Site-Specific Polysialylation of an Antitumor Single-Chain Fv Fragment

A. Constantinou,† A. A. Epenetos,‡ D. Hreczuk-Hirst,†,‡ S. Jain,‡ M. Wright,§,† K. A. Chester,§ and M. P. Deonarain*,†

Division of Cell & Molecular Biology, Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, Exhibition Road, London, SW7 2AZ, U.K., Lipoxen plc, 2 Royal College Street, London, U.K. NW1 0NH, and UCL Cancer Institute, University College London, 72 Huntley Street, London, U.K. WC1E 6BT. Received December 2, 2008; Revised Manuscript Received March 12, 2009

Protein pharmacokinetic modulation is becoming an important tool in the development of biotherapeutics. Proteins can be chemically or recombinantly modified to alter their half-lives and bioavailability to suit particular applications as well as improve side effect profiles. The most successful and clinically used approach to date is chemical conjugation with poly(ethylene glycol) (PEGylation). Here, therapeutic protein half-life can be increased significantly while retaining biological function, reducing immunogenicity and cross-reaction. Naturally occurring alternatives to such synthetic polymers could have major advantages such as lower side effects due to biodegradability and metabolism. Polysialic acid (PSA) has been investigated as a pharmacokinetic modulatory biopolymer with many successful examples in preclinical and clinical development. Single-chain Fvs (scFvs) are a choice antibody format for human therapeutic antibody discovery. Because of their small size, they are rapidly eliminated from the circulation and often are rebuilt into larger proteins for drug development and a longer half-life. Here we show that chemical polysialylation can increase the half-life of an antiplacental alkaline (PLAP) and ant carcinoembryonic antigen (CEA) scFv (F1 and MFE-23, respectively) 3.4–4.9-fold, resulting in a 10.6–15.2-fold increase in blood exposure. Amine-directed coupling of the MFE-23 scFv reduced its immunoreactivity 20-fold which was resolved by site-specific polysialylation through an engineered C-terminal thiol residue. The site-specifically polysialylated MFE-23 scFv demonstrated up to 30-fold improved tumor uptake while displaying favorable tumor:normal tissue specificity. This suggests that engineering antibody fragments for site-specific polysialylation could be a useful approach to increase the half-life for a variety of therapeutic applications.

INTRODUCTION

Protein drugs are often compromised by limitations arising from their complex molecular structure. Factors such as proteolytic degradation, uptake by cells of the reticulendothelial system, renal removal, and immunocomplex formation all lead to rapid elimination from the blood before effective concentrations are reached (1). This results in an undesirably short therapeutic window. The over-riding factors which contribute to these pharmacokinetic limitations are size, stability, and immunogenicity (2).

Polymer conjugation using poly(ethylene glycol) (PEGylation) has been one of the most successful approaches to improving protein pharmacokinetics (3). PEG is a neutral polymer that can bind water molecules, forming a ‘watery cloud’ around the protein (4). This gives the conjugate a larger hydrodynamic volume compared to its true molecular weight, affecting its pharmacokinetics and pharmacodynamics in the body (4). In addition to its size, the protein surface is modified and biological epitopes are shielded from potential immune responses or degradation. A number of PEGylated proteins have been approved for clinical use, e.g., PEGasys (PEG-interferon) for the treatment of chronic hepatitis C infections (5), and Cimzia (certolizumab pegol), a PEGylated anti-TNFα Fab for Crohn’s disease (6). However, since PEG is a synthetic polymer, there are concerns about its metabolism and immunogenicity. Uptake by cells of the reticulendothelial system has been reported, and PEG can accumulate in lysosomes, leading to toxicity (7). Repeated administration of PEG conjugates resulted in the production of anti-PEG antibodies (8). More recently it was shown that PEG-infusion-related side effects include unwanted complement activation (9). Taken together, the uncertainty in the metabolism and immune responses has led to the search for natural alternatives.

Molecules which are inconspicuous to the innate and adaptive immune systems are more likely to survive for prolonged periods in the circulation. Polysialic acid (PSA): polymers of N-acetylneuraminic (sialic) acid (10) is one such molecule and offers a natural alternative to PEG. PSA is a human polymer found almost exclusively on NCAM (neural cell adhesion molecule) where it plays an antiadhesive function in brain development (11). The highly hydrophilic nature of PSA results in similar hydration properties to PEG, giving it a high apparent molecular weight in the blood. This increases circulation time since no receptors with PSA specificity have yet been identified (12).

