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RESEARCH ARTICLE

Asymmetric Peptide Dendrimers are Effective Linkers for Antibody-Mediated Delivery of Diverse Payloads to B Cells in Vitro and in Vivo

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ABSTRACT

Purpose Safe, targeted delivery of therapeutics remains a focus of drug/gene delivery, the aim being to achieve optimal efficacy while minimising off-target delivery. Dendrimers have a vast array of potential applications and have great potential as gene and drug delivery tools. We previously reported the development of peptide dendrimers that effectively complexed DNA and that have distinct advantages over conventional spherical dendrimers. Here, to expand the application of peptide-based low generation dendrimers we tested their capacity to be transformed into linkers for antibody-based targeting of diverse payloads.

Methods Peptide-based low-generation asymmetric dendrimers were generated and conjugated to partially-reduced antibodies specific for B cell surface antigens or an irrelevant antigen. Preservation of antigen binding by the antibodies and targeting of the conjugated dendrimers carrying a small molecule (biotin) or plasmid DNA payloads was tested.

Results Peptide-based low generation dendrimers were efficiently and site-specifically conjugated to antibodies with retention of antigen-binding capacity. Altering the branching termini of dendrimers facilitated delivery of diverse payloads *in vitro* and *in vivo*.

Conclusions We propose that safe, non-toxic peptide dendrimers, which are readily synthesised and modifiable for a variety of applications, form the basis of a new family of biocompatible "linkers" with substantial potential for targeted delivery applications.

KEY WORDS B cell · Dendrimer; DNA · Monoclonal antibody · Targeting

ABBREVIATIONS

PAMAM	Polyamidoamine
PPI	Polypropylenimine
PEI	Polyethylenimine
PAGE	Polyacrylamide gel electrophoresis
2MEA	2-mercaptoethanolamine
MAb	Monoclonal antibody
FITC	Fluoroscein isothyocyanate
PE	Phycoerythrin
APC	Allophycocyanin

INTRODUCTION

Dendrimer technology has emerged as a versatile tool, being applied across a wide variety of fields, ranging from disciplines as diverse as photoelectronics, to medicine [1, 2] From a clinical perspective, the safe and effective delivery of therapeutic payloads such as genes or small molecule agents using dendrimers has received considerable attention over the past decade [3, 4] Commercially-available high generation (G4 – G8) spherical dendrimers (*e.g.* PAMAM, PPI, PEI) serve this purpose well, at least in the *in vitro* setting. For *in vivo* applications, high generation spherical dendrimers suffer from a number of drawbacks, most notably significant cytotoxicity, due to their high cationic charge density [5] Furthermore, spherical dendrimers present a major challenge when attempting to tether targeting-ligands, as the surface-active groups possess identical reactivity, making site-selective

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coupling of therapeutics impractical, with attempts typically laborious and resulting in a heterogeneous mixture of end products, complicating purification of the target [6–8]. Additionally, such dendrimers are constructed from synthetic monomers that when metabolised can give rise to toxic by-products. Low-generation peptide-based asymmetric dendrimers on the other hand are designed with all of these shortcomings in mind, representing an exciting viable alternative to currently-available spherical dendrimer systems.

The laborious and inefficient nature of solution phase synthesis, particularly in the case of highly dendritic molecules renders commercial dendrimers, such as PAMAM, PEI and PPI prohibitively expensive. Low-generation peptide-based asymmetric dendrimers are, on the other hand, readily prepared using well-established and high-yielding solid phase synthesis methodologies alongside orthogonal protecting group chemistries [9]. With a view to specifically testing the capacity of asymmetric dendrimers to serve as "targetable" delivery agents we constructed peptide-based asymmetric dendrimers featuring a dual-protected and thiol-reactive functionality on their peptide backbone to permit conjugation with a targeting-ligand, in this case, a reduced monoclonal antibody (mAb) that specifically binds B cells. For *in vitro/vivo* tracking purposes the branched head groups of the dendrimer were terminated with biotin as a "model therapeutic" which allowed dendrimer distribution to be traced using streptavidin-fluorochrome complexes. Furthermore, to assess the broader applicability of this system for delivery of macromolecules mAb-dendrimer conjugates were complexed with biotinylated DNA and their distribution traced.

Here we report that asymmetric peptide dendrimers could be readily and reliably conjugated with partially reduced mAb and that the resulting conjugates were preferentially bound by cells "targeted" by the conjugated mAb. Using this approach we demonstrate targeted delivery of a small molecule (biotin) payload *in vitro* and *in vivo* and delivery of DNA *in vitro*.

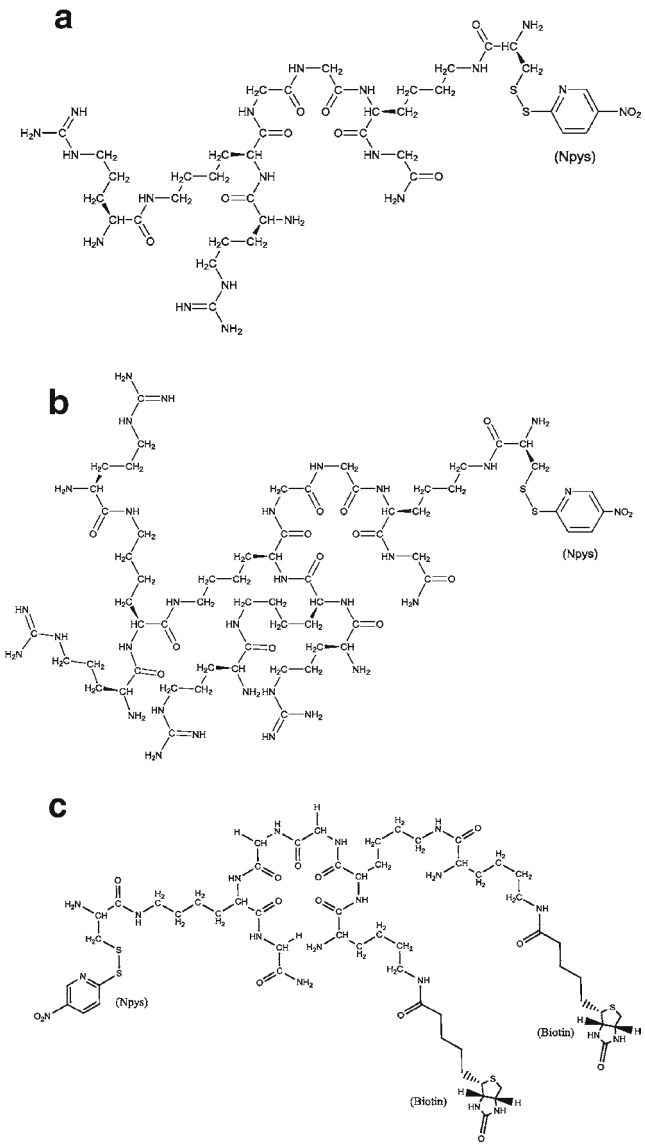


Fig. 1 Chemical structures of dendrimers. **a** 4^{+}Cys (Npys) Arg (dendrimer (4^{+})), Mw 1014.20 Da. **b** 8^{+}Cys (Npys) Arg (dendrimer (8^{+})), Mw 1582.93 Da. **c** 2^{+}Cys (Npys) Lys (biotin) $_2$ (dendrimer (biotin)), Mw 1409.62 Da

