptides were also reported, yet with no remarkable selectivity between αvβ3 and α5β1. Later, the same group reported the synthesis, structural analysis and biological evaluation of pentapeptides containing the constrained *cis*-β-aminocyclopropanecarboxylic acid (β-Acc).[89] The cyclic pentapeptide **c(RGD-(+)-β-Acc-V) (Table 1)** exhibited a very high activity for αvβ3 (20 nM) in a cellular adhesion assay of WM115 cells to FN and good selectivity against α5β1 (1.5 μM, 75-fold) of K562 cells on FN. In 2008, Pramanik *et al*. reported that a lipopeptide with the tetrapeptide sequence RGDK could selectively target genes to the α5β1 integrin receptor *in vitro*.[90]

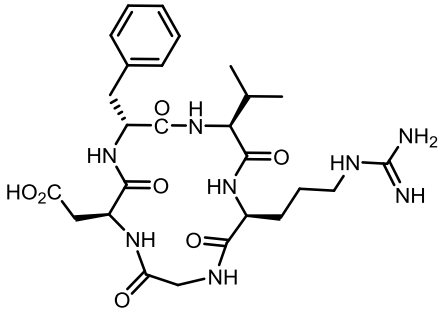
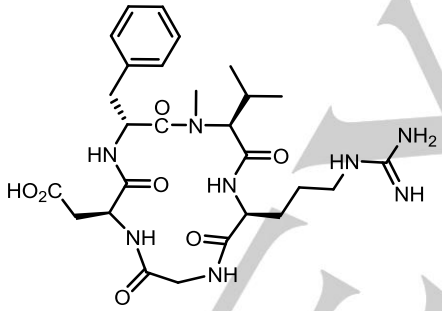
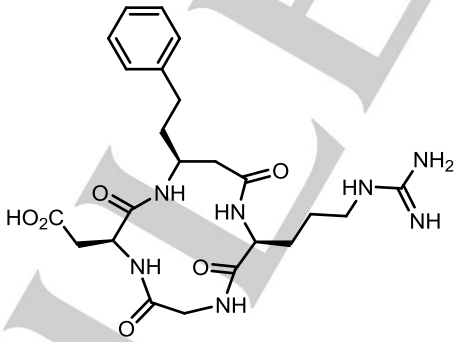
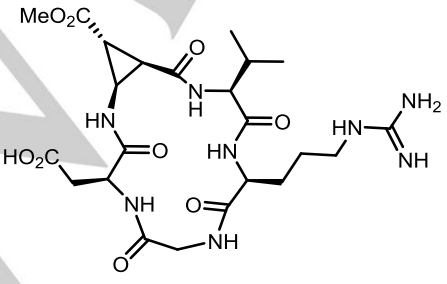
These studies illustrate that the development of peptides with selectivity between integrins αvβ3 and α5β1 has been limited. In this regard, cyclic peptides containing the isoDGR sequence represent one of the few reported examples of peptides capable of achieving high binding affinities and outstanding selectivity between these two receptors. The isoDGR motif, which results from the deamidation of asparagine at the 5th type repeat I module of FN, was identified by the group of Corti as an unexpected integrin binding motif in that protein.[91] Based on these findings, we designed head-to-tail cyclic peptides containing the isoDGR motif and the spatial screening procedure was applied.[21a] In these peptides, the isoDGR sequence was flanked by one Gly and one aromatic amino acid (reported as crucial for binding with αvβ3).[72b] The aromatic residue was introduced in either the L- or D-configuration, enabling the adoption of different peptide conformations. Interestingly, the relative position of the flanking residues determined the binding affinity towards αvβ3 or α5β1. This was illustrated by the ***c*(phg-isoDGR-G)** peptide, which exhibited an affinity for α5β1 in the nanomolar range (IC_{50} = 19 nM) but was inactive for αvβ3. In contrast, shifting the position of the flanking residues in ***c*(G-isoDGR-phg)** yielded the opposite biological behavior **(Table 1)**. Such selectivity was corroborated with docking studies and cellular tests using α5β1- and αvβ3 expressing fibroblasts.[21a]

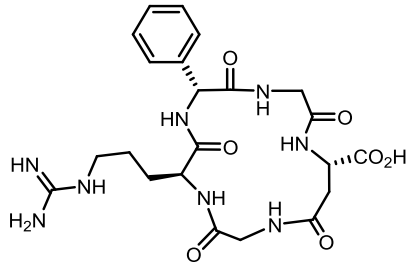
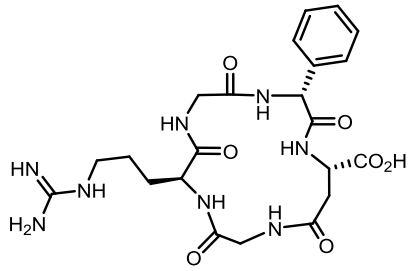
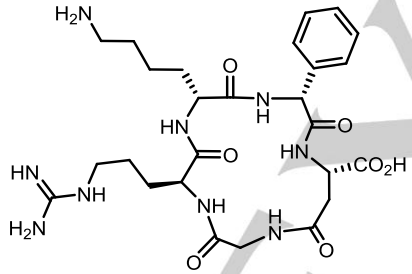
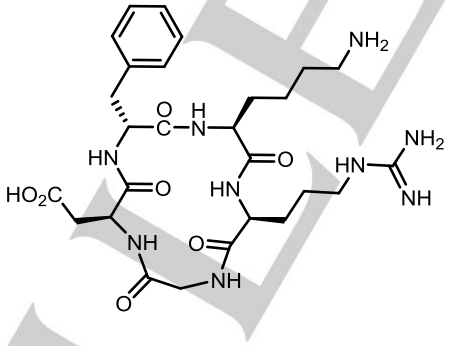
Follow-up studies based on the α5β1-selective peptide, ***c*(phg-isoDGR-G)**, were done by substituting the Gly by other L- and D-amino acids.[21b] From the designed library, the best compound was *c*(phg-isoDGR-w), which displayed an increased affinity for α5β1 (IC_{50} = 5.5 nM) while keeping the selectivity against αvβ3. Interestingly, this peptide also exhibited a moderate affinity for the αvβ6 subtype (IC_{50} = 92 nM). The introduction of D-Lys instead of D-Trp in ***c*(phg-isoDGR-k)** further increased the activity for αvβ6 to 19 nM, retaining an excellent activity for α5β1 and selectivity against αvβ3. This peptide was functionalized with a thiol and anchored to a nanopatterned gold surface to study the adhesion and behavior of REF52 cells.[21b]

The recent determination of the x-ray structure of the head groups of α5β1 has shed further light into the different binding modes of RGD ligands to αv and α5 subunits.[24] In particular, it was shown that the guanidine group of Arg binds to the αv-

subunit of αvβ3 only via side-on interactions with Asp218. In contrast, binding of Arg to α5 in α5β1 is established through side-on and end-on interactions with Asp218 and Gln221, respectively. This has allowed shifting the selectivity between the two receptors by different *N*-methylation patterns of the guanidinium group.[92] For example, methylation in N_ω abrogated end-on interactions and totally prevented binding to α5, which in turn increased selectivity for αvβ3 and αvβ6 receptors.

