Supplementary Material (ESI) for Chemical Communications

Supplementary information

TUMOR ANGIOGENIC VASCULATURE TARGETING WITH PAMAM DENDRIMER–RGD CONJUGATES

Rameshwer Shukla, Thommey P. Thomas, Jennifer Peters, Alina Kotlyar, Andrzej Myc and James R. Baker Jr.*

Contents:

General:

All solvents and reagents were purchased from Aldrich and used as received. Alexa Fluor-NHS ester was obtained from Molecular Probes and RGD-4C peptide was synthesized by SynPep corp. All the reactions were carried out under N_2 atmosphere unless stated otherwise. NMR spectra were recorded on a Bruker AVANCE DRX 500 instrument. UV spectra were recorded using Perkin Elmer UV/VIS Spectrometer Lambda 20.

Synthesis of G5 dendrimer. The PAMAM G5 dendrimer was synthesized and characterized at the Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan. The synthesized dendrimer has been analyzed and the molecular weight was found to be 26,530 g/mol by GPC and the average number of primary amino groups was found to be 108 by potentiometric titration.

Synthesis of G5-Ac: G5 amine dendrimer (0.265 g, 0.0099 mmol), triethyl amine (0.088 g, 0.8635 mmol) were dissolved in 30 mL anhydrous MeOH. After 30 min stirring acetic anhydride (0.0712 g, 0.6974 mmol) in anhydrous MeOH (15 ml), was added dropwise while stirring. Reaction mixture was allowed to stir overnight at room temp. After evaporation of solvent the residue was dissolved in H_2O and dialyzed in 10,000 MWCO regenerated cellulose dialysis bags initially against PBS buffer followed by water. The partially acetylated dendrimer was lyophilized to give a colorless powder (0.270g, 91.2%). The average number of acetyl groups (80) has been determined based on ^1H NMR calibration curve drawn by plotting a ratio of acetyl protons and sum of all methylene protons *vs* degree of actetylation.

G5-Ac-AF: G5-Ac (0.0345 g, 1.237 X 10^{-3} mmol) was dissolved in 3.0 ml PBS. Alexa Fluor 488-NHS ester (3.0 mg, 4.665 X 10^{-3} mmol) was dissolved in 0.6 mL DMSO and added dropwise to reaction mixture while stirring under N_2. The reaction mixture was allowed to stir overnight. Concentration of the reaction mixture by membrane filtration and further purification on a G-25 Sephadex column gave the dendrimer conjugate which was further purified by membrane filteration and lyophilized to give orange powder (0.029 g, 80.5 %).

G5-Ac-AF-RGD: To a solution RGD-4C (0.003g, 2.41 X 10^{-3} mmol) in DMSO was added EDC (0.001g, 5.230 X 10^{-3} mmol) and HOBt (0.001g, 7.35 X 10^{-3} mmol), the mixture was allowed to stir for 2h. The above mixture was added to a solution of G5(Ac)-AF (0.020 g, 6.422 X 10^{-4} mmol) in PBS buffer and stirred for 18h. The reaction mixture was concentrated, gel filtered on G-25 sephadex column, purified by membrane filtration and lyophilized (0.018 g, 86.9%).

Cell Culture and In Vitro Experiments

Cells were obtained American Type Tissue Collection (Manasass, VA). All the cells were supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin and were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. HUVEC cells were grown in RPMI medium supplemented with heated-inactivated FBS, 120 µg/mL endothelial cell growth supplement and heparin 10 mg/ml. Jurkat and KB cells were grown in RPMI medium with and without folic acid respectively. The L1210 cells were maintained in DMEM medium.

Flow Cytometry:

The standard fluorescence of the dendrimer solutions was quantified using a Beckman spectrofluorimeter. For flow cytometric analysis of the uptake of the targeted polymer, cells were trypsinized and suspended in PBS containing 0.1% bovine serum albumin (PBSB) and analyzed using a Becton Dickinson FACScan analyzer. The FL1 fluorescence of 10 000 cells was measured, and the mean fluorescence of gated viable cells was quantified.

Confocal Microscopy:

HUVEC cells were seeded at a density of 5 x 10^5 cells/plate on glass bottom culture dishes (Mattek, Ashland, MA) two days prior to the experiment. Before each experiment, cells were washed three times in Dubelco's phosphate buffered saline (pH = 7.4) and incubated with 0, 30, 60, 90 and 100 nm solution of G5-Ac-AF-RGD. The cells were fixed with 2% p-formaldehyde for 15 min at room temperature, washed and mounted with a coverslip using Prolong Gold (Molecular Probes, Eugene, OR). Cells were

imaged on an Olympus FluoView 500 laser scanning confocal microscope with a 60X, 1.4 NA objective and pinhole settings resulting in an optical section thickness of 0.5 μm.

Surface Plasmon Resonance spectroscopy :

The affinity of G5-Alexa Fluor-RGD conjugate was tested on human integrin αvβ3 purified protein (Chemicon International, Inc. Temecula, CA) a BIAcore X instrument (BIAcore AB, Uppsala, Sweden). The running buffer for protein immobilization was HBS-EP (150 mM NaCl, 3 mM EDTA, 10 mM HEPES, 0.005% P20, pH 7.4).
The loading was performed according to previously described method (Löfås and Johnsson, J. Chem. Soc. Chem. Commun. 21, 1526-1528.). The carboxyl groups on the sensor surfaces were activated with an injection of a solution containing 0.2 M *N*-ethyl-*N'*-(3-diethylamino-propyl) carbodiimide (EDC) and 0.05 M *N*-hydroxysuccinimide (NHS). The specific surfaces were obtained by injecting integrin. This protein was diluted in 10 mM acetate buffer at pH 4 and used at concentration of 80 μg/ml. The binding of the purified ligand was measured at a concentration of 10065 RU (arbitrary resonance units). The RGD at a concentration of 500 μM diluted in running buffer was injected at a flow rate of 5 μl/min for 180 s (total volume 30 μl) at 25°C. The specific binding signal was obtained by subtracting the background signal, routinely obtained by injection of the RGD sample over an activated-deactivated surface. Regeneration of the surfaces was achieved by injection of 5 μl of 10mM glycine-HCl, pH 2.5. After regeneration of the surface, the G5-AF-RGD conjugate (1μM) was injected at a flow rate of 5 μl/min for 180 s (total volume 30 μl) at 25°C. The data for both analytes were analyzed by global fitting to a bivalent binding model using the BIAevaluation 3.2

software (BIAcore AB). The apparent equilibrium dissociation constants (K_D) were calculated from the ratio of the dissociation and association rate constants (k_{off}/k_{on}). The binding capacity of the surface depends on the levels of immobilized ligand (integrin). A theoretical R_{max} value was calculated using the formula:

R_{max}= (analyte MW/ligand MW)*immobilized amount * stoichiometric ratio

for RGD free peptide

R_{max}= (33875/1243)*10065*1 = 52 RU (experimental 10 RU)

for PAMAM dendrimer-RGD conjugate

R_{max}= (33875/237000)*10065*2 = 2877 RU (experimental 1500 RU)

A theoretically calculated R_{max} is often higher than experimentally derived R_{max} for the same interaction. There are many potential explanations for this, e.g. that the ligand is not fully active or that there is steric hindrance of the interaction.