MATERIALS AND METHODS

Dendrimer Synthesis and Analysis

Dendrimers Figure. 1a,b were synthesised and analysed as previously described [9]. Briefly, the growing peptide dendrimer, with *C*-terminus covalently tethered to an insoluble support allowed quantitatively coupling, through sequential addition of excess *N*-terminus protected amino acids. Coupling efficiency was monitored using a simple colorimetric test performed *in-situ* [10]. Biotin Fig. 1c was introduced onto the dendrimer head group using the previously described active-ester coupling strategy [9] and Fmoc-Lys (Biotin)-OH (NovaBiochem, Germany). In the case of Npys, it was introduced onto the peptide backbone of a Cys (Trt) residue upon simultaneous cleavage of the peptide off-resin. Here, the resin cleavage mixture (TFA: DCM:H_2O; 95:2.5:2.5) was mixed with a 10-fold excess (relative to resin loading) of 2,2'-dithiobis (5-nitropyridine). The resin was incubated with the mixture for 4 h followed by filtration and TFA removal *in vacuo*. The mixture was then co-evaporated with toluene (3×15 mL) and triturated with ice cold diethyl ether (3×50 mL). Dendrimers (crude) were reconstituted in water and lyophilized followed by purification (using preparative RP-HPLC where fractions identified with the target molecular ion, as determined *via* mass spectrometry only were pooled) and analysed using 1D & 2D-NMR and/or ESI^{+}-MS (Supplementary Information).

Antibody Reduction and Conjugation

Monoclonal antibodies RA3-6B2 (antiCD45R) [11], 1D3 (anti-CD19) [12] and MAC4 (anti-phytochrome C) [13] (all rat IgG2a) were purified from hybridoma supernatants grown in-house. mAb (1.87 mg/mL final concentration in 2MEA) were reduced with the noted concentration of 2MEA (Pierce Thermo Scientific, Rockford, IL) in 0.1 M phosphate buffer/ 10 mM EDTA (pH7.2) at 37°C for the times specified. Fully reduced mAb was prepared by incubating mAb with 2-Me in a boiling water bath for 5 min. After incubation reducing agents were removed by passage through a Zeba de-salt column (Pierce Thermo Scientific, Rockford, IL) according to manufacturer's instructions. Free thiols were detected using Ellman's assay in accordance with the manufacturer's recommendation (Pierce Thermo Scientific, Rockford, IL) using cysteine hydrochloride monohydrate as a standard. A sample of standard or reduced mAb was incubated with Ellman's reagent for 15 min at R.T. and A_{412} determined. Free thiols per mAb were calculated from the standard curve using GraphPad Prism (GraphPad Software, Inc). For alkylation, partially reduced mAb was incubated with iodoacetamide according to manufacturer's instructions (Pierce Thermo Scientific, Rockford, IL). SDS-PAGE was performed under non-reducing conditions using 10% PAGE gel. Samples (~25 μL) and a protein ladder (PageRuler, Fermentas) were run for approximately 1.5 h at 100 V. Gels were stained in Coomassie blue stain for ~1 h and destained overnight.

Reduced mAb were incubated with asymmetric dendrimers (dissolved in 0.1 M phosphate buffer, pH 7.2) for 3–4 h at R.T. with shaking. Conjugation was monitored spectrophotometrically by determining the concentration of free Npys (A_{390}) released as a stoichiometric product of conjugation. Npys release was calculated as a proportion of the initial total dendrimer-bound Npys (*i.e.* % released). The number of dendrimer molecules per mAb molecule was then calculated by dividing the proportion of Npys release (%) by the molar excess of dendrimer (10-fold) *i.e.* Npys release (%) ÷10.

Complexation of DNA by MAb:Dendrimer

Plasmid DNA (pEGFP-C1, Clonetech, Mountain View, CA) was expanded in *E. coli* and purified using an Endotoxin-Free Mega Prep kit (Qiagen, Germantown, MD). DNA was biotin-labelled using Label IT Tracker Intracellular Nucleic Acid Localization Kit (Mirus Bio LLC, Madison, WI) according to manufacturer's instructions. Biotinylation of DNA was then confirmed by agarose gel electrophoresis after binding with streptavidin. For complexation of DNA by mAb:dendrimer conjugates, plasmid DNA was diluted to 1 mg/mL in 20 mM phosphate buffer and incubated with RA3-6B2-dendrimer (8^+) at 20:1 N:P ratio under previously defined optimum conditions [9].

Flow Cytometry

All animal experiments were approved by the University of Queensland Animal Ethics Committee and 6–8 week-old male C57BL/6 mice (Animal Resources Centre, Perth, Australia) were used in all studies. Fluorochome-conjugated and biotin-conjugated mAb were prepared in house or purchased from Biolegend (San Diego, CA). Mice were euthanized by CO_2 narcosis and the spleens or mesenteric lymph nodes collected. Single cell suspensions were prepared as described previously [14]. For *in vivo* injection, mAb or mAb:dendrimer diluted in phosphate buffered saline was injected intravenously (i.v.) *via* the lateral tail vein. Mice were euthanized 3 h or 24 h later as indicated and spleen or mesenteric lymph node cell suspensions prepared and stained for flow cytometry. For flow cytometry, single cell suspensions were resuspended in PBS/1% BSA (FACS wash) and incubated with normal rat serum and antiCD16/32 mAb (5 min on ice) to block non-specific staining, then stained with fluorochrome-conjugated mAb (30 min on ice) and washed prior to flow cytometric analysis. Where stated, unreduced antibody, reduced antibody or dendrimer-conjugated antibody at the indicated concentrations were incubated with spleen cells (30 min on ice), prior to addition of fluorochrome-labelled antibody. Where blockade of antibody binding was tested, mAb used for staining was the same clone as that used for blockade. To visualise biotin-labelled dendrimers or DNA, cells were washed and incubated with streptavidin-PE for 30 min on ice and washed prior to analysis. For intracellular staining of biotin-labelled DNA, cells and cultured for 4 h with mAb-dendrimer/DNA at which point cultures were supplemented with 10% FCS. After culturing for 24 h, cells were harvested, washed, surface stained as required, fixed with 4% paraformaldehyde for 30 min at room temperature, washed, and then treated with 0.02% Triton ×–100 for 10 min at room temperature. Cells were washed twice and blocked with 3% BSA for 30 min at room temperature and washed again. Cells were then stained with streptavidin-PE for 30 – 45 min at room temperature, washed three times. All mAb and staining reagents were used at pre-optimised concentrations. Cytometric data was collected using a FACS Canto cytometer (BD, San Jose, CA). Typically, 100,000 events were acquired and analysed using FACS Diva software (BD, San Jose, CA). Where required, background staining (typically< 10%) in the presence of unlabelled DNA was subtracted when biotinylated DNA was used.