Table 1: Structure of representative cyclic RGD peptides with affinity for αvβ3 and/or α5β1 integrins

Compound	Structure	IC_{50} **αvβ3** [nM]	IC_{50} **α5β1** [nM]	Ref
***c*(RGDfV)**		4.9[a]	n.r.[b]	20a, 72b
***c*(RGDf(*N*Me)V) (Cilengitide)**		0.58[c]	13.2[c]	16a, 77
***c*(RGD-β-hPhe)**		63[d]	>1000[d]	87, 88
***c*(RGD-(+)-β-Acc-V)**		20	1500	89

***c*(G*iso*DGRphg)**		89	406	21a
***c*(phg*iso*DGRG)**		>1000	19	21a
***c*(phg*iso*DGRk)**		>1000	8.7	21b
***c*(RGDfK)**		2.6[e]	133[e]	72b, 21b

[a] In the original publication [ref 20a] the activity of this peptide was measured in terms of inhibition of cell adhesion to VN and laminin P1 fragment. The activity towards αvβ3 using isolated integrins was reported later in [ref 72b]. [b] n.r. = not reported. [c] In the original publication [ref 16a] only the activity for αvβ3 and αIIbβ3 was measured. IC_{50} values for other integrins αvβ5 and α5β1 were reported later in [ref 77]. [d] The activity towards αvβ3 was reported in [ref 87]; for α5β1 in [ref88]. [e] In the original publication [ref 72b] the activity of the peptide for αvβ3 was reported as the ratio Q = IC_{50}[peptide]/IC_{50}[GRGDSPK]. IC_{50} values for both αvβ3 and αvβ3 have been recently published in [ref 21b].

3.2. RGD-based Peptidomimetics

The development of cyclic RGD peptides has been accompanied with the design and synthesis of totally non-peptidic antagonists, aiming at improving the activity and selectivity profiles obtained by peptidic ligands.[20c,93] Undoubtedly, the first crystal structure of the extracellular segment of the αvβ3 integrin in 2001 was a major breakthrough for the design of selective integrin ligands.[22a] One year later, the crystal structure of the extracellular segment of integrin αvβ3 complexed with *cilengitide* was also elucidated.[22b] This work gave important insights into the binding modes of integrin ligands and served as basis for docking studies of drug candidate molecules. As the crystal structure of α5β1 was unknown, we published in 2005 a three dimensional model of

this subtype based on homology modeling of the experimental three dimensional structure of αvβ3 in its bound conformation.[23] Since the binding pockets of αvβ3 and α5β1 have strong similarities (αv:α5 53% identity; β3:β1 55% identity in the integrin's head group),[23] this model, together with the previously published crystal structures, paved the way for the rational design of selective ligands. In this section, we will only focus on the development of peptidomimetic ligands with the capacity to differentiate between these two closely related subtypes. Integrin ligands which have been tested only for one of these subtypes as well as biselective integrin ligands, active for both αvβ3 and α5β1, are beyond the scope of this review and have been described elsewhere.[94]

Besides some peptidic ligands with low micromolar activities for α5β1 and isoDGR peptides, the first highly active α5β1 ligand was the small non-peptidic molecule **SJ749 (M1, Table 2)**. This molecule was developed by conformational restriction of an αvβ3 antagonist. It contains a spiro-oxazoline scaffold and exhibits an excellent activity for α5β1 (IC_{50} = 0.18 nM) and at least a 200-fold selectivity against αvβ3.[95] Docking studies of **M1** into the α5β1 binding pocket revealed key specific interactions with the receptor, which were responsible for its high activity **(Figure 2A)**.[23] **M1** was able to show inhibition of angiogenesis by affecting adhesion and migration of endothelial cells,[96] inhibited tumor cell proliferation[97] and facilitated cell apoptosis in a functional p53 background in the human glioblastoma cell line U87MG.[98]

The first rationally designed selective peptidomimetics were developed by us[99] and *Jerini AG*[100] at the same time. The design of these compounds was based on previous docking studies into the crystal structure of αvβ3[101] and on the homology model of α5β1 in complex with **M1 (Figure 2A)**.[23] Comparing the two binding pockets, two regions seemed to be especially suitable for achieving selectivity between αvβ3 and α5β1: In the β-subunit (β3)-Arg214 and (β3)-Arg216 are replaced by (β1)-Gly217 and (β1)-Leu219, respectively. The substitution of both Arg by smaller residues expands the available space in this site of the α5β1 binding pocket, which, in comparison to the αvβ3 integrin, allows the introduction of bulky moieties into the ligands core structure. Secondly, the α5 subunit turned out to be less acidic owing to the mutation of (αv)-Asp150 to (α5)-Ala159. Furthermore, the replacement of (αv)-Thr212 by (α5)-Gln221 results in a different geometry of this binding region, which offers the opportunity to gain selectivity by modification of the basic moieties.[99] As mentioned above, the modulation of selectivity between these two receptors via *N*-alkylation of the guanidinium group of Arg has recently been achieved.[24,92]

On the basis of these observations, we synthesized a series of peptidomimetics derived from a tyrosine scaffold, which had already been successfully employed into other integrin ligands.[102] The most potent α5β1 targeting ligand (**M2, Table 2**) was active for α5β1 in the subnanomolar range (IC_{50} = 0.7 nM) and displayed good selectivity against the αvβ3 integrin.[99] Docking studies revealed an optimal fitting of this compound into the α5β1 binding pocket (**Figure 2B**). Conversely, the removal of the two methyl groups in the aromatic moiety allowed the fitting into the αvβ3 binding pocket and led to the highly active αvβ3 integrin ligand (**M3, Table 2**) (IC_{50} = 1.2 nM) with remarkable selectivity against α5β1. [99]

Stragies et al. from *Jerini AG* reported the development of highly active compounds derived from a virtual combinatorial library.[100] Since the starting compound (**M4**) of these series was α5β1-active (IC_{50} = 3.7 nM) but showed little selectivity against αvβ3 (IC_{50} = 16 nM), variation of the hydrophobic side chain on R^1 (see **Table 2**) was investigated. In this regard, replacement of the sulfonamide with an amide led to a remarkable drop in αvβ3 binding activity (IC_{50} ~30 000 nM), while completely maintaining the activity for α5β1 (**M5**, **JSM6427**). The fact that integrin α5β1 has a bulkier binding pocket seems to explain its capacity to accommodate both ligands, whereas the more sterically restricted region in αvβ3 only allows the binding of the conformation adopted by the sulfonamide group, where the substituents are twisted 90° about the SO_2-N bond in comparison to the planar amide.[99,100]

M5 inhibited choroidal neovascularization in a dose dependent manner in monkey and rabbit models[103] and was investigated for the treatment of age related macular degeneration (AMD) in clinical phase I. However, its therapeutic use appears to have been discontinued.[11b] The introduction of a 4-methoxygroup at the 2-aminopyridine ring led to one compound (peptidomimetic **M6**, **Table 2**) with even higher activities for α5β1 and still good selectivity against αvβ3. The very high α5β1-binding affinity of this compound was confirmed in a cellular adhesion assay with HEK293 cells.[100] Further investigations based on substituted 2-aminopyridine units linked to five- or six-membered heterocyclic ring systems and a phenylalanine moiety, yielded compounds with good α5β1-selectivity.[104]

Based on the observation that glycine can be replaced by aza-glycine in RGD-containing linear peptides with preservation of biological activity and selectivity,[105] and its successful incorporation into αvβ3-active peptidomimetics,[106] this approach was also applied to develop α5β1-ligands. Noteworthy this strategy yielded compounds with very high affinity for α5β1 (IC_{50} < 1 nM) and outstanding selectivity against αvβ3 in comparison to the tyrosine scaffold.[107] The optimal selectivity profiles (i.e. by a factor of 6000 and higher) are due to the rigidity of the diacylhydrazone scaffold compared to the rather flexible tyrosine. Additionally, arylguanidyl and alkylguanidyl groups were used as basic moieties instead of the previously used 2-aminopyridine.[99] Nonetheless, we were able to show that only the C-terminal moiety of the molecule is responsible for selectivity. The substitution of aza-glycine in **M7** with glycine (**M8**) had little effect on the biological activity and led to a highly active (IC_{50} = 0.86 nM) α5β1-ligand with remarkable selectivity against αvβ3 (IC_{50} = 9600 nM) (**Table 2**).[107] Due to this excellent integrin subtype selectivity and its straightforward synthesis, this ligand was later used for a number of biological investigations and functionalized for different purposes.[32,81a,108]

