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Application of a trifunctional reactive linker for the construction of antibody–drug hybrid conjugates

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ABSTRACT

A flexible, trifunctional poly(ethylene glycol)-succinamide-Lysine-Lysine-maleimide (PEG-SU-Lys-Lys-mal) linker was employed to simultaneously allow biotin tagging and cell-surface targeting through an integrin $\alpha_4\beta_1$-binding peptidomimetic that was regiospecifically conjugated to an IgG1-derived Fc fragment with an engineered C-terminal selenocysteine residue. The resulting antibody derivative mediates Fc receptor binding by virtue of the Fc protein and selectively targets cancer cells expressing human integrin $\alpha_4\beta_1$. The PEG-SU-Lys-Lys-mal linker may have general utility as an organic tether for the construction of antibody–drug conjugates.

The development of antibody–drug conjugates has been undertaken for the treatment of cancer, in part because this approach may improve selectively and pharmacokinetics (PK).[1,2] Tissue-specificity and increased serum half-life are typically governed by the antibody component, while the cytotoxic or radioactive drug cargo provides the therapeutic effect. Alternate immunoconjugates, termed 'chemically programmed antibodies' (cpAbs), have also been described that employ cell-targeting by the drug cargo rather than by the antibody.[3–5] An important advantage of cpAbs over traditional immunoconjugates is that a single antibody can be directed to multiple targets via conjugation to different antigen-specific peptides or small molecules. Such an approach expands the versatility of a given antibody while endowing the small molecule with the effector functions and PK characteristics of an antibody.

To broaden the scope of immunoconjugate-based chemotherapy, we recently reported a genre of cpAbs that does not require antibody-variable domains.[6] Instead, while the antigen-specific small molecule provides target specificity, an IgG1-derived Fc fragment improves the PK properties of the small molecule and allows alternative routes of administration such as interaction with the neonatal Fc receptor (FcRn).[6] In addition, an engineered C-terminal selenocysteine (Sec) residue on the Fc protein (Fc–Sec) insures site-specific attachment of a single drug molecule. To achieve this, we designed a flexible trifunctional poly(ethylene glycol)-succinamide-Lysine-Lysine-maleimide (PEG-SU-Lys-Lys-mal) linker that simultaneously allows cell-targeting, regiospecific conjugation to the Fc protein and conjugate detection. For cell-targeting we employed LLP2A **1** (Fig. 1) a recently developed peptidomimetic that binds with high affinity and specificity to the cell-surface protein integrin $\alpha_4\beta_1$ (IC_{50} = 2 pM).[7]

Integrin $\alpha_4\beta_1$ has been shown to promote metastasis and angiogenesis in a variety of cancers, and it plays a key role in the onset of drug-resistance that can lead to relapse following chemotherapy for acute myelogenous leukemia (AML).[8–10] Although targeting integrin $\alpha_4\beta_1$ is not without its risks,[11] studies suggest that integrin $\alpha_4\beta_1$ antagonists may be particularly valuable therapeutic agents for the treatment of hematologic malignancies, such as multiple myeloma and AML.[10,12] We wondered whether conjugation of LLP2A to Fc–Sec could overcome undesirable PK characteristics of LLP2A while maintaining its potency and selectivity.[6,13]

In conjugating **1** to Fc–Sec the linking segment needed to be sufficiently long to allow **1** to bind to integrin $\alpha_4\beta_1$ without steric interference from the relatively large Fc protein. For this reason PEG-SU was chosen because it can be extended in a modular fashion depending on the number of PEG-SU units employed. Additionally, the *N*-Fmoc and *N*-Boc protected forms of PEG-SU can be used in solid-phase syntheses.[14] A PEG-SU dimer was utilized in the Fc–Sec–LLP2A conjugate, since it had been previously shown that **1** retains its affinity for integrin $\alpha_4\beta_1$ when indirectly