PSA is also synthesized by bacteria such as Neisseria meningitidis and some Escherichia coli where it is synthesized as a capsule (10). Bacterial PSA is chemically and immunologically similar to human PSA and is naturally degraded by sialidases (e.g., neuraminidase); unlike PEG, it is metabolized as a natural sugar molecule by tissue sialidases (13). Pioneered by Gregoaidis et al. (14, 15), PSA has been developed for
Site-Specific Polysialylation of an Fv Fragment

Vol. 20, No. 5, 2009

Bioconjugate Chem., 2009 925

Cloning of MFE-23-cys scFv Construct. The pUC119 expression plasmid containing the scFv MFE-23 gene was used as a template to produce the mutant MFE-23-cys construct (21). Oligonucleotides incorporating restriction sites for recombinant subcloning, as well as mutations to include a C-terminal thiol group, were used to flank the scFv gene and allow PCR amplification of the mutant scFv MFE-23-cys. The forward primer was LMB3; 5′-CAGGAAACACGTTAGAC-3′, while the reverse primer cyssMFE; 5′-GGCAGCAGCCCAAGT-GCGGCCGCAACAGCTATGAC-3′ contained a realigned Neo I site (underlined) and (Gly)6Cys peptide (bold). The PCR product was subcloned into the pHEN2 vector as an Neo I/Not I fragment and DNA sequence verified.

Production and Purification of F1, MFE-23, and MFE-23-cys scFvs. F1 was used as a non-CEA binding negative control scFv. F1 binds to human placental alkaline phosphatase (PLAP) and was selected from a phage display library [Wright, Ph.D. thesis, University of London, 2003]. Protein expression of F1, MFE-23, and MFE-cys scFvs was initiated by growing single colonies of newly transformed E.coli HB2151 bacteria in 2TY media with 0.1 mg/mL ampicillin overnight shaking at 250 rpm and 37 °C. When a culture OD600nm of 0.6–0.8 was reached, IPTG was added to a final concentration of 0.1 mM. The induced culture was then left to grow overnight at 30 °C and 250 rpm during which time protein expression occurred. Induced cultures were centrifuged at 10,000 rpm, and the supernatant from which protein was to be purified was supplemented with benzamidine (final concentration 2 mM) and kept on ice where possible to minimize protein degradation. The supernatant was concentrated 10-fold by ultrafiltration and dialyzed into phosphate-buffered saline (PBS). The scFv protein was purified by immobilized metal affinity chromatography using PALON according to the manufacturer’s instructions. Pure protein was eluted with 200 mM imidazole and stored at 4 °C. ScFv proteins were further purified using a HiLoad 10/30 Superdex-75 HR gel filtration column equilibrated with PBS and compared to previously run protein standards. Samples were further analyzed for the presence of protein by Coomassie staining and Western blotting. The presence of a functional thiol in the MFE-23-cys scFv was confirmed by coupling a fluorescein maleimide (Pierce Chemicals) reporter group, electrophoresing by SDS-PAGE, and visualizing the protein band by fluorescence imaging compared to nonthiol containing MFE-23 scFv (data not shown) or by binding to CEA-positive LS174T cells and analyzing by flow cytometry (data not shown).

Chemical Polysialylation. The PSA used in this work was colominic acid (linear α-(2→8) linked N-acetylneuraminic acid) purified from the capsular coating of E. coli K1, supplied as a 11 kDa polymer (average molecular weight), which is equivalent to 1.5–5 units of sialic acid. The average molecular weight (nominal mass) of these polymers was determined by gas-phase chromatography by Viscotech Europe Ltd. (14). For the purpose of F1 and MFE scFvs-PSA conjugation by reductive amination, PSA (colominic acid) was oxidized with 0.1 M periodate as described (14, 17). The oxidized material was dialyzed extensively against a 0.01% ammonium carbonate buffer (pH 7.4) at 4 °C, concentrated by reverse dialysis on a bed of polyethylene glycol (8 kDa), lyophilized, and stored at −80 °C until required. Oxidized PSA was added to 5 mL of protein (0.2 mg/mL) to give a 2-fold molar excess in a glass tube. The sample was mixed gently and supplemented with 20 mg of NaCNBH3. The tube was sealed and rotated at 37 °C for 48 h to allow the conjugation reaction to occur. For thiol conjugation, scFv samples were dialyzed extensively against 50 mM Tris-HCl buffer pH 7.0 concentrated to 0.5 mg/mL and made up to a final volume of 950 μL. Protein was reduced by addition of 50

Clinical use. Reductive amination of the nonreducing end of the oxidized PSA allows chemical conjugation via primary amine groups on proteins, and the therapeutic benefits of PSA conjugation have been demonstrated with aspariginase (16) and insulin (14) for the treatment of leukemia and diabetes, respectively. Recent clinical data from trials with polysialylated insulin (SulixPen) and erythropoietin (ErepoPen) showed that these biopharmaceuticals were well tolerated and had improved pharmacokinetics [http://www.lipoxen.com/news.aspx]. More recently, we have shown that Fab fragments can be chemically polysialylated with a range of different lengths and ratios of linear PSA chains (17). Polysialylation of an antitumor Fab resulted in a 5-fold increase in blood exposure (bioavailability) with a corresponding 3-fold increase in tumor uptake compared to the unmodified Fab (17).