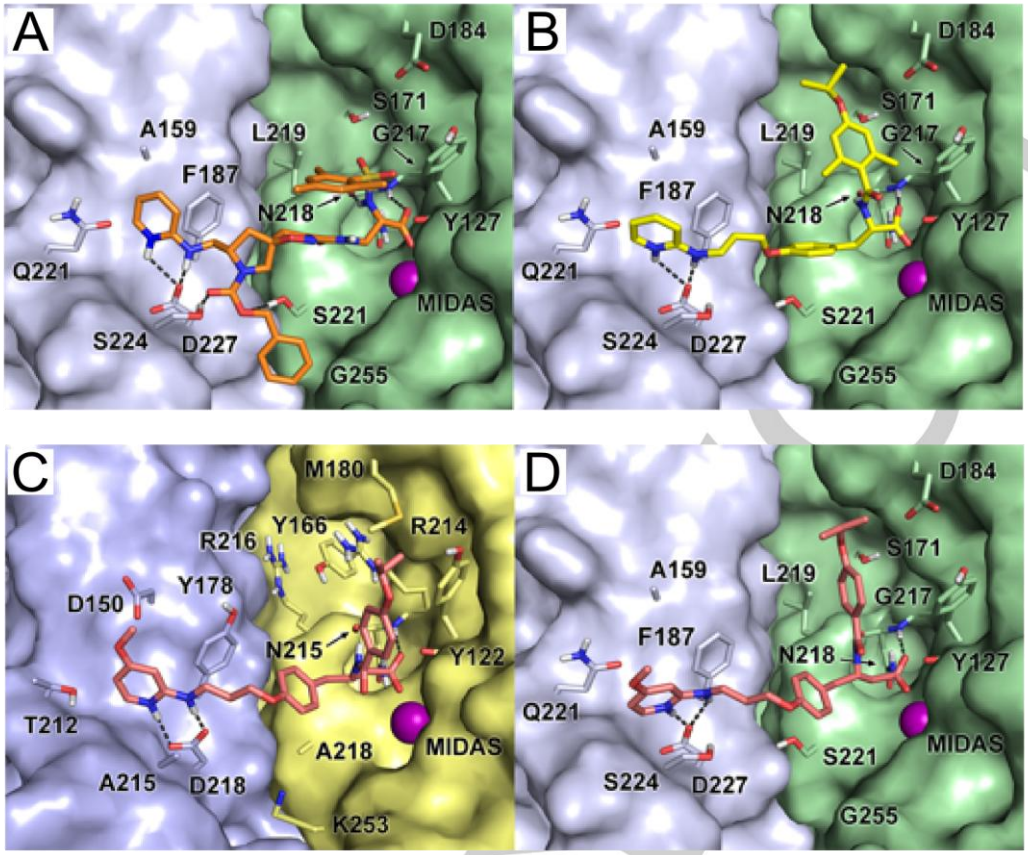


Figure 2. Binding modes of selected peptidomimetics into integrin binding pockets. Docking of (A) **SJ749** (**M1**) (orange sticks) and (B) **M2** (yellow sticks) into the α5β1 binding pocket. Both compounds show an optimal fitting in the receptor. Docking of **M11** (pink sticks) into the αvβ3 (C) and α5β1 (D) binding pockets. The preferential fitting of this compound into αvβ3 results in an increased selectivity towards this receptor. The α5 and β1 subunits are represented as light blue and green surfaces, while the αv and β3 subunits are represented as blue and yellow surfaces, respectively. Receptor amino acid side chains important for the ligand binding are shown as sticks. The metal cation at the MIDAS is depicted as a magenta sphere.

One year later, we reported for the first time the successful replacement of the carboxylic acid with an isosteric group, by using hydroxamic acids.[109] Even though no super-active ligands were obtained, we were able to develop highly active αvβ3 ligands (IC_{50} values up to 5 nM) with good selectivity against α5β1 (1-2 orders of magnitude) and provided deep insights into the binding modes of these integrin antagonists. Compounds **M2** and **M9** only differ in the nature of the acid coordinating the metal at the metal-ion-dependent adhesion site (MIDAS) but show opposing selectivity for the two integrin subtypes (see **Table 2**). The ligand containing the carboxylic acid is a highly active α5β1-ligand, whereas in the hydroxamic acid ligand the selectivity is shifted to αvβ3 with strongly reduced α5β1 activity. Docking models could reveal that the reason for that is the increased distance between the acidic and basic groups, favoring αvβ3 affinity.[109] This principle could also be proven for other analogues based on the same scaffold, such as **M10** (**Table 2**), which showed higher αvβ3 affinity and improved selectivity against α5β1.

To investigate the precise roles of these receptors in biological processes there was a high demand of αvβ3-specific ligands. Extensive research was thus stimulated to develop novel selective ligands as cyclic RGD peptides showed in general no satisfactory selectivity against α5β1. Based on the three dimensional structure of the αvβ3 binding pocket and the pharmacophoric requirements of already published peptidomimetics, we were able to develop novel αvβ3 subtype-specific compounds. The backbone of these RGD ligands is presented as β-homotyrosine, which was shown to be essential for selectivity against α5β1 and αIIbβ3.[99] Due to the steric and electrostatic demands of the amino acid residues presented in the binding region of the α-subunits, a 4-methoxypyridine residue was incorporated into the backbone as basic moiety and mimic for Arg.[110] (**M11** and **M12**, **Table 2**, **Figure 2C,D**).

Previous studies revealed an enhanced affinity profile towards αvβ3 by introducing the aromatic moiety via a sulfonamide group instead of a carboxamide bond.[100] This effect was based on the relative structural orientation of these chemical groups and differential fitting in the integrin binding pocket. However, both compounds **M11** and **M12** showed subnanomolar activities for αvβ3 (**M11**, IC_{50} = 0.86 nM; **M12**, IC_{50} = 0.65 nM) with almost no difference in the affinity profile due to an optimized fitting of the

ligands into the αvβ3 integrin binding pocket (**Figure 3**).[110] These compounds or their functionalized derivatives were potent enough to be used for *in vitro* and *in vivo* applications.[32,81a,108] Furthermore, the ligands showed dose dependent anti-angiogenetic effects on spontaneous, basic fibroblast growth factor (bFGF)- and VEGF-induced capillary sprouting in a rat aorta ring system, and also induced an antitumor effect in mice bearing WEHI-164 fibrosarcomas.[108b]

Other groups have also described ligands with αvβ3/α5β1 selectivity. The group of DeGrado reported the design and synthesis of a library of αvβ3-selective antagonists based on a diaminopropionic acid scaffold.[111] The most active compound from these series, **M13**, showed high αvβ3-affinity (IC_{50} = 1.1 nM) and high selectivity against α5β1 (**Table 2**). Two highly active and selective αvβ3 inhibitors have also been reported by SmithKline.[112] The compounds are derivatives of benzodiazepine and could show excellent pharmacokinetic profiles in rats. Biological evaluation in an isolated receptor assay showed αvβ3-affinities (IC_{50} values) of 1.2 and 0.9 nM for **M14** and **M15**, respectively (**Table 2**). Their high potential was confirmed in a cell adhesion assay of αvβ3-expressing HEK-cells. The α5β1 activity was reported to be of 110 nM and 1000 nM, respectively. Very recently, Galletti *et al*. reported the development of α5β1/αvβ3 active peptidomimetics based on a β-lactam scaffold. The most active and selective compound **M16** exhibited an EC_{50} of 11 nM in an assay using αvβ3 expressing SK-MEL-24-cells. Contrarily, when α5β1-expressing K562 cells were used, the EC_{50} dropped to 763 nM, converting this compound in a relatively selective αvβ3 inhibitor.[113]

4. Coating of surfaces with αvβ3 or α5β1-selective ligands

4.1 General considerations on surface coating

Immobilizing bioactive molecules onto a biomaterial surface represents a critical step that needs to be carefully designed. Even ligands with high affinity for integrins may fail to support cell adhesion if their binding to the surface and/or accessibility to integrin receptors are not optimal. The following considerations should be taken into account:

1) **Method of immobilization**. Integrin ligands can be coated on the surface of materials by simple physical adsorption.[25b,114] This method, also known as physisorption, relies on the establishment of non-covalent interactions (e.g. electrostatic interactions, van der Waal forces and hydrogen bonds) between the ligand and the substrate. Although this procedure is commonly used to immobilize proteins and large molecules, it is based on weak interactions and does not ensure a stable binding for small molecules. Moreover, the adsorption of the molecules takes place in a non-specific manner, which may affect their optimal conformation or hinder motifs required for the activity. For these reasons, a chemical anchoring to the biomaterial is preferable.[25b,114] Covalent immobilization offers much higher stability, which is important for clinical applications. Moreover, coating molecules can be functionalized with anchoring groups, which provide chemoselective binding to the surface without affecting the pharmacophoric properties of the molecule.