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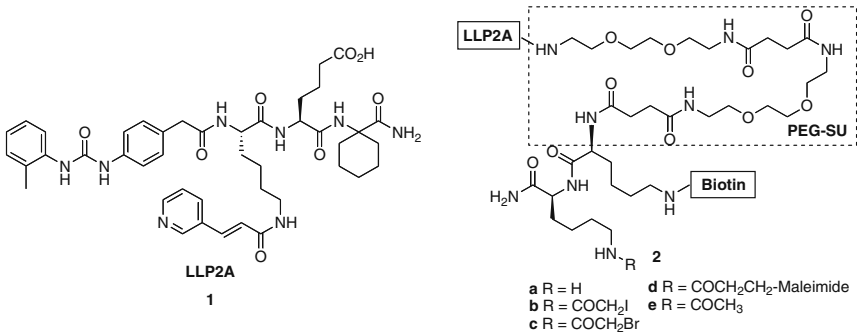


Figure 1. Structure of LLP2A (**1**) and trifunctional linker **2** showing sites of attachment for biotin and LLP2A, with R showing the intended site of Fc–Sec attachment.

coupled to streptavidin through a biotin attached via this linker.[7] The versatility of PEG-lysine-based linkers is also known.[7,15] For our purposes, two lysine residues were introduced at the C-terminal end of the PEG-SU spacer to provide primary amines as attachment points for (1) auxiliary functionality and (2) an alkylating agent that could be used for conjugation to the Sec residue of the Fc protein. Inclusion of biotin as the auxiliary functionality yielded the prototype construct LLP2A-(PEG-SU)$_2$-Lys(N^{ε}-Biotin)-Lys(N^{ε}-R)-amide (**2**), where R would be the nucleophile acceptor suitable for conjugation with the Sec residue of the Fc protein (Fig. 1).[6,16]

The solid-phase preparation of final products **2b**–**2e** (Fig. 1) required the previously reported acids **3**–**5** (Fig. 2).[7] Coupling of N^{ε}-Mmt-N^{α}-Fmoc-L-Lys to Rink amide MBHA resin followed by N^{ε}-biotin-N^{α}-Fmoc-L-Lys using standard Fmoc protocols provided the intermediate **6** (Scheme 1). Two subsequent coupling cycles

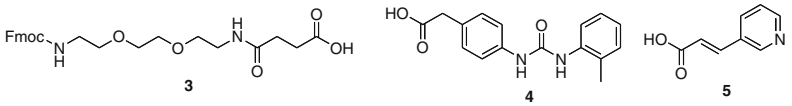
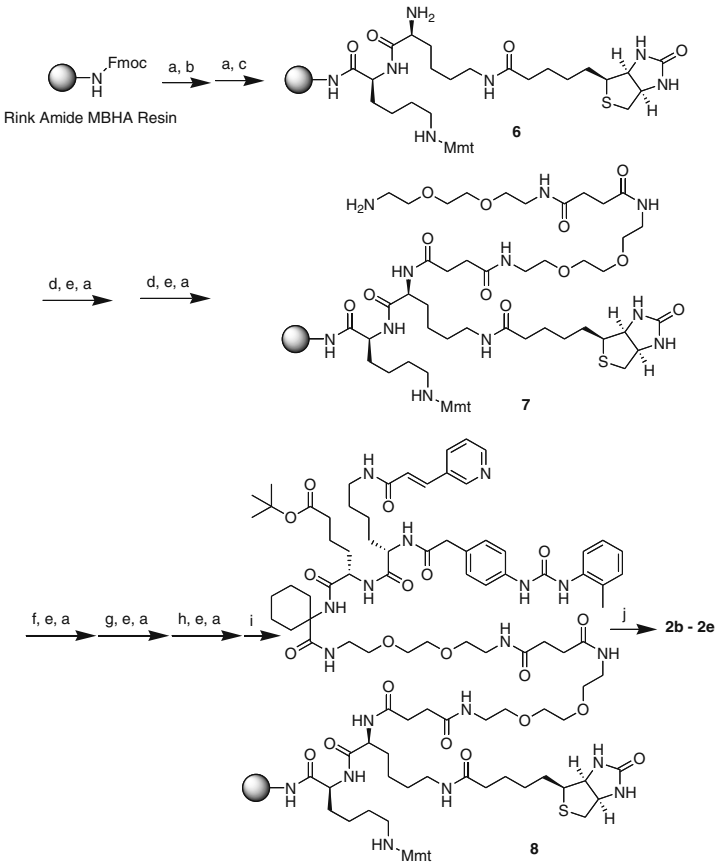


Figure 2. Structures of reagents used in the solid-phase synthesis of **2a**–**2e**.



