# Tumor angiogenic vasculature targeting with PAMAM dendrimer–RGD conjugates†

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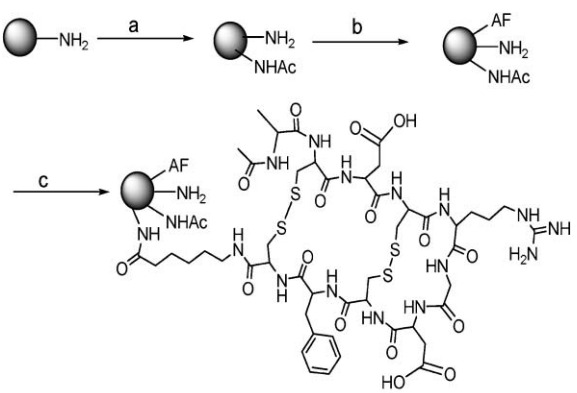
**PAMAM dendrimer–RGD-4C peptide conjugate was synthesized and *in vitro* targeting efficacy to integrin receptor expressing cells was studied by flow cytometry and confocal microscopy.**

Drug targeting is critical for effective cancer chemotherapy. Targeted delivery enhances the chemotherapeutic effect and spares normal tissues from the toxic side effects of these powerful drugs. Antiangiogenic therapy prevents neovascularization by inhibiting proliferation, migration and differentiation of endothelial cells.[1] The identification of molecular markers that can differentiate newly formed capillaries from their mature counterparts paved the way for targeted delivery of cytotoxic agents to the tumor vasculature.[2–4] The $\alpha_V\beta_3$ integrin is one of the most specific of these unique markers. The $\alpha_V\beta_3$ integrin is found on the luminal surface of the endothelial cells only during angiogenesis. This marker can be recognized by targeting agents that are restricted to the vascular space during angiogenesis.[5,6] High affinity $\alpha_V\beta_3$ selective ligands, Arg-Gly-Asp (RGD) have been identified by phage display studies.[7] The doubly cyclized peptide (RGD-4C, containing two disulfide linkages *via* four cysteine residues) and a conformationally restrained RGD bind to $\alpha_V\beta_3$ more avidly than peptides with a single disulfide bridge or linear peptides. There has been growing interest in the synthesis of polymer–RGD conjugates for gene delivery,[8] tumor targeting[9] and imaging applications.[10]

PAMAM dendrimers are biocompatible, nonimmunogenic, water soluble and have been coupled to many biological molecules such as proteins, synthetic drugs and small molecules.[11] Dendrimers can be coupled to multiple $\alpha_V\beta_3$ selective ligands (RGD-4C) to target tumor associated capillary beds and allow the delivery of cytotoxic agents to kill the new vessels. In this report we describe the synthesis of RGD-4C conjugated to fluorescently labeled generation 5 dendrimer. Additionally we have studied the binding properties and cellular uptake of these conjugates. Amine terminated dendrimers are reported to bind to the cells in a non specific manner owing to positive charge on the surface. In order to improve targeting efficacy and reduce the non specific interactions, amine terminated G5 dendrimers were partially surface modified with acetic anhydride (75 molar%) in the presence of triethylamine as base.[12] The conjugate was purified by dialysis against PBS buffer initially and then against water. The use of 75 molar excess of acetic anhydride leaves some amine groups for further modification and prevents problems arising out of aggregation, intermolecular interaction and decreased solubility. The degree of acetylation and purity of acetylated G5 dendrimer (G5-Ac) were monitored using $^1$H NMR spectroscopy, which shows a distinct signal for the terminal NHCOC$H_3$ protons of the dendrimer at $\delta$ 1.85. The $^{13}$C NMR spectrum also showed a new peak at $\delta$ 174.4 for the carbonyl carbon of the acetyl group. The degree of acetylation was measured by comparing the ratio of $NHCOCH_3$ protons and the sum of all methylene protons in the dendrimer to a calibration curve as described previously.[12]

For detection of conjugates by flow cytometry or confocal microscopy a detectable fluorescent probe is needed. We used Alexa Fluor 488 (AF) as fluorescent label because it is significantly brighter than fluorescein conjugates and is also much more photostable. The partially acetylated dendrimer was reacted with 5 molar equivalents of Alexa Fluor-NHS ester as described in the manufacturer's protocol to give fluorescently labeled conjugate (G5-Ac-AF) (Scheme 1). This conjugate was purified by gel filtration and subsequent dialysis. The conjugate shows an absorption peak at $\lambda_{max}$ 498 nm. The $^1$H NMR spectrum of the conjugate shows broad peaks for aromatic protons of the dye molecule. The peaks of AF were significantly broadened upon conjugation to the dendrimer and also the peak at $\delta$ 6.86 was shifted downfield to 7.02 ppm. In the $^{13}$C NMR spectrum an additional peak due to amide carbonyl at $\delta$ 174.7 apart from the aromatic carbons from dye was observed. The number of dye molecules was estimated to be ~3 per dendrimer by $^1$H NMR and




a)$Ac_2O$, $Et_3N$ b) AF-NHS ester c) RGD4C, EDC, HOBt

**Scheme 1** Synthesis of G5-Ac-AF-RGD.

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UV-vis spectroscopy as described in the manufacturer's protocol (Molecular Probes).