The use of recombinant antibody fragments has been increasingly investigated for therapeutic applications, as they are versatile and offer many advantages over current therapies using whole immunoglobulins (18). Because of their smaller size and lower molecular complexity, antibody fragments such as single-chain Fvs (scFvs) can be easily selected from combinatorial libraries and more readily expressed in a bacterial expression system, leading to rapid and inexpensive production (19). For therapy in vivo, the smaller size allows for faster tissue penetration; however, the targeted absolute uptake of such species may be limited by the subsequent faster clearance rate through glomerular filtration (20). We hypothesized that chemical polysialylation of a scFv would result in more favorable pharmacokinetic properties. The approach of modulating antibody pharmacokinetics by chemical polysialylation has not been applied to these molecules. Normally, antibody fragments are rebuilt into whole human immunoglobulins or constructed into fusion proteins which generally increases antibody size and hence decreases blood clearance (20). However, polysialylation may have advantages in terms of time and complexity as well as the advantages over chemical PEGylation already described.

The scFv used in our studies was MFE-23, a well characterized anti-CEA (carcinoembryonic antigen) scFv with a Kd of approximately 2 nM (21, 22). MFE-23 has been previously investigated in clinical trials for imaging (23, 24) and therapeutic use (25, 26). We compared the effectiveness of random, amine-directed chemical conjugation of PSA (14, 17) with site-specific, thiol-directed chemical conjugation. The latter method presents a number of advantages in the control of polymer attachment because it is distant from the antigen binding regions and limited only to hydrophilic and therefore normally surface-exposed thiol sites which generally are not involved with protein conformation and bioactivity.

Our results show that both amine-directed and site-specific polysialylation resulted in scFv-immunoconjugates with extended pharmacokinetic parameters, improved tumor uptake, and improved kidney to blood ratios. However, the amine-directed conjugation method resulted in reduced immunoreactivity. This problem was overcome by site-specific polysialylation on an engineered C-terminal thiol peptide.

EXPERIMENTAL PROCEDURES

Materials. Polysialic acid of 11 kDa was from Sigma. CEA antigen was supplied by Calbiochem; CEA-positive LS174T cell-line supplied by the EACC and grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 50 IU/mL of penicillin and streptomycin (Invitrogen). Unless stated, immunochemicals were from Sigma. Mouse anti-PSA-NCAM monoclonal IgM was from Chemicon. Carrier-free 125I-NaCl was supplied by Amersham Pharmacia Biotech. Bacterial cultures were grown with 2TY media supplemented with 0.1 mg/mL ampicillin (Sigma) for selection purposes.
µL of TCEP-HCl to give a final concentration of 2.5 mM and incubated for 90 min at room temperature. Reactive maleimide-PSA (made by the addition of 5-fold molar excess of N-[β-maleimidopropionic acid] hydrazide (Pierce) to colomonic acid aldehyde dissolved in 0.1 M sodium acetate, vortex mixed, wrapped in foil and incubated at 37 °C for 2 h on a rotary mixer followed by ethanol precipitation) was added to the reduced protein sample to give a 25 molar excess of PSA/protein, mixed gently, and incubated at room temperature for 2 h. In both methods, the conjugated material was purified from free PSA by addition of (NH₄)₂SO₄ to give a 70% saturated solution. The sample was rotated for 5 min at room temperature followed by 30 min on ice. The mixture was then centrifuged at 5000 rpm for 5 min, and the resulting pellet resuspended in 1 mL of 100% saturated (NH₄)₂SO₄. The precipitation procedure was then repeated and the pellet dissolved in minimal PBS followed by extensive dialysis. Finally, the conjugated sample was analyzed by SDS-PAGE and characterized for polysialylation efficiency by resorcinol assay. A scheme of the coupling is shown in Figure 1.