Scheme 1. Reagents and conditions: (a) 20% piperidine in DMF; (b) Fmoc-Lys(Mmt)-OH, HOBt, DIC; (c) Fmoc-Lys(Biotin)-OH, HOBt, DIC; (d) **3**, HOBt, DIC; (e) 10% (w/v) 1-acetylimidazole in DMF; (f) Fmoc-Ac$_6$c-OH, HATU, DIEA; (g) Fmoc-Aad(OBu^t)-OH, HATU, DIEA; (h) Fmoc-Lys(Dde)-OH, HOBt, DIC; (i) i—**4**, HOBt, DIC; ii—2% NH_2NH_2 in DMF; iii—**5**, HOBt, DIC; (j) i—1% TFA in CH_2Cl_2; ii—3-maleimidopropionic acid, HOBt, DIC; iii—TFA/*i*-Pr_3Si/H_2O (98:2.5:2.5).

with *N*-Fmoc-PEG-SU (**3**) gave the resin-bound intermediate **7**, onto which was constructed the LLP2A sequence.[7] Treatment of the resulting resin **8** with 1% TFA in CH_2Cl_2 removed the acid-labile N^{ε}-Mmt-Lys group without cleavage from the resin. Subsequent acylation of the resulting free amine followed by resin cleavage (95% TFA) yielded the peptides **2a**–**2e**. However, it was found that the *N*-haloacetamide acylation products proved difficult to obtain in pure form. Iodoacetamide-containing **2b** could not be prepared using standard protocols,[17,18] and although **2c** could be prepared in low yield, it could not be obtained in pure form, even following preparative reverse-phase HPLC.

In contrast to **2b** and **2c**, peptide **2d** was obtained with relatively few side products. One significant side product resulted from deletion of the Aad residue from **2d** (approximately 30%) due to incomplete coupling of residues amino-proximal to the sterically hindered aminocyclohexanecarboxylic acid (Ac_6c). However, pure **2d** could be obtained through a two-stage protocol that involved the initial solid-phase synthesis of **2a** followed by a final solution-phase coupling step to yield **2d**. This two-stage route provided pure **2d** in sufficient quantity for cell-based assays. A more efficient solid-phase route resulting in improved coupling of residues amino-proximal to the Ac_6c, was achieved using 2-(1H-7-azabenzotriazol-1-yl)-tetramethyluronium hexafluorophosphate (HATU).[19] Introducing a capping cycle (1-acetylimidazole) also facilitated the separation of deletion by-products (Scheme 1).

The chemoselective alkylation of Sec residues by **2d** was examined using a variety of Fc constructs. Inclusion of a biotin handle within **2d** allowed facile detection of Fc covalent adducts by avidin pull-down experiments followed by ELISA visualization. This showed that conjugation only occurred when the Sec residue was present in the Fc protein (Fig. 3A).[6,20] The Fc–Sec–**2d** conjugate was then incubated with integrin $\alpha_4\beta_1$-expressing cells. The cells were washed and then treated with Cy5-labeled rabbit anti-human IgG. Under these conditions, cells would only fluoresce if both the Fc and LLP2A components of the hybrid construct were present. As shown in Figure 3B, cells treated with the Fc–Sec–**2d** conjugate were detected by flow cytometry while cells treated with Fc–Stop (Fc protein minus the Sec residue) or **2e** alone, did not exhibit such dual specificity (Fig. 3B).[21]

Competition experiments with an anti-integrin α_4 mAb (purchased from Serotec) indicated that the Fc–Sec–**2d** hybrid and the mAb exhibit identical/overlapping epitopes as evidenced by the diminished binding of Fc–Sec–**2d** to lymphoma cells in the presence of the Serotec mAb (Fig. 3C).[22] In contrast, the binding of an anti-integrin α_4 mAb from a different source (R&D Systems) could not be competed with the Serotec mAb, suggesting that the two mAbs recognize different epitopes on integrin $\alpha_4\beta_1$. These results indicate that the binding of the Fc–Sec–**2d** hybrid to the cells examined is mediated by the peptidomimetic LLP2A (**1**) and not by the Fc protein portion, and that the targeting specificity of the parent peptidomimetic for integrin α_4 is retained in the Fc–Sec–**2d** hybrid.