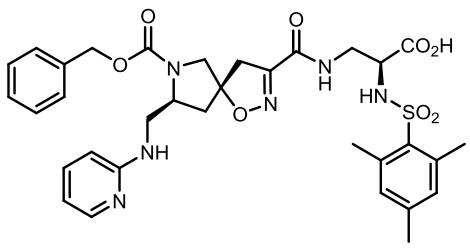
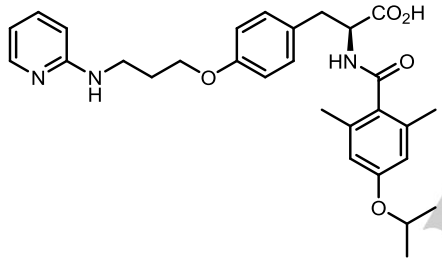
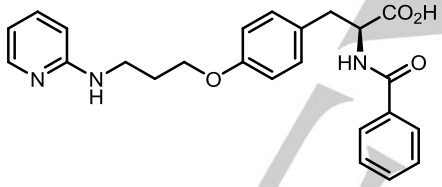
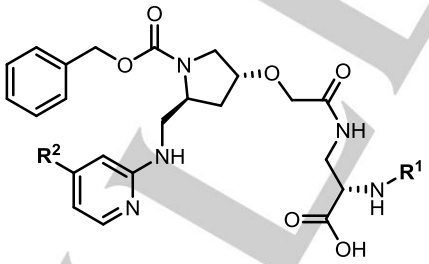
2) **Anchoring unit**. The anchor moiety should allow a strong binding to the surface. For this purpose, the surface can be modified (i.e. by silanization) to expose a wide range of functional groups that can be used to anchor the integrin ligands in a highly chemoselective manner. Moreover, the chemistry of the surface can be exploited to select substrate-specific anchors. For example, thiols bind to gold and phosphonates to titanium oxide (and other metal oxides) with high affinity. This topic has been covered with great detail in the literature.[25a,115]

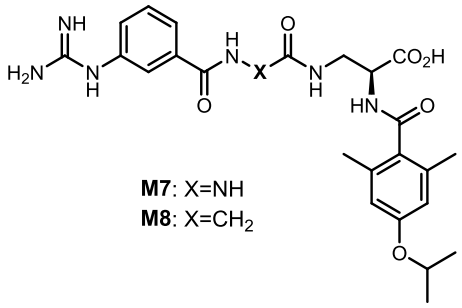
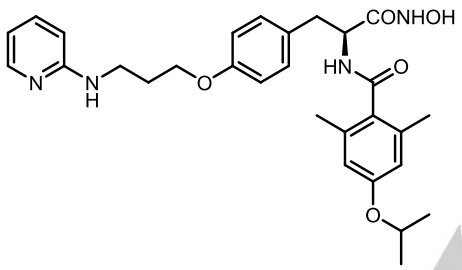
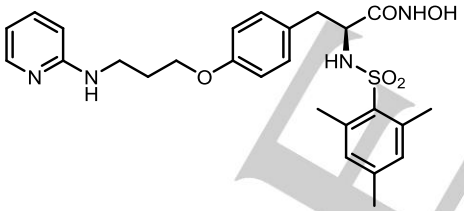
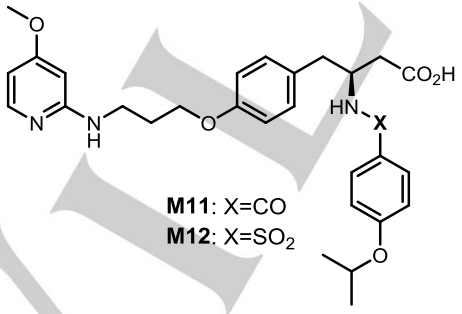
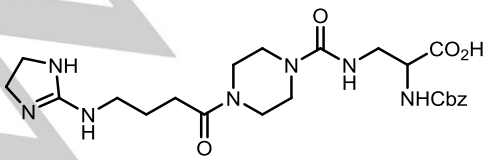
3) **Spacer units**. Though often underestimated, the use of a chemical spacer is a crucial element in the coating system. In particular, the importance in keeping a minimum distance (i.e. > 3.5 nm) between the RGD motif and the surface has been highlighted to engage integrin-mediated adhesion.[116] This distance can be achieved by using chemical spacers, which ensure a correct accessibility of the peptide and an adequate interaction with integrin receptors.[116,117] Chemical groups typically used as spacer units include polyglycine, aminohexanoic acid and polyethylene glycol (PEG). Choosing the right spacer might be a difficult task, since not only an optimal length but also other physicochemical properties need to be carefully considered. For instance, the hydrophilic or hydrophobic nature of the spacers and their conformation in solution play important roles as well. In a recent study we showed that polyproline helices can also be used as spacers.[118] Polyproline chains prefer an extended conformation in solution, whereas PEG chains adopt coiled conformations, which make difficult to assess the exact distance between the biomaterial and the integrin binding peptide.

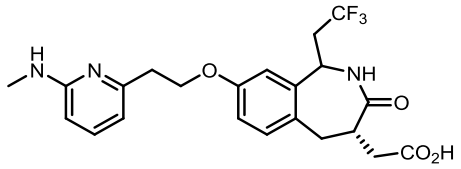
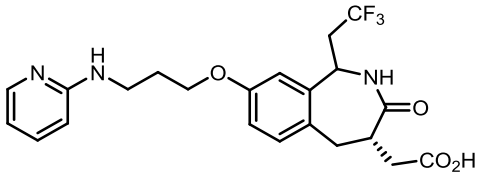
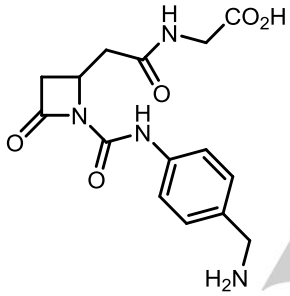
4.2. Coating with RGD peptides and proteins

The discovery of the RGD motif as a universal cell-recognition sequence was accompanied by a series of early studies in which synthetic peptides containing this sequence were used to promote cell attachment on different surfaces.[18] Since then, the RGD motif has been widely used to coat biomaterial surfaces, aiming at improving their bioactivity and conferring cell-instructing properties. Hence, RGD-biofunctionalized materials have been investigated for a myriad of biomedical purposes, including bone, neural and cardiovascular applications.[25a,119] In addition to RGD peptides, over the last years many other cell binding motifs (integrin-dependent or not) have been described. These findings have notoriously increased the molecular tools available for surface functionalization and expanded the initial potential of RGD peptides. For a comprehensive review of the state of the art in this field, the reader is referred to the current literature.[25b,115,120]

Table 2: Structure of representative RGD-based peptidomimetics with affinity for αvβ3 and/or α5β1 integrins

Compound	Structure	IC_{50} **αvβ3** [nM]	IC_{50} **α5β1** [nM]	Ref
M1		49	0.18	95
M2		279	0.7	99
M3		1.2	264	99
M4 **M5** **M6**	 **M4**: R^1= SO_2-mes; R^2 = H **M5**: R^1 = CO-mes; R^2 = H **M6** R^1 = CO-mes; R^2 = OMe	16 ~ 30000 3400	3.7 3.5 0.54	100

M7 **M8**		> 4750 9600	0.96 0.86	107
M9		13.5	40	109
M10		4.8	132	109
M11 **M12**		0.86 0.65	127 108	110
M13		1.1	660	111

M14		1.2[a]	110[a]	112
M15		0.9[a]	1000[a]	112
M16		11[b]	763[b]	113

[a] Integrin binding activities in [ref 112] are given as K_i values. [b] Integrin binding activities in [ref 113] are expressed as EC_{50} values using cells expressing either αvβ3 (SK-MEL-24) or α5β1 (K562).