Statistical analyses

Data was plotted and statistical analyses performed using GraphPad Prism (GraphPad Software, Inc).

RESULTS

Construction of Npys-Dendrimers

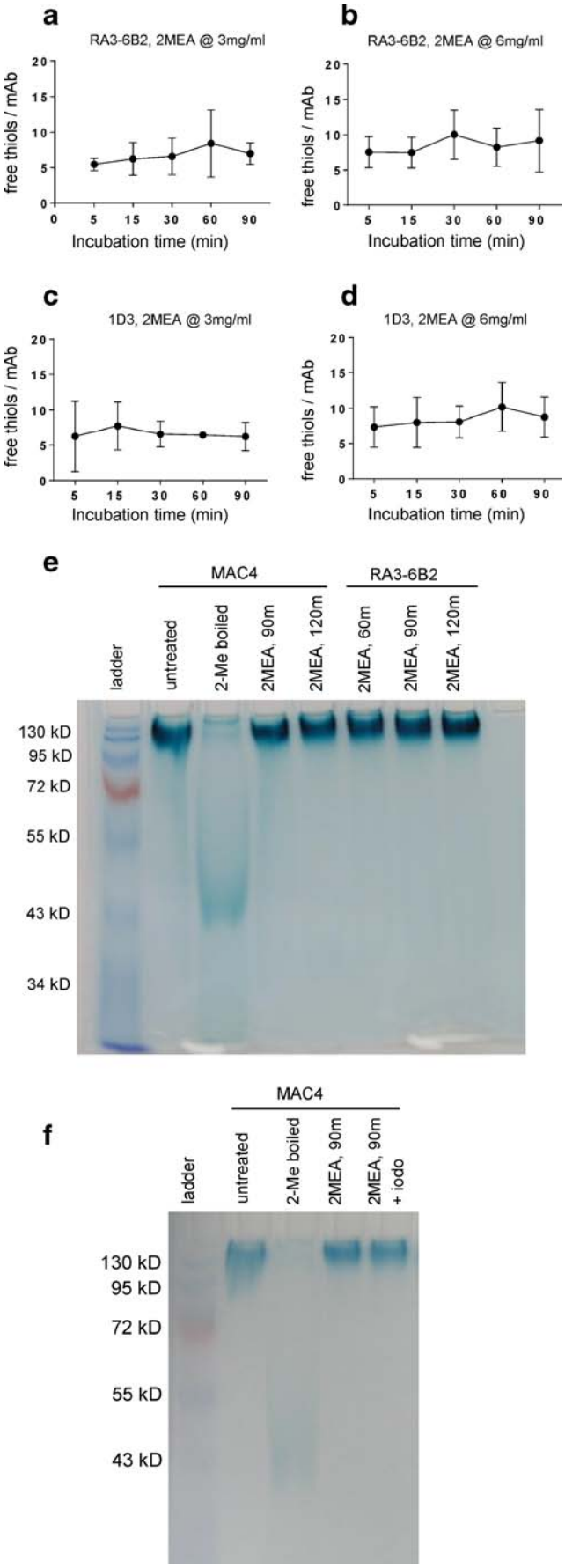
Asymmetric peptide dendrimers were synthesised as previously described [9] with 4 (dendrimer (4^+)) or 8 (dendrimer (8^+)) terminal arginine amino acids but with incorporation of a nitropyridyl sulfenyl (Npys) group distal to the branching termini Fig. 1a,b. A dendrimer was also constructed with biotin molecules on alternate branching termini (dendrimer (biotin)) as "payload" Fig. 1c. Synthesis and purity of each dendrimer was confirmed using 1D & 2D-NMR, and/or ESI^+-MS (Supplementary Information).

Partial Reduction of Monoclonal Antibodies

Prior to conjugation with Npys-dendrimer, monoclonal antibody (mAb) reduction to liberate reactive free thiols was undertaken. To optimise conditions for free-thiol generation, mAb specific for the B cell surface antigens CD45R (RA3-6B2) and CD19 (1D3) were partially reduced by incubation with 2-mercaptoethanolamine (2MEA) at 3 or 6 mg/mL for defined times and after removal of free 2MEA, the number of free thiols liberated was determined. Partial reduction with 2MEA liberated approximately 6–10 free thiols per antibody molecule for both RA3-6B2 and 1D3 mAb Fig. 2a,d. While there was a trend toward liberation of a higher average number of free thiols per antibody molecule with 6 mg/mL 2MEA compared to 3 mg/mL 2MEA, overall this difference was not significant Fig. 2a,d. Incubation times did not significantly alter the average number of free thiols liberated per antibody molecule with 5 min incubation yielding similar results to all other times tested up to a maximum of 90 min Fig. 2a,d. Overall, 5 min reduction with 3 mg/mL 2MEA provided the most reproducible results for RA3-6B2 with liberation of approximately 6 free thiols per mAb molecule Fig. 2a and results did not differ substantially for 1D3 Fig. 2c. In keeping. with the theoretical number of 32 free thiols per molecule that could be liberated from rat IgG2a [15], full reduction by boiling with 2-Me liberated 14–30 (20.9 ± 5.8, mean $\pm$ SD, $n=6$) free thiols for the mAb tested here (data not shown). Overall, these data suggest that an average of 4–6 free thiols were liberated per mAb molecule consistent with, on average, 2–3 disulfide bonds being cleaved per antibody molecule by partial reduction with 2MEA. Under the mild conditions of 2MEA-mediated partial reduction, "hinge region" disulfide bonds are preferentially reduced rather than intrachain disulfide bonds or those between heavy and light chains [15]. Given the extent of disulfide bond cleavage calculated for partial reduction it was unlikely that full cleavage of all 4 IgG2a hinge region disulfide bonds, that would result in intermolecular cleavage of the 2 heavy chains, had occurred here. However, to confirm this, RA3-6B2 or MAC4, an isotype-matched mAb specific for a non-B cell antigen (phytochrome C) were either partially reduced (2MEA, as indicated) or fully reduced (2-Me, boiled) and electrophoresed using non-reducing SDS-PAGE. While unreduced mAb demonstrated little migration Fig. 2e, lane 2), mAb boiled with 2-Me appeared almost entirely cleaved into heavy (H) and light (L) chain monomers (Fig. 2e lane 3, only heavy chain visible) consistent with full reduction. In contrast, RA3-6B2 or 1D3 subjected to extended partial reduction showed negligible migration, consistent with preservation of tertiary structure (Fig. 2E lanes 4–8). In fact, migration may have been reduced, consistent with a reported increase in the molecular radius of partially reduced mAb [15]. To rule out the possibility that re-oxidation of reduced disulfide bonds was restoring mAb structure prior to electrophoresis, partially reduced mAb was alkylated with iodoacetamide and then electrophoresed. Alkylation did not alter the migration profile for partially-reduced mAb Fig. 2f. Overall, the data show mild reduction liberated an average of 5–6 free thiols per mAb molecule without detectable antibody cleavage.