In conclusion, the trifunctional linker **A**–(PEG-SU)$_n$Lys(N^{ε}–**B**)Lys(N^{ε}–**C**), where **A** is a targeting moiety, **B** is auxiliary functionality such as a fluorescent tag and **C** is a nucleophile acceptor, is well-suited for the preparation of immunoconjugates in which a small synthetic molecule governs cell-targeting.[3,5] In the current study, this linker allowed the conjugation of a LLP2A-biotin construct to Fc–Sec in a chemoselective manner. Subsequent in vitro

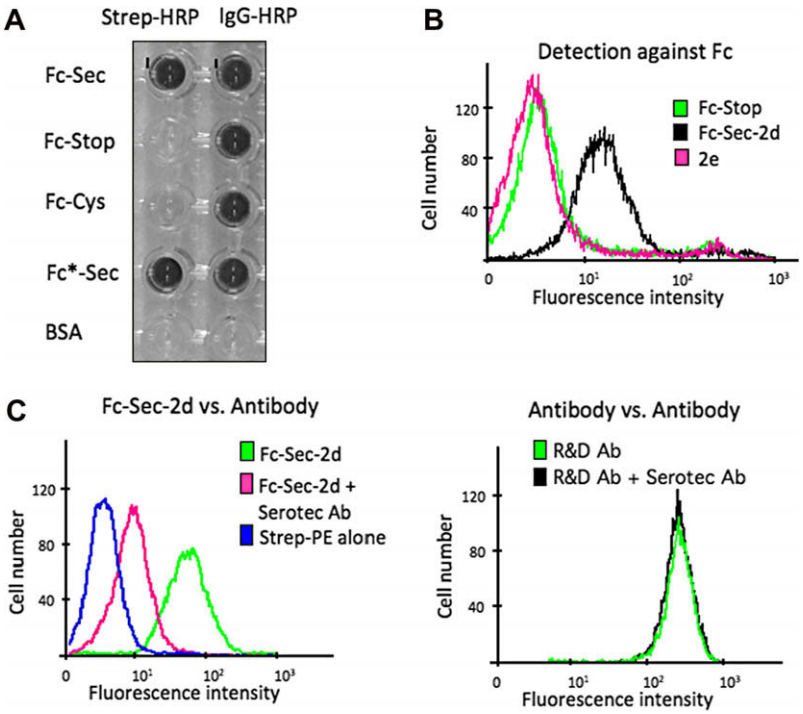


Figure 3. In vitro evaluation of Fc–Sec–**2d** conjugate. (A) ELISA assay examining conjugation of Fc proteins to reagent **2d** as detected by HRP-coupled streptavidin (left column) and with HRP-coupled donkey anti-human IgG polyclonal antibodies as control (right column). Fc–Sec, Fc protein with C-terminal Sec; Fc–Stop, Fc protein without C-terminal Sec; Fc–Cys, Fc protein in which the Sec residue has been replaced with Cys. Fc*–Sec–His, Fc protein with mutation Asn297Ala that reduces binding to the Fc receptor; BSA, bovine serum albumin negative control. (B) Flow cytometry histogram. Following reaction with **2d**, Fc–Sec and Fc–Stop were incubated with cells expressing integrin $\alpha_4\beta_1$ (HEK 293F cells), followed by staining with Cy5-conjugated rabbit anti-human IgG. Compound **2e** (approximately 70% pure) was used as a negative control. (C) Flow cytometry histogram. Fc–Sec–**2d** or a biotinylated mouse anti-human integrin $\alpha_4\beta_1$ antibody (R&D Systems) were incubated with cells from the human Burkitt's lymphoma cell line Raji that were pre-incubated with either a monoclonal mouse anti-human integrin α_4 (Serotec Ab) or with flow cytometry buffer alone. Streptavidin-coupled phycoerythrin (Strep-PE) was used for detection and as a negative control (blue line).

analysis revealed that all three components of the linker system—targeting agent (**1**), tag (biotin), and antibody fragment (Fc–Sec), were fully functional, with the affinities of the parent **1** and Fc protein for integrin $\alpha_4\beta_1$ and Fc receptor, respectively, being retained.[6] The trifunctional PEG-SU-Lys-Lys-maleimide linker may have more general utility as an organic tether for the construction and evaluation of antibody–drug conjugates.