Despite the versatility of RGD-based synthetic peptides, their use in biomaterials has found three major limitations. i) Peptides often display lower cell adhesive potential than full-length ECM proteins, mainly because they lack synergistic or complimentary domains present in native proteins and required for optimal cell signaling. ii) Linear peptides possess high conformational freedom and therefore fail to exhibit receptor selectivity. iii) Linear peptides and large cyclic peptides are susceptible to enzymatic cleavage and consequently easily degradable *in vivo*. As a result of these drawbacks, translation of promising *in vitro* data to successful *in vivo* outcomes has not been possible.[25b,120a] Alternatively, the use of ECM proteins appears as a most intuitive way to mimic the complexity of cell-matrix interactions. However, their use remains controversial and several disadvantages have also been reported including unwanted inflammatory responses, risk of infections, short biological half-life and rapid clearance.[25b,120a] These shortcomings are matter of extensive debate in the field and have urged the finding of newer strategies for surface functionalization.[121] Moreover, none of these classical strategies has achieved integrin-subtype selectivity. In the following, three representative approaches to improve the activity and selectivity of ECM-based molecules for surface coating are presented.

4.2.1. Coating with cyclic RGD peptides

As described above, the limited biological profiles of linear RGD peptides can be significantly improved by the use of cyclic counterparts.[68] Moreover, small cyclic peptides are stable against enzymatic cleavage, especially when they contain D-amino acids and/or *N*-methylated amide bonds. Structure-activity relationship studies of the stem αvβ3-binding ***c*(RGDfV)** (**Table 1**) peptide revealed that the amino acid at the 5th position (i.e. Val) was not essential for its integrin-binding activity.[72b] Such finding was of great value for applications in surface coating. For instance, replacement of valine by lysine in ***c*(RGDfK)** retains the integrin binding activity of the peptide but provides a new functional group that can be further functionalized (**Table 1**).[72b] This has allowed the production of αvβ3-binding cyclic peptides containing different spacer-anchor systems for coating a variety of surfaces. Given the importance of this integrin in bone biology, this peptide has been widely used to coat implant materials, showing improved levels of osteoblast adhesion *in vitro*[116,117a,122] and bone formation *in vivo*.[116b,123] As previously mentioned, a cyclic RGD has also recently been described to promote osteogenic differentiation on MSCs.[46] However, the fact that cyclic RGD peptides also

display some affinity for α5β1 does not allow ascribing the aforementioned biological effects univocally to the αvβ3 subtype.

4.2.2. Coating with engineered protein fragments

The production of protein fragments of the ECM by recombinant methods has also been exploited to achieve integrin selectivity, but mainly towards α5β1. The group of Garcia engineered a recombinant fragment of FN spanning the 7th to the 10th type III repeats of the protein (FN-III_{7-10}). This fragment, which contains the RGD sequence and the synergy motif PHSRN, directed α5β1-dependent adhesion of osteoblast-like cells, their spreading and assembly of FAs on functionalized surfaces.[35a] In a subsequent study, this fragment also showed enhanced values of osteoblast adhesion in comparison with a linear RGD peptide and the oligopeptide RGD-G_{13}-PHSRN. Interestingly, whereas cell adhesion on surfaces functionalized with FN-III_{7-10} was shown to be mediated by α5β1, binding of cells on the surfaces coated with the peptides was αvβ3-dependent.[35b] The lack of α5β1-binding activity for the RGD-G_{13}-PHSRN construct could be explained by the great flexibility of the polyglycine spacer, which may not match the optimal distance between the RGD and PHSRN motifs adopted in the context of the protein fragment and required for integrin binding.[124] Immobilization of FN-III_{7-10} on titanium surfaces also promoted differentiation of bone marrow stromal cells into osteoblasts and improved implant osseointegration *in vivo* compared to surfaces coated with linear RGD peptides[42b] or full-length FN.[43a] In a recent study, this fragment also improved osseointegration of stainless steel screws on healthy and osteoporotic rats.[43b] Other authors have also shown an enhanced osteogenic differentiation of human MSCs on surfaces functionalized with a recombinant fragment derived from the 9th and 10th type III domains (FN-III_{9-10}), compared to surfaces modified with the 10th type III domain of FN (FN-III_{10}), which does not contain the synergy binding site for α5β1.[37] Noteworthy, the extent of osteoblastic differentiation for each fragment was correlated with their selectivity towards α5β1. As previously discussed, these studies highlight a crucial role for α5β1 in cell adhesion, proliferation and differentiation, in comparison to a somewhat more modest role for αvβ3 in these processes. However, these studies used as comparison linear RGD peptides, which show low affinity for αvβ3. The positive biological outcomes obtained with highly αvβ3-active cyclic RGD peptides suggest a more important function for this integrin. Comprehensive studies in this regard are missing.

4.2.3. Coating with multiple peptide motifs

The combination of distinct peptides motifs to exert integrin selective effects is another interesting strategy. It takes advantage of the favorable properties of synthetic peptides compared to proteins, while it improves their activity and specificity. In this regard, we have recently introduced a novel peptide-based platform with the capacity to simultaneously present two distinct bioactive sequences on the surface of biomaterials.[125] In a proof of concept study, the combination of the RGD and PHSRN in this platform supported a very homogeneous spreading of osteoblasts all over the surface. In contrast, cell spreading on surfaces coated with a mixture of the two motifs was not homogeneous, probably due to the random orientation and spacing of the RGD and PHSRN sequences, which did not match the spatial conformation required for binding for α5β1.[124]

4.3. Coating with RGD-based peptidomimetics

The previous examples illustrate the extensive effort devoted to install integrin-selective activity on the surface of biomaterials. However, the majority of approaches in the literature still focus on RGD-containing peptides and proteins with relatively poor integrin receptor selectivity. This has hampered the dissection of integrin roles in cell behavior and also has resulted in frustrating pre-clinical outcomes. It is thus surprising that integrin-selective peptidomimetics are rarely applied as surface coating molecules: besides their capacity to exhibit excellent integrin-binding activity and subtype selectivity, non-peptidic ligands are devoid of the intrinsic pharmacokinetic limitations of peptides and proteins.