Asymmetric Peptide Dendrimers are Efficiently Conjugated to Partially Reduced MAb

The peptide dendrimers prepared with a thiol-reactive Npys group on their backbone were ready for conjugation to partially reduced mAb. An advantage of this approach is that Npys liberation during mAb-conjugation can be used to validate the extent of conjugation. We first tested whether dendrimers functionalised in this way conjugated to reduced mAb. When partially-reduced RA3-6B2 was mixed with a 10-fold molar excess of Npys-dendrimer an immediate yellow colour change was observed, indicative of Npys detachment from the dendrimer and mAb-dendrimer conjugation occurring. After 3–4 h incubation the extent of mAb-to-dendrimer conjugation was calculated. Although partial reduction and free-thiol liberation had been relatively consistent after incubation of mAb with 2MEA for 5, 15, 30, 60 or 90 min Fig. 2 we tested whether the period of incubation with 2MEA impacted the extent of mAb:dendrimer conjugation. We found a similar extent of conjugation of RA3-6B2 to dendrimer ($\mathbf{4}^+$) irrespective of the incubation time of mAb with 2MEA (Table I) although the extent of conjugation was marginally higher for mAb reduced at 6 mg/mL of 2MEA this was not statistically significant. As this was a minor difference and conjugation of mAb reduced for 5 min with 3 mg/mL 2MEA carried, on average, approximately 1.3 dendrimer molecules we opted to continue further studies using partial reduction of mAb with these conditions. Consistent with expectations, fully-reduced mAb bound significantly more dendrimer (Table I). We next compared the dendrimer (4^+) Fig. 1a and dendrimer (8^+) Fig. 1b and found that both yielded relatively similar results regardless of whether



◀ **Fig. 2** Partial reduction of mAb liberates free thiols without mAb cleavage. **a-d** mAb were incubated with 2MEA (3 mg/mL or 6 mg/mL as noted) at 37°C for the times indicated. Liberation of free thiols was estimated using Ellman's assay. Data show number of free thiols per mAb molecule (mean ± SD). E) mAb were incubated with 2MEA 3 mg/mL at 37°C for the indicated times or fully reduced by boiling with 2-Me. Reduction products were resolved by non-reducing SDS-PAGE. F) mAb were incubated with 2MEA 3 mg/mL at 37°C for 90 min or fully reduced by boiling with 2-Me. Where indicated, reduction productions were alkylated with iodoacetamide (+iodo) and prior to resolving with non-reducing SDS-PAGE. Data shown are pooled from a minimum of 4 separate experiments (A-D) where $n = 4$-5 (A, B) or 1–5 (C, D) for individual points or representative of 3 experiments (E,F).

conjugated to RA3-6B2 or 1D3 and, on average, 1.5 to 3 dendrimer molecules were conjugated per mAb molecule Fig. 3a,b. This indicated that approximately one quarter to one third of available free thiols on the reduced mAb were reacting with Npys-dendrimer.

Antibody Retains Antigen-binding Specificity After Partial Reduction and Dendrimer Conjugation.

Using a competitive inhibition assay, we next tested retention of antigen binding by mAb after partial reduction and following conjugation to dendrimer. Here, the capacity of mAb-dendrimer to inhibit binding of fluorescently-labelled mAb of identical specificity was compared with native mAb. Spleen cells were incubated with or without graded concentrations of RA3-6B2 or RA3-6B2-dendrimer, washed and then incubated with fluorescently-labelled RA3-6B2 (RA3-6B2-FITC) and an antiCD19-APC mAb to enable the proportion of B cells binding RA3-6B2-FITC to be quantified. Incubation with unlabelled RA3-6B2 at either 10 or 50 μg/mL effectively blocked RA3-6B2-FITC binding as did a mixture of unreduced RA3-6B2 and dendrimer (4^+) Fig. 3c. Some non-specific interaction between unconjugated dendrimer (8^+) and FITC-labelled RA3-6B2 may have occurred at higher dendrimer concentrations Fig. 3dcme, fifth column. RA3-6B2 when reduced and conjugated with dendrimer (4^+) Fig. 3c or dendrimer (8^+) Fig. 3d was equally as effective as unconjugated mAb at blocking subsequent RA3-6B2-FITC binding when used at 10 μg/mL indicating that the antigen-binding

Table I Extent of mAb:dendrimer conjugation is not altered by mAb reduction times.

Reduction time	2-Me, boiled	2MEA, 3 mg/mL	2MEA, 6 mg/mL
	extent of conjugation (dendrimer per mAb molecule, mean ± SD)		
5 min	4.6 ± 1.6[1]	1.49 ± 0.97	1.57 ± 0.29
30 min	N.D.[2]	1.43 ± 0.31	1.60 ± 0.36
90 min	N.D.	1.43 ± 0.21	1.53 ± 0.57

$n = 3$ for all groups, [1] $p < 0.05$ compared to 3 mg/mL, $p < 0.01$ compared to 6 mg/mL; [2] N.D.: not determined.