Acknowledgments

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19. Solid-phase synthesis of LLP2A-(PEG-SU)$_2$-Lys(N^{ε}-Biotin)-Lys(N^{ε}-CO(CH$_2$)$_2$-maleimide)-amide (**2d**). Rink amide MBHA resin (0.259 g, 0.150 mmol, 0.58 mmol/g loading) was allowed to swell in DMF for 6 h, and then deprotected with 20% piperidine in DMF. A solution of Fmoc-Lys(Mmt)-OH (0.469 g, 0.750 mmol), HOBt (0.115 g, 0.750 mmol), and DIC (116 μL, 0.750 mmol) was added to a suspension of the resin in DMF, and the resulting mixture was agitated (overnight). The resin was filtered, washed, deprotected, and then reacted (overnight) with Fmoc-Lys(Biotin)-OH (0.446 g, 0.750 mmol), HOBt (0.115 g, 0.750 mmol), and DIC (116 μL, 0.750 mmol) in DMF as described above. Incomplete coupling (as indicated by a Kaiser test) resulted in a second cycle of coupling (1.5 h) with PyBrop (0.210 g, 0.450 mmol), DIEA (156 μL, 0.90 mmol), and Fmoc-Lys(Biotin)-OH (0.281 g, 0.450 mmol) in DMF. Two additional cycles of deprotection and coupling were then carried out with **4** [Fmoc-PEG-SU (see Ref. 11)] using the HOBt/DIC protocol described above. After each coupling step, unreacted amino groups on the resin were capped with 1-acetylimidazole (8 mL, 10% w/v in DMF, overnight or 2× 2 h). The resin was Fmoc-deprotected and treated with a solution of Fmoc-Ac$_6$c-OH (0.274 g, 0.750 mmol), HATU (0.285 g, 0.750 mmol), and DIEA (209 μL, 0.155 g, 1.20 mmol) (2 h, overnight), and then capped with 1-acetylimidazole as described above. The resin then underwent additional cycles of deprotection, coupling, and capping (1-acetylimidazole) with Fmoc-Aad(OBu^t)-OH (HATU/DIEA protocol), followed by Fmoc-Lys(Dde)-OH (HOBt/DIC protocol). The resin was Fmoc-deprotected and then reacted with **5** (2-(4-(3-*o*-tolylureido)phenyl)acetic acid [0.426 g, 1.5 mmol (see Ref. 7)], HOBt (0.230 g, 1.5 mmol), and DIC (232 μL, 1.5 mmol) (overnight). The resin was treated with hydrazine hydrate (8 mL, 2% in DMF, 5 min, 10 min) to remove the Dde protecting group, and then it was reacted with **6** (trans-3-(3-pyridyl)acrylic acid) (HOBt/DIC protocol, 5 h, overnight). The resin was then washed well with DMF, MeOH, and DCM, and swollen in DCM for 2–3 h. The Mmt protecting group was removed with 1% TFA in DCM (8 mL, 3× 5 min, TFA/TIS/DCM 1:5:94), and the resin was allowed to swell in DMF for 1.5 h. A solution of 3-maleimidopropionic acid (0.254 g, 1.5 mmol), HOBt (0.230 g, 1.5 mmol), and DIC (232 μL, 1.5 mmol) was added to the resin, and the resulting suspension was shaken (3 h). The resin was filtered, washed sequentially with DMF, MeOH, DCM, diethyl ether, and then dried under high vacuum (overnight). The resin was cleaved with 95% TFA (10 mL, TFA/TIS/H$_2$O 95:2.5:2.5) (2 h), precipitated with diethyl ether, and purified by RP-HPLC [80% H$_2$O (0.1% TFA) to 90% MeCN (0.1% TFA), using a concave gradient over 35 min]. Product **2d** was obtained as a white solid (95% pure by LC–MS): ^{1}H NMR (DMSO-d_6), 8.99 (s, 1H), 8.75 (s, 1H), 8.55 (d, J = 4.2 Hz, 1H), 8.20–8.12 (m, 3 H), 8.02–7.98 (m, 2H), 7.91 (s, 1H), 7.88–7.82 (m, 3H), 7.74–7.72 (m, 2H), 7.47–7.43 (m, 2H), 7.36 (d, J = 