**Determination of PSA:Fab Ratios.** The resorcinol assay was based on the method by Svennerholm (27) and allowed the amount of colomonic acid present in a sample relative to known standards to be measured. The resorcinol agent (0.25 mM cupric sulfate, 0.2% resorcinol, 80% concentrated HCl made up with distilled water) was freshly prepared, and at least five colomonic acid standards were prepared within the 40–400 µg/mL range. In triplicate, 100 µL of standard and test samples was added to 100 µL of resorcinol agent. The tubes were sealed, and the mixture was heated at 50 min at 95 °C. Once the tubes had cooled, 400 µL of ethanol was mixed in. Finally, the absorbance at 560 nm was measured for 250 µL of each sample and standards in a microplate assay. The standard values were used to construct a calibration curve from which the colomonic acid content of the test samples could be determined. A molar value was determined according to the mean PSA length used based on the method by Svennerholm (27).

**Western Blot Analyses.** Protein gels for Western analysis were transferred to a nitrocellulose membrane, blocked in PBS/5% Marvel milk protein, and then incubated with either anti-His₉ specific (Qiagen) or anti-PSA specific (Chemicon) antibodies followed by antimouse HRPO conjugates (Sigma). The blot was developed using enhanced chemiluminescence and exposed to high performance autoradiography film.

**Gel Filtration of Proteins and Conjugates.** Gel filtration was carried out using a Superdex-200 column equilibrated with PBS run at 1.5 mL/min at 4 °C on a Biologic DuoFlow system. Native molecular weight standards were used to calibrate the column. Eluted protein were monitored at absorbencies of 280 nm (protein) and 214 nm (peptide bonds).

**Protein Radioiodination.** Protein samples were radioiodinated using Pierce iodination tubes. To precoated tubes, 100 µL of the Tris iodination buffer was added followed by 10 µL (1.0 mCi) Na¹²⁵I. The activated iodide was then removed and added directly to the protein solution (1 mL of 0.2–0.5 mg/mL) for iodination. The reaction was allowed to take place for 8 min. To end the reaction, 50 µL of scavenging buffer (10 mg/mL tyrosine in PBS) was added and mixed. After 5 min incubation, the iodinated sample was dialyzed against PBS.

**Bioactivity by ELISA Analysis.** Flat-bottom 96-well ELISA plates coated with CEA antigen at 2 µg/mL in PBS were used. After three washes with PBS/0.1% Tween-20 and three PBS washes, the plate was then blocked with 5% PBS/Marvel for 1 h at 37 °C and washed again. Binding activity of the anti-CEA scFvs and conjugates were established by serially diluting samples across test wells in 1% Marvel/PBS for 1 h at 37 °C. Following a repeat of the wash steps, detection of bound scFv was made by incubating with ‘one-step’ HRP conjugated anti-His antibody for 1 h at 37 °C. For the purpose of PSA detection, the plate was incubated first with primary anti-PSA IgM followed by secondary anti-IgM HRP for 1 h at 37 °C. In both cases, incubation with detection antibodies was terminated by washing the plates as described above and binding detected using BM Blue POD substrate. The development was stopped with 1 M H₂SO₄ and the absorbance measured at 450 nm. Sigmoidal curves were fitted to ELISA data to determine Kd values using SigmaPlot using the one-site saturation ligand-binding equation.

**Pharmacokinetic and Biodistribution Studies.** Female nude BALB/c mice, 6–8 weeks old (Harlan UK), were used for in vivo studies. All in vivo research was carried out under a UK Home Office project license PPL 70/5833. Human tumor xenografts were set up by injecting mice subcutaneously into the left flank with 0.1 mL containing up to 10 million LS174T cells. Tumour growth was monitored and took 3–4 weeks to reach the required 5–8 mm diameter for subsequent work. For tissue analyses, 50 µL radiolabeled samples (5–10 µg) were injected intravenously into the mouse tail vein. At the appropriate time points (typically 0.5, 2, 6, 24, and 48 h) mice were culled by exsanguination under terminal anesthesia. Immediately, blood was collected by cardiac puncture and...
transferred to a sample tube. The mouse was then dissected, and tissues were collected for biodistribution analysis. Radioactivity was counted using a gamma counter. Values were expressed as a percentage of the initial injected dose per gram (% id/g). For studying in vivo blood clearance pharmacokinetics, data values were fitted using SigmaPlot to equations that conform to the two-compartmental intravenous model of clearance, which takes into account the biexponential clearance phases, distribution phase and elimination phase, of single intravenous doses. This is described by the exponential decay, double, four-parameter equation

\[ y = ae^{-bt} + ce^{-dt} \]

where the distribution phase clearance rate \( t_{1/2}R \) can be determined by \( \ln 2/b \), and the elimination clearance rate \( t_{1/2}/beta_{two} \) can be determined by \( \ln 2/d \). The area under the curve values for blood clearance were determined using the macros available within SigmaPlot to represent protein blood bioavailability.