Probably one of the first examples reporting the use of an RGD peptidomimetic for surface coating was described by Marchand-Brynaert and coworkers at the end of last century.[126] In these studies, an RGD peptidomimetic based on a tyrosine scaffold (**Table 3**, **C1**) grafted on a poly(ethylene terephthalate) (PET) membrane supported the adhesion of adenocarcinoma epithelial cells (Caco-2) to similar levels than PET surfaces grafted with an RGDS peptide, but lower than surfaces coated with FN. Unfortunately, the activity of this compound was only evaluated for αIIbβ3 and thus the affinity for other subtypes is unknown. Although the authors showed that this compound could adopt a conformation similar to that of ***c*(RGDfV)**, thus indicating a potential activity for αvβ3,[126a] the fact that this compound failed to inhibit the binding of αvβ3-expressing cells to VN[126b] suggested a low affinity for this receptor. Also the flexibility of the compound could be associated with poor receptor selectivity. Nonetheless, this work demonstrated for the first time that cell binding properties of synthetic peptidomimetics could be recapitulated on the surface of biologically relevant materials. To increase the affinity for αvβ3, the guanidine function was rigidified with an isonipecotic group and the α-amino substituent replaced by a bulkier hydrophobic moiety (**C2** and **C3**, **Table 3**). Such modifications resulted in affinities for this integrin in the nanomolar range, however, selectivity against αIIbβ3 could not be attained.[127] In accordance with the improvement in αvβ3 activity, these compounds efficiently improved Caco-2 adhesion on PET surfaces, and inhibited integrin-mediated binding of these cells onto VN-coated materials. Noteworthy, cell adhesion capacity was higher compared to an RGDS control peptide.[128] In follow-up studies, exchanging the relative positions of the Arg surrogate and the spacer yielded highly active αvβ3 antagonists, which showed selectivity versus αIIbβ3 (**C4**, **Table 3**).[129] Grafting of these compounds on PET surfaces improved the adhesion of human endothelial cells.[129,130]

In another example, an Arg-Lys dipeptide, in which the C-terminus of Arg was bound to the ε-amino group of Lys, was produced as an RGD mimic (**C5**, **Table 3**).[131] This compound was very stable to enzymatic degradation in comparison to the linear RGD peptide. When immobilized on dextran-coated surfaces it promoted extensive adhesion and spreading of BALB/c-3T3 cells comparable to substrates grafted with GRGDSP. However, on the basis of the flexibility of this construct, modest integrin affinity and lack of receptor specificity are expected.

Squaramide-based RGD mimics have also been described. Immobilization of **C6** on self-assembled monolayers on gold substrates mediated stronger rates of cell adhesion, more mature stress fibers and higher numbers of FAs compared to a linear RGD control. These biological effects could be attributed to the increased rigidity of the squaramide moiety, which in turn would increase the affinity of the ligand for αvβ3. However, the authors did not check integrin binding affinity for their ligands.[132]

In parallel to these studies, we reported in 2004 the first example of a highly αvβ3-binding non-peptidic ligand selective against αIIbβ3 for surface coating (**C7**, **Table 3**).[133] This compound exhibited stimulated osteoblast adhesion on titanium to similar levels than the cyclic ***c*(RGDfK)** peptide, being also the first report to describe the functionalization of a metallic implant material with a non-peptidic ligand. In this study the importance of a suitable spacer was again illustrated, since the use of shorter linkers significantly reduced the adhesion of osteoblasts on the surfaces. At the time of this study there were still no α5β1-selective ligands reported and the affinity for this integrin was not studied.

The first example of surface functionalization with non-peptidic ligands capable of discriminating between αvβ3 and α5β1 was also reported by us one decade later,[108a] and was the result of our extensive work in the development of integrin-selective peptidomimetics (see Section 3). In this study, αvβ3- (**C8**) or α5β1-specific (**C9**) ligands (derived from the stem compounds **M11** and **M8**, respectively, **Table 2**) were immobilized on nanostructured gold surfaces and their capacity to mediate integrin-dependent cell adhesion was analyzed with genetically modified fibroblasts expressing either αvβ3 or α5β1. Noteworthy, αvβ3-expressing fibroblasts exclusively adhered on surfaces coated with **C8**. In contrast, surfaces functionalized with compound **C9** only supported the adhesion and spreading of fibroblasts expressing α5β1 (**Figure 3** and **Figure 4A**), providing striking evidence on the integrin-selectivity displayed by the surfaces. Noteworthy, integrin-mediated cell adhesion could be triggered through one specific integrin. Thus, this work opened new prospects to elucidate the role of these two subtypes in cell adhesion and other biological processes (see **Figure 4**).

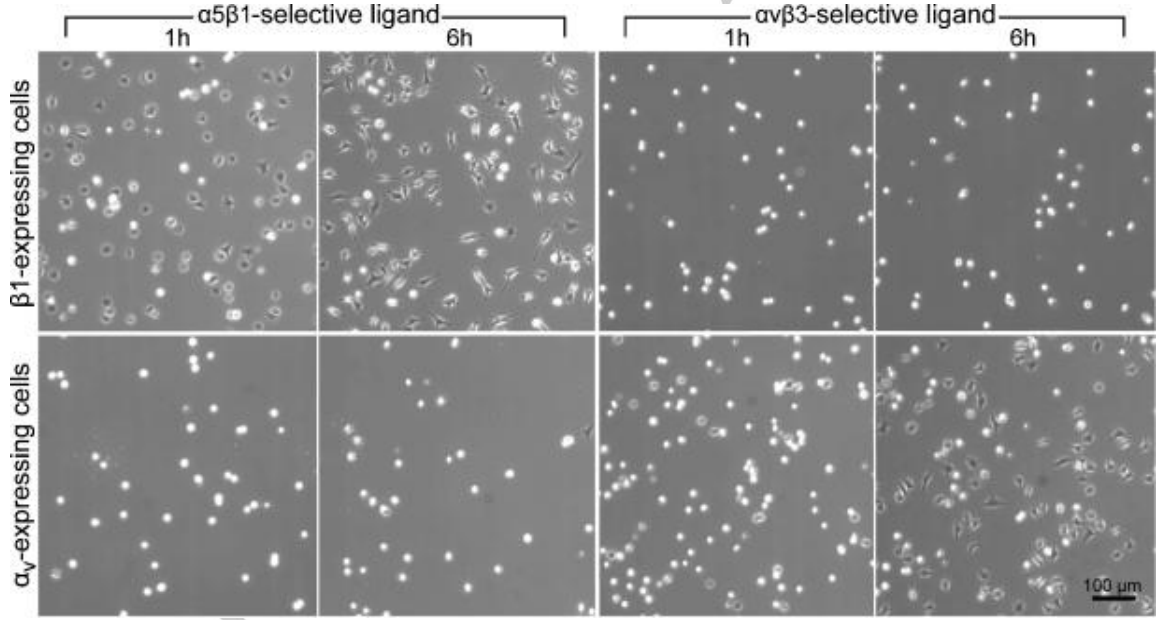


Figure 3. Cell adhesion assay. αvβ3-expressing fibroblasts adhere and spread on surfaces coated with compound **C8** (αvβ3-selective) but not on surfaces coated with **C9** (α5β1-selective). The opposite behavior is observed when cells expressing α5β1 are used. From [ref108a].