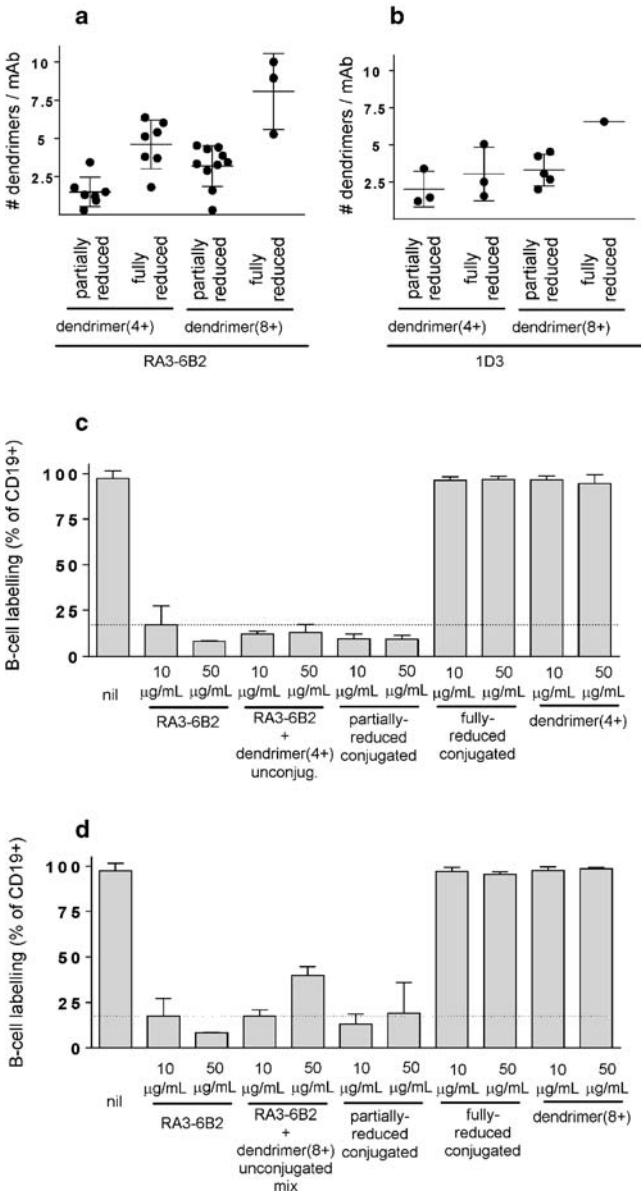


Fig. 3 Antigen-binding is retained by mAb when conjugated to dendrimer. **a**, **b** RA3-6B2 **a** or 1D3 **b** was partially reduced (2MEA, 3 mg/mL, 5 min, 37°C) or fully reduced (2-Me, boiled) and incubated with a 10-fold molar excess of dendrimer (4^+) or dendrimer (8^+) for 4 h at room temperature. Npys release was determined by spectrophotometry (A_{390}) the extent of conjugation (# dendrimers/mAb molecule) calculated. C, **d** RA3-6B2 was partially reduced (2MEA, 3 mg/ml, 5 min, 37°C) or fully-reduced (2-Me, boiled) and conjugated to dendrimer (4^+) **c** or dendrimer (8^+) **d**. Unreduced RA3-6B2, Npys-dendrimer, a mix of unreduced mAb and dendrimer, partially-reduced mAb conjugated to dendrimer or fully-reduced mAb conjugated to dendrimer as indicated was added to a suspension of spleen cells at 10 μg/mL or 50 μg/mL (mAb equivalent concentration) and incubated at 4°C for 30 min followed by addition of FITC-labelled RA3-6B2 for an additional 30 min. Cells were washed and analysed by flow cytometry. Bars show mean ± SD of data from pooled individual experiments ($n = 3$-7 for RA3-6B2 (A), $n = 1$-5 for 1D3 (B) or pooled from 3 experiments ($n = 3$ for all groups (C,D). Data are individual results pooled from 3 separate experiments. Dotted line denotes level of blockade at 10 μg/mL of RA3-6B2 (C,D)

capacity of the mAb had not been compromised by partial reduction and conjugation with dendrimer. In contrast, and as expected, fully-reduced (2Me, boiled) dendrimer-conjugated mAb did not block subsequent RA3-6B2-FITC binding, consistent with disruption of mAb structure by complete reduction, nor did Npys-dendrimer alone. In preliminary experiments, similar results were observed for dendrimer-conjugated with 1D3 mAb, although this was not extensively characterised (data not shown).

Antibody-conjugated dendrimers are efficiently delivered to target cells

Next, we wished to confirm whether dendrimers, when conjugated with mAb, were in fact targeted to specific cell types, as this would provide confirmation of their potential for use as "linkers" for targeted delivery. To achieve this we synthesised a low-generation asymmetric dendrimer constructed with two biotin molecules tethered to alternating head groups of the branch termini (dendrimer (biotin)) as "payload" Fig. 1c. This Npys-containing dendrimer (biotin) was then conjugated to RA3-6B2 or the irrelevant specificity isotype-matched mAb (MAC4) using the optimal conditions derived earlier. Spleen cells were incubated with mAb-dendrimer (biotin) conjugates, washed, incubated with antiCD19-APC and antiCD8-FITC to identify B cells and T cells respectively, washed again and then incubated with streptavidin-PE to identify the bound biotin payload.

Visualisation of biotin revealed a high level of co-localisation of RA3-6B2-dendrimer (biotin) to $CD19^+$ B cells at low concentrations of mAb-dendrimer (equivalent to 0.5 µg/mL of mAb; Fig. 4a. Furthermore, biotin co-localisation to $CD19^+$ B cells was equivalent or better than that observed for biotinylated-RA3-6B2 mAb at the lowest concentrations tested, indicating comparable binding between native mAb and mAb-dendrimer (biotin) conjugates. In contrast, MAC4-dendrimer (biotin) conjugates made using an irrelevant isotype-matched antibody showed a significantly lower degree of co-localisation with $CD19^+$ B cells, labelling a typically low proportion of cells Fig. 4a. This data indicated that biotinylated-dendrimer, when conjugated to a B-cell specific mAb, was delivered to B cells with high specificity. To further define specificity, co-localisation of biotin with T cells was also analysed. In this case, mAb-dendrimer (biotin) conjugates were not targeted to T cells regardless of the specificity of the mAb used Fig. 4b. Overall this indicated that dendrimer conjugated to an anti-B cell antibody was specifically and efficiently targeted to B cells.

B-cell Targeted MAb-dendrimer Conjugates Deliver a Small Molecule Payload *in Vivo*

We next tested *in vivo* delivery to B cells using RA3-6B2-dendrimer (biotin). To first establish whether RA3-6B2

targeted B cells *in vivo*, mice were injected i.v. with RA3-6B2-biotin and 3 h later spleen cells were collected and analysed by flow cytometry for the presence of biotin. B cells were effectively labelled with RA3-6B2-biotin when as little as 5–10 μg was injected *e.g.* Fig. 4c. Next, mice were injected with RA3-6B2-dendrimer (biotin) or MAC4-dendrimer (biotin) at the equivalent of 1, 5, 10 and 50 μg mAb and 3 h later spleen and mesenteric lymph node collected and analysed. When 50 μg of RA3-6B2-dendrimer (biotin) was injected, >25% of B-cells in the spleen and mesenteric lymph nodes were found to stain with streptavidin-PE Fig. 4c,d indicating delivery of dendrimer-bound biotin. In keeping with the more limited access of blood-borne substances to lymph nodes [16], labelling in mesenteric lymph nodes Fig. 4e,f was somewhat reduced relative to spleen. A high specificity of "targeting" was observed as few B cells were stained with streptavidin-FITC following injection of MAC4-dendrimer (biotin) and delivery of biotin to $CD8^+$ T cells was also minimal Fig. 4d,f. Overall, the data clearly showed that the RA3-6B2-dendrimer (biotin) conjugates delivered biotin *in vivo*, and specifically to the targeted B-cell population.

DNA complexation by mAb-dendrimer conjugates does not prevent binding to B cells

To determine whether complexing mAb-dendrimer conjugates with DNA altered binding to target cells RA3-6B2-dendrimer (8^+) conjugates were incubated with DNA at a 20:1 N:P ratio under conditions optimised for dendrimer (8^+) [9]. The resultant complexes were then tested for their capacity to block subsequent binding of fluorescently-labelled RA3-6B2, to indicate retention of antigen binding. Under these conditions, RA3-6B2-dendrimer (8^+)/DNA complexes blocked subsequent RA3-6B2-FITC binding equivalent to native RA3-6B2 Fig. 5a. As we intended to again use biotin as an indicator of delivery, DNA was biotinylated (DNA (biotin)) and compared with unbiotinylated DNA (DNA). No difference was found in retention of antigen-binding capacity between RA3-6B2-dendrimer (8^+) complexed with DNA (biotin) or DNA Fig. 5a. Overall, this demonstrated that complexation of mAb-dendrimer with plasmid DNA did not impair antigen binding capacity of the conjugated mAb indicating that such complexes have potential applications in targeted delivery of DNA.