The RGD peptide used in this study (RGD-4C) has a conformationally restrained RGD sequence that binds specifically with high affinity to $\alpha_V\beta_3$. The RGD binding site in the heterodimeric $\alpha_V\beta_3$ integrin is located in a cleft between the two subunits. In order to keep the binding portion of the peptide exposed to the target site, we used an ε-Aca (acylhexanoic acid) spacer to conjugate the peptide to the dendrimer. A protonated $NH_2$ terminus of the RGD-4C peptide is not essential for biological activity, therefore the $NH_2$ terminus was permanently capped with an acetyl group.[13] An active ester of the peptide (5 equivalents) was prepared by using EDC in a DMF/DMSO solvent mixture in presence of HOBt, and then this was added dropwise to the aqueous solution of the G5-Ac-AF.[14] The reaction times are 2 and 18 h, respectively. The partially acetylated PAMAM dendrimer conjugated with Alexa Fluor and RGD peptide, G5-Ac-AF-RGD was purified by membrane filtration and dialysis. The $^1$H NMR of the conjugate shows overlapping signals in the aromatic region for both the Alexa Fluor and phenyl ring of peptide apart from the expected aliphatic signals for the dendrimer overlapping with some aliphatic signals from the peptide. The UV-vis spectrum of the conjugate shows a characteristic peak for conjugated peptide at $\lambda_{max}$ 275 nm. The number of peptides was calculated to be 2–3 peptides per dendrimer based on MALDI-TOF mass spectroscopy and Ellman assay‡. MALDI-TOF MS has been a widely used technique for characterization of surface functionalization of heterogeneously functionalized dendrimers.[15] The cellular uptake of dendrimer–RGD-4C conjugate was measured in Human umbilical vein endothelial cells (HUVEC) that express a high cell surface $\alpha_V\beta_3$ receptor. In brief, HUVEC cells were cultured in RPMI medium supplemented with endothelial cell growth factor. The cells were treated with different concentrations of G5-Ac-AF-RGD conjugate and the uptake was monitored by flow cytometry. As shown in Fig. 1, flow cytometric analysis showed a dose-dependent and saturable binding to the HUVEC cells whereas control compound G5-Ac-AF showed minimal binding.

We also tested the binding of this conjugate to several different cell lines with varying levels of integrin receptor expression using flow cytometry (Fig. 2). The conjugate showed different binding affinities to various cell lines with HUVEC cells binding to the conjugate most effectively followed by Jurkat cells. The human

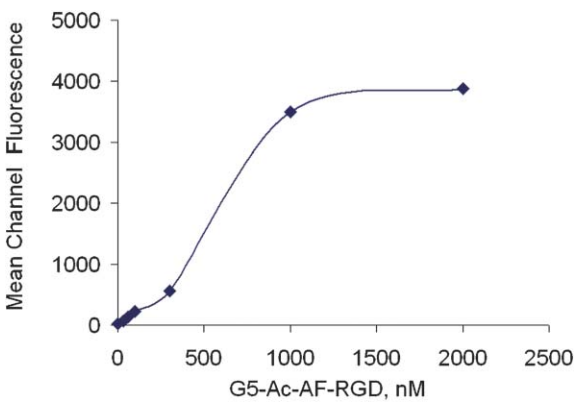


**Fig. 1** Binding G5-Ac-AF-RGD conjugate to HUVEC cells.

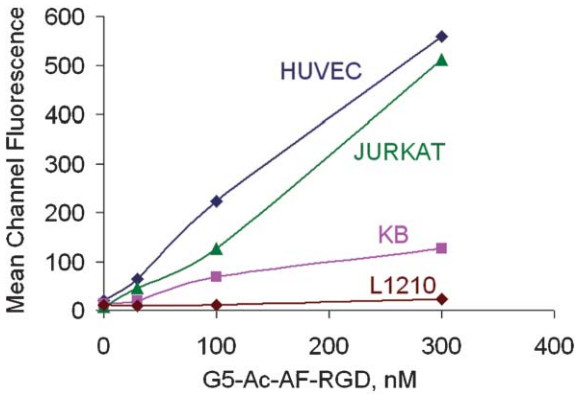


**Fig. 2** Binding of G5-Ac-AF-RGD conjugate to various cell lines.

lymphocyte cell line Jurkat has previously been reported to have a large number of integrin receptors and was able to bind to RGD-4C peptide.[16] The L1210 mouse lymphocyte line failed to bind the conjugate, whereas the KB cells showed only moderate binding.