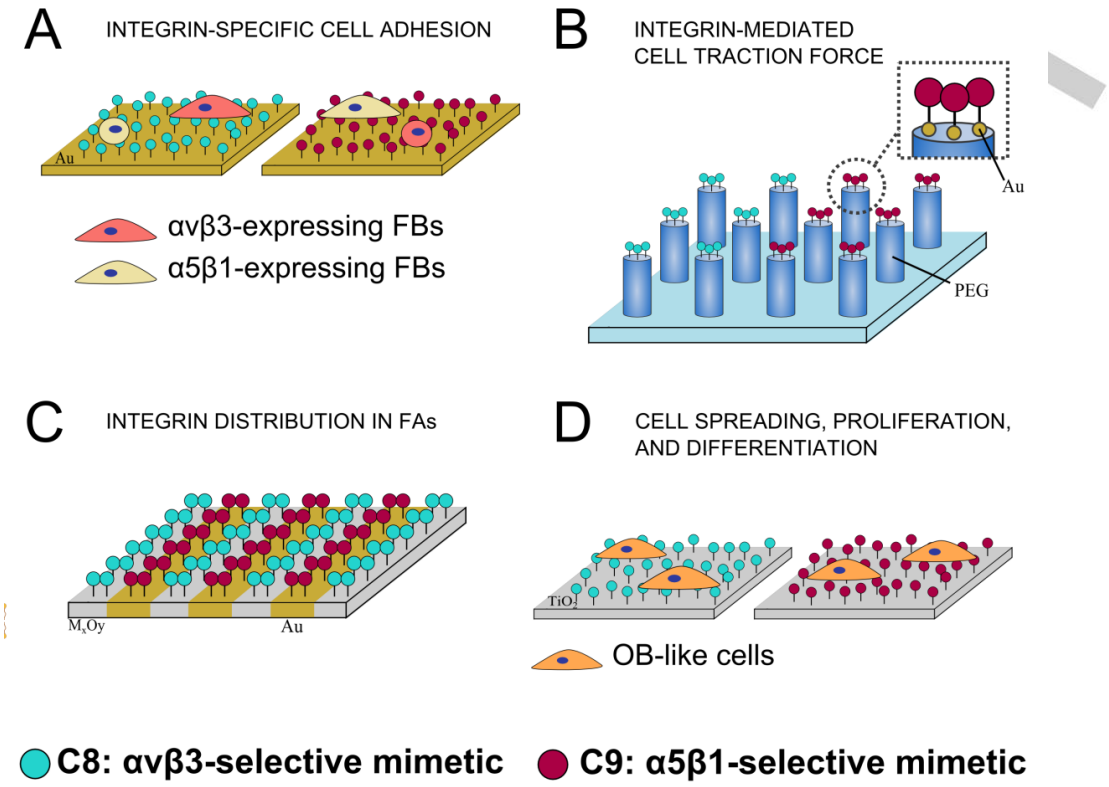


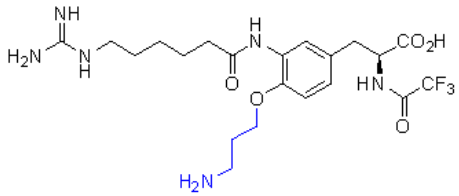
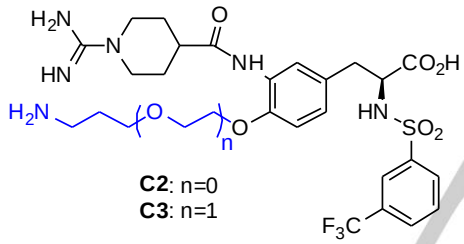
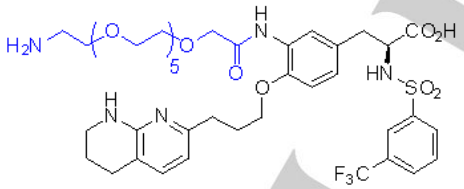
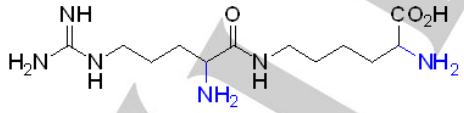
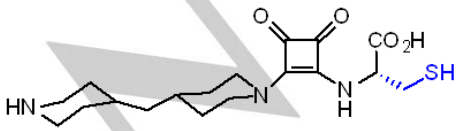
Figure 4. Application of αvβ3-selective (peptidomimetic **C8**) and α5β1-selective (peptidomimetic **C9**) ligands. These molecules can be used for biophysical studies of integrin-mediated cell adhesion (A) and cell traction forces (B), as well as to investigate integrin distribution (segregation vs. co-localization) in FAs (C). Moreover, integrin-selective peptidomimetics can also be used to install osteointegrative properties on the surface of implant materials (D).

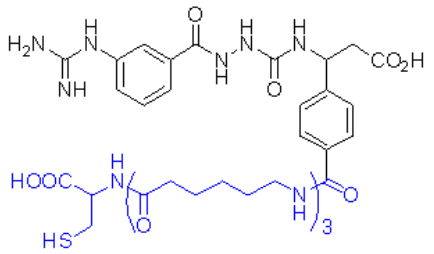
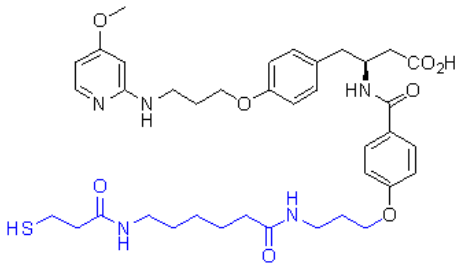
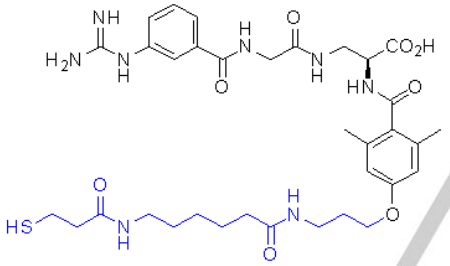
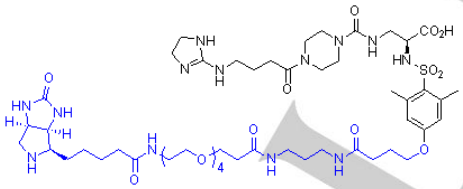
For example, in a subsequent study these compounds were bound to PEG-based micropillars, covered at the top with gold nanoparticles, to investigate the contribution of αvβ3 or α5β1 integrins to cell traction forces (**Figure 5B**).[32] Force measurements after seeding rat embryonic fibroblasts on these pillars revealed that cells binding to the pillars via α5β1 exerted higher forces on the pillars than cells binding through αvβ3, in good correlation with previous findings.[31] Integrin-mediated cell adhesion could also be achieved on titanium surfaces coated with the same compounds but functionalized with phosphonates as anchor molecules.[117b] To this end, a molecular toolkit based on click chemistry was established, which enables the modification of a large variety of surfaces in a straightforward manner. The possibility to use different anchors to coat surfaces of distinct chemistry has recently been exploited to construct binary micropatterned arrays in an orthogonal fashion (**Figure 5C**).[134] Thus surfaces containing alternating stripes of gold and metal oxide (i.e. Fe_2O_3 or TiO_2) were functionalized with the αvβ3- and α5β1-selective mimetics, respectively. To achieve an orthogonal attachment the αvβ3 ligand contained a thiol group, while the α5β1 ligand contained a phosphonate anchor. This strategy further allowed the incubation and segregation of integrins on the surface, obtaining arrays of αvβ3 integrins on the gold stripes and of α5β1 on the Fe_2O_3/TiO_2 stripes. Such binary system allowed the study of integrin distribution during FAs. Interestingly, on gold surfaces (αvβ3-selective) osteosarcoma U2OS cells showed clusters of αvβ3, whereas on metal oxide surfaces (α5β1-selective) colocalization of both αvβ3 and α5β1 in clusters was observed. These findings suggest that α5β1 activation may promote diffusion and recruitment of αvβ3 integrins through an inside-out signaling. Such intimate crosstalk between these two integrin subtypes warrants further investigations.

The group of Kiessling has also recently reported the use of peptidomimetics to study integrin-specific cell behavior on surfaces.[135] In this study, an αvβ3-inhibitor[136] was biotinylated (**C10**, **Table 3**) and immobilized on streptavidin-coated surfaces. The resulting surfaces were highly affine for αvβ3, (i.e. no binding of integrins αvβ5 or α5β1 was detected) and enhanced M21 melanoma cell adhesion and activated αvβ3-signaling. As a whole, these recent findings demonstrate that coating of surfaces with integrin-selective peptidomimetics can be used to unravel the specific role of integrin subtypes in cell adhesion processes.

Moreover, this strategy can also be used to dictate cell behavior on the surface of biomaterials. In this regard, we have recently coated titanium surfaces with compounds **C8** or **C9**, respectively, and studied the behavior of osteoblast-like cells on the surfaces.[137] For the first time, it was shown that integrin-binding peptidomimetics are able to support and promote all the biological processes required to ensure a reliable osseointegration of an implant material: the immobilization of these molecules on titanium significantly enhanced the attachment, spreading, proliferation, ALP production and mineralization of osteoblast-like cells (**Figure 5D**). Remarkably, the biological activity exhibited by these molecules was comparable to that observed on surfaces coated with native proteins of the ECM. These results are remarkable because they show an unprecedented biological activity for low-molecular-weight ligands, and demonstrate that the activity of complex ECM proteins can be recapitulated by synthetic integrin-binding ligands.