DNA is Delivered to B Cells *In Vitro* by MAb-dendrimer Conjugates

Next, to assess delivery of DNA *in vitro*, RA3-6B2-dendrimer (8^+) was complexed with DNA (biotin) and incubated with spleen cells. After washing, the cell suspension was incubated with streptavidin-PE and to identify the extent to which RA3-6B2-dendrimer (8^+)/DNA (biotin) complexes had bound to B cells antiCD19-APC was used to identify B cells. Background binding of streptavidin-PE determined for dendrimer or RA3-6B2:dendrimer conjugates complexed with DNA was subtracted and the proportion of B cells and non-B cells binding streptavidin-PE determined by flow cytometry. The proportion of B cells binding RA3-6B2-dendrimer (8^+)/DNA, based on PE fluorescence, increased with increasing RA3-6B2-dendrimer (8^+)/DNA (biotin) concentration Fig. 5b. When comparing B cells, which would be targeted, with non-B cells (CD19-ve) which would not, the results revealed that a significantly greater proportion of B cells (targeted) had stained with streptavidin-PE indicating targeted DNA delivery Fig. 5b. In 4 of 7 experiments where RA3-6B2-dendrimer (8^+)/DNA (biotin) was used at 50 μg/mL (mAb equivalent) close to 10% of B cells were bound. However, overall, despite some "targeting" effect being observed the overall proportion of B cells bound by RA3-6B2:dendrimer/DNA was low. Overall, the data indicated that RA3-6B2-dendrimer/DNA (biotin) complexes bound to B cells, achieving targeted delivery but the efficiency of delivery was either low or the detection method used, insensitive.

Given the low level of DNA (biotin) detected with surface staining of B cells, we pondered whether the surface antigen, CD45R, once bound by RA3-6B2-dendrimer (8^+) DNA (biotin) might be internalised. Therefore, to test this and to further the depth of the analyses of targeted delivery we assessed the level of DNA accumulating within B cells when delivered as RA3-6B2-dendrimer (8^+)/DNA (biotin) complexes in cell culture. Spleen cells were cultured in the presence of RA3-6B2-dendrimer (8^+)/DNA (biotin) complexes for 24 h. The cells were then washed, surface stained with antiCD19-APC to identify B cells, permeabilized and incubated with streptavidin-PE in order to detect internalised DNA (biotin) and cytometric analysis was performed as described above. When permeabilized, few non-B cells were found to bind streptavidin-PE after incubation with dendrimer (8^+)/DNA (biotin) complexes regardless of whether or not these were conjugated with RA3-6B2 Fig. 5c. In contrast, 10-20% of B cells incubated with RA3-6B2-dendrimer (8^+)/DNA (biotin) for 24 h bound streptavidin-PE when permeabilized. This data suggests that the RA3-6B2:dendrimer conjugates complexed with DNA (biotin) were preferentially binding B-cells and, in this case, most likely internalized to deliver DNA (biotin) intracellularly.

DISCUSSION

The aim of the present work was to prepare asymmetric peptide dendrimers with a thiol-reactive (Npys) moiety on their backbone and demonstrate conjugation with a targeting ligand, in this case a reduced mAb. We demonstrated that this