From this study it is evident that this conjugate shows variable specificities for cell lines having different levels of cell surface integrin receptor expression. The binding seen by flow cytometry was confirmed by confocal microscopic analysis. HUVEC cells treated with G5-AF-RGD-4C (0, 30, 60, 100 nm) concentrations were washed and fixed with p-formaldehyde, the nuclei were counterstained with DAPI. It is evident from the appearance of fluorescence in confocal microscopic images in Fig. 3 that the uptake increases with the increasing concentration of the conjugate.

We found that addition of excess free peptide to inhibit the uptake of the conjugate by HUVEC cells blocked the uptake to a significant level indicating receptor mediated uptake of the conjugate (Fig. 4). However, the incomplete inhibition of uptake could be attributed to very high avidity of dendrimer conjugate when compared with free peptide.

In order to ascertain if polyvalent interaction shows stronger binding when compared to monovalent interaction, the binding

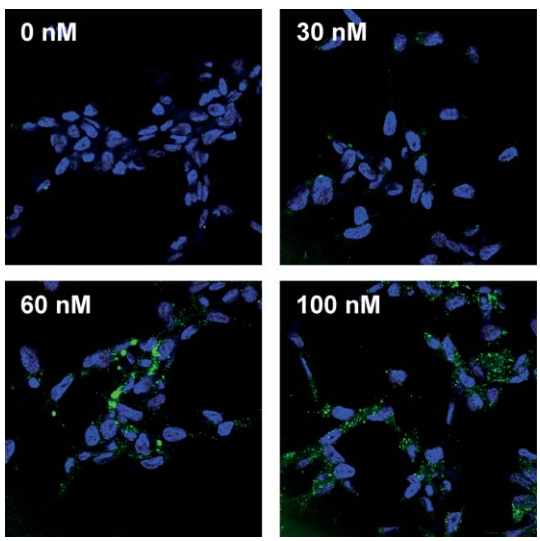


**Fig. 3** Dose dependent binding of G5-Ac-AF-RGD conjugate to HUVEC cells determined by confocal microscopy.

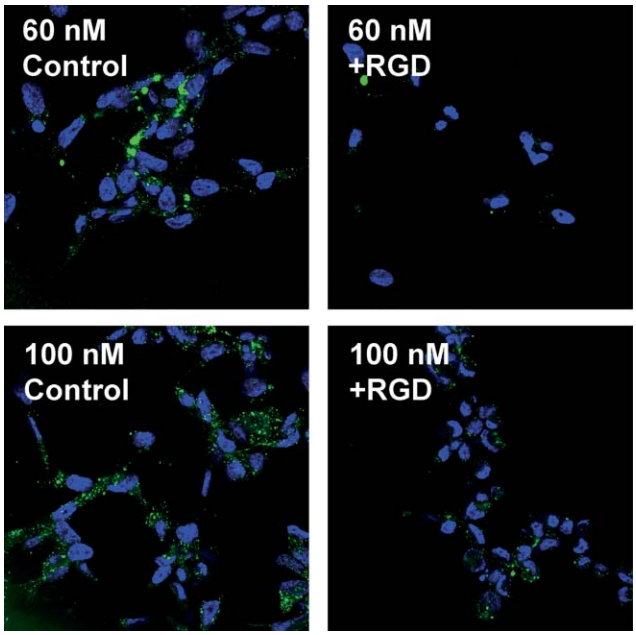


**Fig. 4** Inhibition of uptake of G5-Ac-AF-RGD in HUVEC cells with free peptide.

affinities of G5-Ac-AF-RGD-4C conjugate and RGD-4C peptide were monitored on human integrin αvβ purified protein (Chemicon International, Inc., Temecula, CA) using a BIAcore instrument (BIAcore AB, Uppsala, Sweden). The obtained data for both analytes were analyzed by global fitting to a bivalent binding model using the BIAevaluation 3.2 software (BIAcore AB). The equilibrium dissociation constants ($K_D$) were calculated from the ratio of the dissociation and association rate constants ($k_{off}/k_{on}$). The binding of the free RGD-4C peptide to the human integrin $\alpha v\beta_3$ was very rapid in reaching a maximum binding of 10 RU. In contrast, the binding of the G5-Ac-AF-RGD-4C conjugate was less rapid, reaching a maximum binding of approximately 1500 RU. Both analytes showed different off-rates. The free RGD-4C peptide rapidly dissociated from the ligand during the washing time with running buffer. The nanodevice dissociation was approximately 522 times slower as compared to the free peptide. This result suggests that multiple peptide conjugation on a single dendrimer exerts a synergistic effect on binding efficacy.

In summary we have synthesized PAMAM dendrimer RGD-4C peptide conjugate which was taken up by cells expressing $\alpha_V\beta_3$ receptors. This conjugate can be potentially used to direct imaging agents and/or chemotherapeutics to angiogenic tumor vasculature.

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## Notes and references

‡ Mass spectra were recorded on a Waters TOfspec-2E, run in delayed extraction mode, using the high mass PAD detector and calibrated with BSA in sinapinic acid. To determine the functionalization of the dendrimer with peptide (*m*/*z* 29650 $[M + H]^+$) of the starting material was subtracted from the (*m*/*z* 32770 $[M + H]^+$) of the product.

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