Table 3: Structure of representative RGD-based peptidomimetics with affinity for αvβ3 and/or α5β1 integrins

Code	Structure[a]	IC_{50} [nM]	Surface	Coating chemistry	Biological results	Ref
C1		αvβ3: n.r. αIIbβ3: 320000	poly(ethylene terephthalate) (PET)	i) oxidation of PET hydroxyl groups to carboxylic acids ii) amide bond formation by carbodiimide chemistry	Increased values of surface occupancy by Caco-2 cells ≈RGD <FN	126
C2 **C3**	 **C2**: n=0 **C3**: n=1	**C2;** αvβ3: 63 αIIbβ3: 11 **C3;** αvβ3: 765 αIIbβ3: 5	PET	i) tosylation of PET hydroxyl groups ii) nucleophilic substitution	Increased values of surface occupancy by Caco-2 cells >RGD <VN	127, 128
C4		αvβ3: 0.7 αIIbβ3: 51	PET	i) activation of PET hydroxyl and carboxyl groups with trifluorotriazine ii) aromatic nucleophilic substitution	Improved adhesion of human endothelial cells >RGD	129, 130
C5		αvβ3: n.m. αIIbβ3: n.m.	dextran-coated TCPS	i) oxidation of dextran hydroxyl groups to aldehydes ii) Nucleophilic addition iii) Schiff base reduction	Enhanced adhesion and spreading of BALB/c-3T3 ≈RGD	131
C6		αvβ3: n.m. αIIbβ3: n.m.	SAMs of PEGylated alkanethiols on gold	thiol addition to squaramate reactive moiety	Faster and stronger cell attachment, mature stress fibers and more focal adhesions >RGD ≈c(RGDfK)	132

C7		αvβ3: 0.72 αIIbβ3: 3150	Ti6Al4V	thiol binding to metal oxide	Enhanced adhesion of MC3T3 mouse osteoblasts	133
C8		αvβ3: 1.8 α5β1: 130	a) Gold b) Ti6Al4V /metal oxide c) TiO_2	a) thiol binding to gold b) the thiol group is replaced by a phosphonate anchor c) binding via Michael-addition to a maleimide containing silane layer	Multiple (see Fig. 5 for details)	108a, 32, 117b, 137
C9		αvβ3: 229 α5β1: 1.5	Idem as C8	Idem as C8	Idem as C8	108a, 32, 117b, 137
C10		αvβ3: 0.47[b]	streptavidin-coated polystyrene	biotin-streptavidin binding	Enhanced adhesion of M21 melanoma cells, activation of integrin αvβ3 ≈cRGD > VN, FN (integrin specificity)	135

[a] The bioactive moiety of the molecules is depicted in black; the spacer-anchor units in blue. [b] Integrin binding activity in [ref 135] is given as K_d value. Binding of αvβ5 or α5β1 was not detected.

5. Summary and outlook

The development of integrin-selective ligands for surface coating has been a long and challenging journey. In this regard, the use of integrin-binding molecules as surface coating agents has evolved over the last 3 decades (**Figure 5**). Early studies focused on the use of RGD-containing peptides and proteins with, in general, relatively low integrin-binding activity and receptor selectivity. The biological profile of these ligands has been improved by different approaches, including the development of cyclic RGD peptides and fragments derived from FN. In 2004 we published the first coating of a surface with a highly active peptidomimetic for αvβ3 and selective against αIIbβ3.[133] Selectivity between αvβ3 and α5β1 on a surface was not achieved until 2013.[108a]

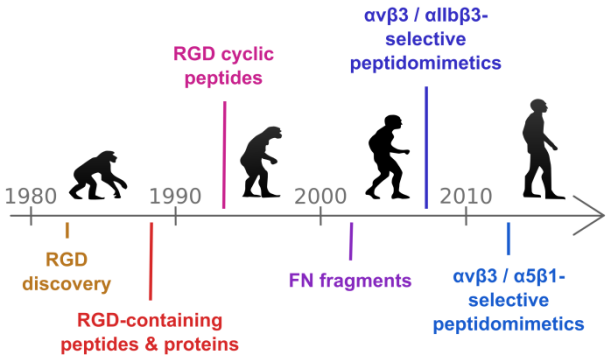


Figure 5. Evolution of surface coating strategies over the last decades. The principal milestones in the field are highlighted.

Peptidomimetics overcome the majority of limitations displayed by peptides and proteins. They exhibit very potent integrin-binding affinities (with IC_{50} values in the sub- to nanomolar range) and excellent selectivity. Additionally, they are highly stable to enzymatic degradation, changes of pH and temperature, and are devoid of immunogenic responses. They can furthermore be immobilized on the surface at high densities and produced in a large scale. It is thus surprising their use has been rarely documented in the literature. This could be attributed, in part, to the fact that their production requires expertise in synthetic organic chemistry, which may discourage scientists from other fields. Moreover, their development often requires comprehensive structure-activity relationship studies and achieving receptor selectivity is not straightforward. Last but not least, the functionalization of the peptidomimetic ligand for surface coating without loss of biological activity is another crucial issue.

This review illustrates these aspects and provides examples of peptidomimetic design and their application in surface coating. Insights from these studies may help to produce novel types of mimetics with improved selectivity profiles and increased affinity for other integrins. The studies described in this review have demonstrated the following:

1. Surfaces with integrin selective peptidomimetics (i.e. capable of discriminating between αvβ3 and α5β1) are very useful tools to elucidate the role of integrins in cell biology. The possibility to selectively engage one integrin subtype opens new prospects in the study of integrin-mediated signaling, for instance in stem cell differentiation, where the specific role of integrin subtypes remains unknown.

2. The capacity to elicit integrin activation can be used to tailor cell specific responses and modulate cell behavior on biomaterials for regenerative purposes. For instance, peptidomimetics have been proposed as promising molecules to improve the osseointegration of implant materials. Their application in the regeneration of other organs and tissues has yet to be explored.

Further studies with these types of molecules are thus warranted. Given the importance of αvβ3 and α5β1 in the osteogenic differentiation of MSCs, integrin selective surfaces could be used to tune the differentiation of these cells into the osteoblastic lineage. Moreover, the functionality of these peptidomimetic-coated biomaterials to promote bone growth has not been proven *in vivo*. Both strategies are currently being investigated in our laboratories.

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Keywords: RGD peptides • peptidomimetics • integrins • surface coating • receptor selectivity

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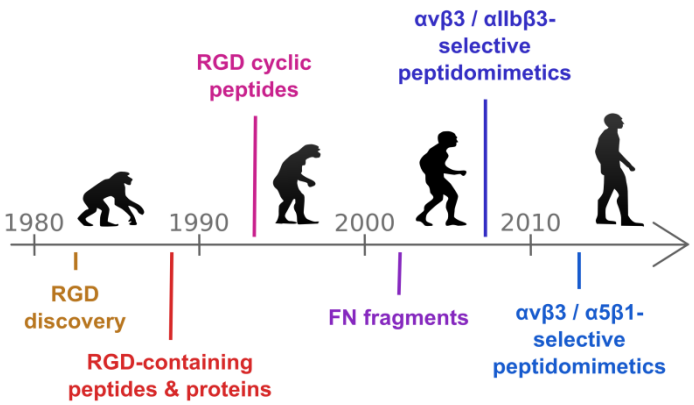

Surface coating has evolved from RGD-based peptides and proteins with relatively poor integrin-binding activity and selectivity to peptidomimetics with high affinity and receptor subtype selectivity. This review highlights the most representative milestones in this amazing journey.

Carlos Mas-Moruno, Roberta Fraioli, Florian Rechenmacher, Steffanie Neubauer, Tobias Kapp, and Horst Kessler**

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The quest towards αvβ3- or α5β1-integrin selective peptidomimetics for surface coating - History, recent advances and future perspectives