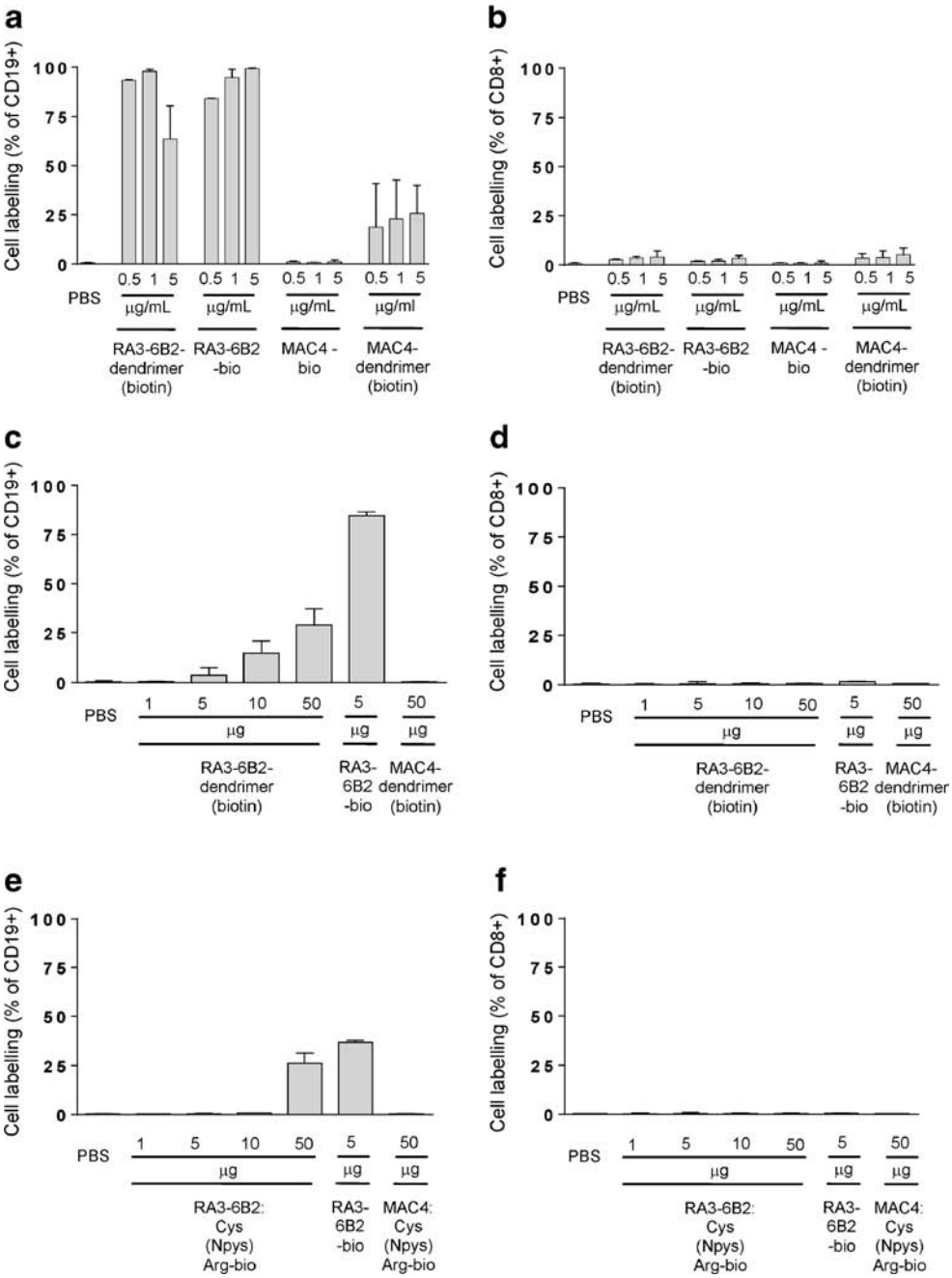


Fig. 4 B cell targeted dendrimers deliver a small molecule payload *in vitro* and *in vivo*. A-F) RA3-6B2 and MAC4 were partially reduced (2MEA, 3 mg/mL, 5 min, 37°C) and conjugated to dendrimer (biotin) and A,B) added to spleen cells at the indicated Ig equivalent concentrations. MAC4-biotin and RA3-6B2-biotin incubations were performed in parallel. Cells were incubated for 30 min on ice, washed and stained with streptavidin-FITC for 30 min, washed and analysed by flow cytometry. C-F) PBS, RA3-6B2-biotin, MAC4:dendrimer (biotin) (50 μg) and RA3-6B2-dendrimer (biotin) (at the indicated Ig equivalents) were injected i.v. and 3 h later spleen (C,D) and mesenteric lymph node (E,F) cells collected and stained with streptavidin-PE and antiCD 19-APC or antiCD8-FITC. Cells were washed and analysed by flow cytometry. Bars show mean ± SD of data pooled from 3 independent experiments (A,B) or 2 separate experiments (C-F) where $n = 3$ (RA3-6B2: dendrimer (biotin)) or $n = 2$ (PBS, MAC4-bio, MAC4-dendrimer (biotin)

'targeted' system could deliver payloads *in vitro* (biotin, DNA) and *in vivo* (biotin) to B cells with high specificity.

Peptide dendrimers hosting an Npys Fig. 1a,c moiety on their backbone and biotin Fig. 1c on the head groups were successfully synthesised by the well-established technique of Fmoc-SPPS. Characterisation using 1D- & 2D-NMR, and ESI^+-MS confirmed the purity and presence of each dendrimer. The dendrimers were designed with targeting in mind, in this case using partially reduced mAb, with tethering of thiols in the mAb-hinge region to the dendrimer backbone to preserve antigen binding capacity following conjugation. Advantages of using partially-reduced mAb include specificity of conjugation between the dendrimer backbone and the hinge region of the mAb. With mAb conjugation directed to the dendrimer backbone, "distal" head groups on the dendrimer remained available to accommodate payload, which was here either chemically-conjugated (biotin) or complexed (DNA (biotin)). An advantage of the Npys-dendrimer approach is its versatility meaning it is suitable for use with a range of high affinity targeting ligands (*e.g.* nanobodies) in the future.

IgG2a contains a total of 16 disulphide bonds, four of which provide the hinge region linkage between heavy chains [15]. An advantage of using 2MEA partial reduction is that hinge-region disulphide bonds are preferentially reduced, thus