8.5 Hz, 2H), 7.16 (d, J = 8.5 Hz, 2H), 7.12 (d, J = 8.0 Hz, 2H), 6.99 (s, 1H), 6.98 (bs, 1H), 6.91 (t, J = 6.4 Hz, 1H), 6.72 (d, J = 15.9 Hz, 1H), 6.41 (bs, 1H), 6.35 (bs, 1H), 4.31–4.26 (m, 1H), 4.26–4.20 (m, 1H), 4.20–4.10 (m, 2H), 4.07–4.01 (m, 2H), 3.65–3.32 (m, 40H), 3.25–3.05 (m, 8H), 3.04–2.91 (m, 4H), 2.82 (dd, J = 5.0 Hz, J = 12.4 Hz, 1H), 2.67 (m, 1H), 2.57 (d, J = 11.5 Hz, 1H), 2.42–2.35 (m, 2H), 2.34–2.27 (m, 4H), 2.23 (s, 3H), 2.17–2.13 (m, 1H), 2.04 (t, J = 7.4 Hz, 2H), 1.87–1.82 (m, 1H), 1.70–1.20 (m, 26H) MS (ESI), m/z: 1904.7 (MH$^+$); 953.4 (M+2H)$^{2+}$, 635.8 (M+3H)$^{3+}$. HRMS (ESI-TOF) Calcd for $C_{92}H_{134}N_{19}O_{23}S$: 1904.9621 (MH$^+$). Found: 1904.9622.
20. Procedures for generating ELISA data shown in Figure 3A. All incubations were for 1 h at 37 °C. To confirm selective biotinylation with **2d** at the Sec interface, each well of a 96-well Costar 3690 plate (Corning) was incubated with 200 ng of conjugated Fc–Sec protein or derivatives thereof (Fc –Sec protein, Fc–Stop protein and Fc–Cys protein that had been exposed to the same conjugation conditions) in 25 μL PBS. Note: conjugation with **2d** was carried out as previously described in Ref. 6 After blocking with 3% (w/v) BSA/PBS, the plate was incubated with either HRP-coupled streptavidin (50 ng/well) or a 1:1000 dilution of HRP-coupled donkey anti-human IgG polyclonal antibodies in 1% (w/v) BSA/PBS. After washing with H$_2$O (10× 200 μL/well), colorimetric detection was performed using 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (Roche) as substrate according to the manufacturer's directions.
21. Procedures for functional analysis of Fc–Sec–LLP2A shown in Figure 3B. Following incubation in 10% (v/v) FCS/PBS (1 h, 0 °C), HEK 293F cells were centrifuged, resuspended in 1% (v/v) FCS/PBS, and aliquots (50 μL) containing 5 × 10^5 cells were distributed into wells of a V-bottom 96-well plate (Corning). The cells were then incubated with Fc–Sec–LLP2A, Fc–Stop, and **2e** (1 h, 0 °C). After washing twice with 1% (v/v) FCS/PBS, the cells were incubated with a 1:20 dilution of Cy5-conjugated rabbit anti-human IgG (1 h, 0 °C). After washing twice as above, the cells were resuspended in FCS/PBS (400 μL 1% v/v) and analyzed using a FACScan instrument (Becton-Dickinson).
22. Procedures for monoclonal antibody competition data shown in Figure 3C. Following incubation in 10% (v/v) FCS/PBS (1 h, 0 °C), Raji cells were centrifuged, resuspended in 1% (v/v) FCS/PBS, and aliquots (50 μL) containing 5 × 10^5 cells were distributed into wells of a V-bottom 96-well plate (Corning). The cells were then incubated with either a monoclonal mouse anti-human integrin α_4 (Serotec, 10 μg/mL) or with FCS (1 h, 0 °C). After washing twice with 1% (v/v) FCS/PBS, the cells were incubated with Fc–Sec–LLP2A or biotinylated mouse anti-human integrin $\alpha_4\beta_1$ antibody (R&D Systems) (both 10 μg/mL) (1 h, 0 °C). Cells were washed and a 1:25 dilution of PE-coupled streptavidin (BD Biosciences) was added. After washing twice as before, the cells were resuspended in FCS/PBS (400 μL 1% v/v) and analyzed using a FACScan instrument (Becton-Dickinson).