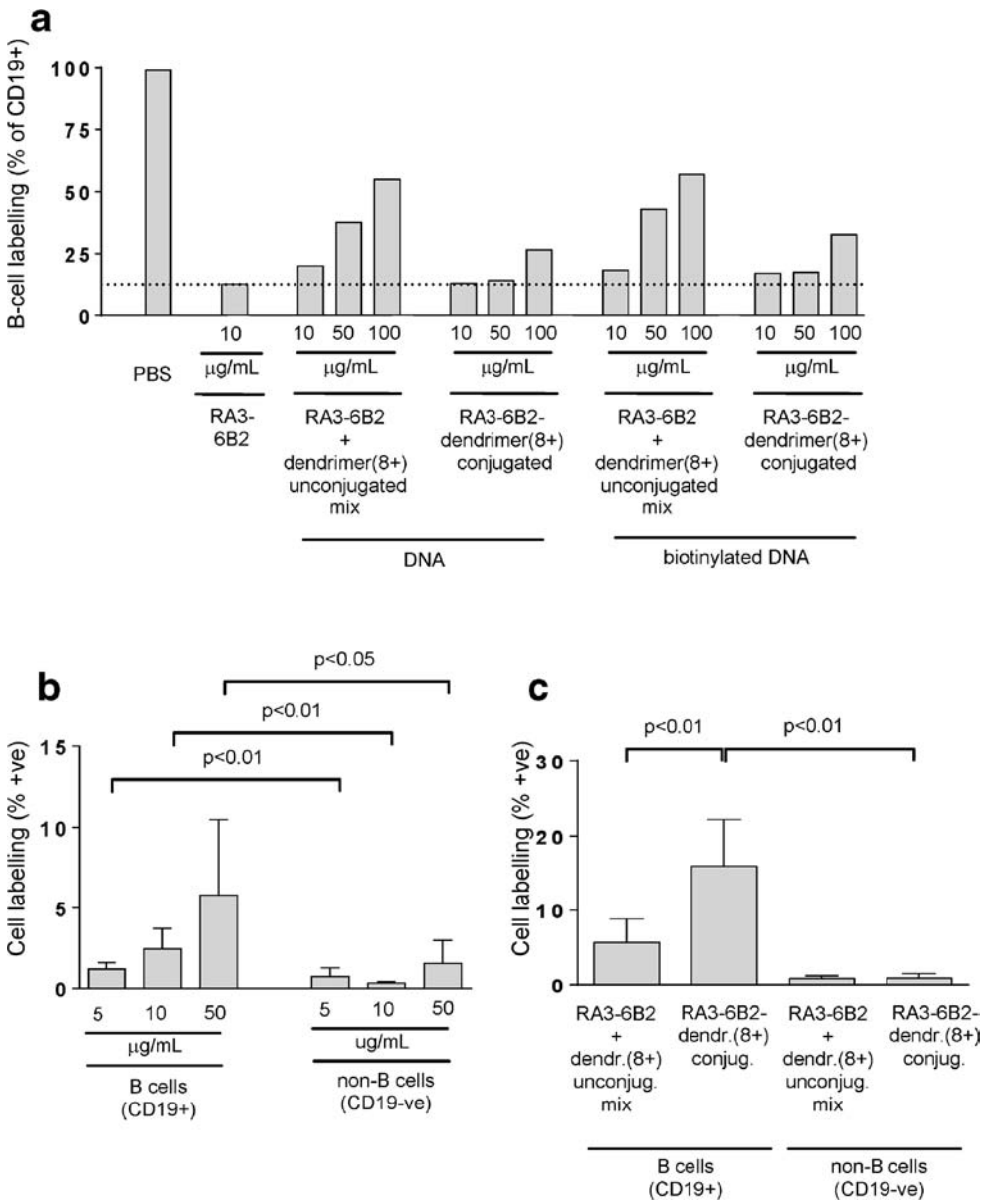


Fig. 5 RA3-6B2 mAb:dendrimer targets DNA delivery to B cells. **a** RA3-6B2 was partially reduced (2MEA, 3 mg/mL, 5 min, 37°C) or left unreduced and mixed with dendrimer (8^+) to generate RA3-6B2-dendrimer (8^+) (conjugated) or an unconjugated mix of the 2 reagents. Conjugated or unconjugated dendrimer was complexed with biotinylated (DNA (biotin)) or unbiotinylated plasmid DNA (DNA) (20:1 N:P ratio) and added to spleen cells at the indicated mAb equivalent concentrations. After 30 min on ice, RA3-6B2-FITC was added for 30 min on ice, cells washed and analysed by flow cytometry. **b** RA3-6B2-dendrimer (8^+) (conjugated) was complexed with DNA (biotin) and added to spleen cells. Cells were washed and incubated with streptavidin-PE to detect cell-associated biotin and anti-CD19-APC to identify B cells. Cells were washed and analysed by flow cytometry. **c** RA3-6B2-dendrimer (8^+) (conjugated) or dendrimer (8^+) (unconjugated) was complexed with DNA (biotin) and added to spleen cells and cultured for 4 h at which point cultures were supplemented with 10% FCS. After culturing for 24 h, cells were harvested, washed and stained with anti-CD19-APC to identify B cells and $CD8^+$ Tcells. After washing, cells were fixed and permeabilized and stained with streptavidin-PE. Cells were washed and analysed by flow cytometry. Data represented are (A) a single experiment from two performed with similar outcomes, (B) pooled from 4–5 experiments ($n = 4$-6) showing mean ± SD or (C) pooled from 3 experiments ($n = 3$) showing mean ± SD

preserving the integrity and antigen binding of the heavy (H)-light (L) chain complexes [17]. Overall, we found on average approximately 2–3 disulphide bonds per IgG2a molecule were cleaved to liberate on average 4–6 free thiols for the IgG2a tested here with 5 min reduction, but this may have been slightly higher with longer reduction times. This indicates that individual H-L complexes were unlikely to be formed and non-reducing PAGE confirmed this. Given the similarities in disulphide reduction profiles we adopted the mildest reduction conditions for our work, employing 2MEA at 3 mg/mL with an incubation time of 5 min and we confirmed that reduction time had only a minor influence on the extent of dendrimer-mAb conjugation. Our findings with RA3-6B2 are in agreement with previous reports

suggesting that reduction products are dictated by the type of mAb and the nature (mild-strong) of reducing reagent, similar to our observations Fig. 3e,f [15]. When conjugation efficiency under a range of conditions was measured, little differences in Npys liberation (% of maximum theoretical amount) were observed. The conjugation process indicated that an average of 2 dendrimer molecules bound per partially-reduced Ig G2a and was in-line with an earlier report where mAb conjugation to drugs using cysteine chemistry had been performed [18].

Importantly for the prospects of mAb- or other- targeting moiety-conjugated peptide dendrimers as *in vitro* or *in vivo* targeting agents, mAb reduction and conjugation did not impair the ability of the targeting mAb to recognise its cognate antigen. This is significant as, i) and in line with previous studies [19], mild reduction retains sufficient appropriate tertiary structure of the mAb for antigen binding, and ii) conjugation with the peptide dendrimer linker does not sterically hinder the antigen binding site of the mAb. As a consequence of this, delivery of the first payload tested, in this case biotin attached to the dendrimer-head groups, was highly specific and efficient both *in vitro* and *in vivo*. This provides strong evidence that a wide range of small molecule payloads such as chemotherapeutics, anti-bacterial and anti-retroviral agents could likely be readily targeted to specific cell populations *in vitro* and *in vivo* using peptide dendrimers as dual-linking and -targeting scaffolds. Further work examining targeting to a broader range of cell types is likely to validate this proposal.

In the final aspect of the study we explored the potential of targeted peptide dendrimers to deliver a complexed macromolecule, in this case biotinylated plasmid DNA (DNA (biotin)), in a targeted manner to B-cells *in vitro*. The initial apparent low levels of DNA (biotin) delivery observed may have been due to internalisation of the targeted CD45R molecule. Indeed, permeabilisation to permit additional visualisation of any internalised DNA (biotin) was consistent with this. Interestingly, although antigen binding by RA3-6B2-dendrimer (8^+)/DNA (biotin) indicated by the blockade assay performed was almost equivalent to that of native mAb Fig. 5a suggesting efficient and complete binding of CD45R on the surface of B cells, only a minority (up to 20%) of B cells appeared to bind RA3-6B2-dendrimer (8^+)/DNA (biotin) Fig. 5b or to take up DNA (biotin) in culture Fig. 5c. Given that our detection method involved tracing biotin-tethered-DNA, it is possible that this underestimates the degree of targeting and/or delivery obtained through a range of factors, including limitations of our detection method. Further development of procedures for detecting the actual degree of DNA delivery to targeted cells is likely to provide greater clarity. Additionally, and while not tested here, expression of reporter gene (s) encoded by delivered DNA will provide further information beyond delivery alone. A key outcome of these studies, however, is the demonstration that mAb, when conjugated to a suitably designed peptide dendrimer and then complexed with DNA retains its antigen-binding capacity. While the data here shows that partial reduction does not impair the antigen binding capacity of mAb, preservation of antigen-binding capacity after complexation of mAb-dendrimer with DNA further indicates the strong potential of the peptide dendrimers described here to act as exceptionally useful linkers, in this case apparently limiting steric hindrance by a complexed macromolecule intended for targeted delivery. Based on our previous demonstrations that peptide dendrimers, as used here, with positively-charged termini effectively complex with plasmid DNA and in doing so condense into compact toroidal-like structures it is feasible to propose that when used as linkers, this permits the targeting ligand, here mAb, to be exposed around the periphery of the structure thereby retaining its capacity to bind cognate antigen.

Here we show that peptide dendrimers are effective linkers between mAb and a small or macromolecular payload for targeted delivery to cells. Whilst mAb have proven themselves as potent, multi-modal therapeutics in their own right [20, 21], their unequalled ability to distinguish cancerous and healthy cells paves the way for their use in the targeted delivery of diverse payloads/delivery systems [22]. To this end peptide dendrimers would be readily modifiable for use as linkers with a range of targeting moieties which could include, nanobodies [23, 24] single chain antibodies [23], growth factors or other proteins. Additionally, a further feature of peptide dendrimers that potentially adds to their versatility is their flexibility in construction. By further increasing the generation of dendrimer it is likely they can, through the capacity to tether multiple "payload" molecules, act as a means to amplify the capacity of individual targeting moieties to deliver payloads.

CONCLUSION

Overall, we conclude that peptide dendrimers represent a family of linkers with great potential to facilitate the effectiveness of targeted diverse payload delivery.

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