RESULTS

Purification and Characterization of scFv MFE-23-cys.

The MFE-23-cys scFv constructed and expressed in E. coli showed a 5-fold lower level (∼1 mg/L) of protein expression than the parental scFv, probably because of the formation of incorrectly folded intermediates and mixed disulfides due to the unpaired cysteine as seen previously (28). As expected, the amount of monomeric MFE-cys scFv was even lower due to the presence of disulphide-linked scFv dimers and, additionally, noncovalent dimers (29) which were resolved by size-exclusion chromatography (see Supporting Information Figure S1). This led to a final yield of MFE-cys scFv monomer of around 0.5 mg/L of bacterial culture. The binding activity of the MFE-23-cys scFv relative to that of the unmodified scFv was determined by ELISA (Figure 2a). Similar \( K_d \) values were determined for the MFE-23-cys and MFE-23 scFvs of 2.5 and 2.4 nM, respectively (derived by fitting a curve using SigmaPlot) which implied that the additional thiol peptide in the MFE-23-cys scFv did not alter its antigen binding affinity. Fluorescein coupling confirmed the functionality and 1:1 stoichiometry of the C-terminal thiol and also demonstrated the ability for thiol conjugates to bind to live cells by FACS (data not shown).

Chemical Polysialylation of scFvs.

F1 and MFE-23 were initially polysialylated by amine coupling at low (1:1 to 1:2) molar ratios. Resorcinol tests showed that the substitution ratios were approximately 1:1.5 (Table 1). However, the amine-coupled polysialylated MFE-23 scFv showed a 20-fold reduced immunoreactivity compared to the unmodified scFv (Figure 2b). This suggested that the process modified one or more key lysine residues in the MFE-23 scFv. Thiol coupling using the maleimide-activated PSA yielded a ratio of ∼1:1 with fully immunoreactive scFv (Figure 2c). Reducing/SDS-PAGE gel analysis confirmed amine- and thiol-coupled scFv conjugates showed a 20-fold reduced immunoreactivity compared to the unmodified scFv (Figure 2b). This suggested that the process modified one or more key lysine residues in the MFE-23 scFv. Thiol coupling using the maleimide-activated PSA yielded a ratio of ∼1:1 with fully immunoreactive scFv (Figure 2c). Reducing/SDS-PAGE gel analysis compared amine- and thiol-coupled scFvs (Figures 3 and 4). Both the amine-coupled MFE-23 and F1 scFvs displayed a broad polysialylated migration pattern, characteristic for a protein conjugate of heterogeneous mass composition (Figure 3). The thiol-coupled MFE-23-cys conjugate produced a less polydispersed immunoconjugate compared to the amine-coupled antibodies, suggesting a more defined molecular species. Both conjugates eluted from a gel filtration column in the excluded/void volume, indicating a larger apparent size (>300 kDa rather than the theoretical size of 41 kDa) or hydrodynamic radius. Proteins of molecular weight greater than 300 kDa are not resolved, and therefore it was not possible to determine the true apparent sizes of the scFv–PSA conjugates (see Supporting...

**Table 1. Mean PSA Coupling Ratio of the Three scFvs Used in This Study**

<table>
<thead>
<tr>
<th>ScFv species</th>
<th>mean PSA:scFv molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.5</td>
</tr>
<tr>
<td>MFE-23 (amine)</td>
<td>1.4</td>
</tr>
<tr>
<td>MFE-23-cys</td>
<td>1.1</td>
</tr>
</tbody>
</table>
The presence of the PSA chain was confirmed by immunodetection of the PSA chain by ELISA (Figure 2d) or Western blotting (Figure 4c) as well as the resorcinol assay (Table 1). In each case, the PSA chain alone was undetectable by ELISA or Western blot unless conjugated to a protein.

**Figure 3.** SDS-PAGE and blotting of amine-conjugated scFv. (A) Coomassie-stained gel of protein markers (M), MFE-23 scFv (1), MFE-23 scFv process control (2), and polysialylated MFE-23 scFv (3). (B) as in A, detected with anti-His6 in a Western blot. (C) Coomassie-stained gel of protein markers (M), F1 scFv (1), and F1-PSA (2). (D) as in B, detected with anti-His6 in a Western blot.

**Figure 4.** SDS-PAGE and blotting of thiol-conjugated scFv. (A) Coomassie-stained gel of protein markers (M), MFE-23 scFv (1), and polysialylated MFE-23-cys scFv (2), free PSA (3). (B) as in A, detected with anti-His6 in a Western blot. (C) as in A, detected with anti-PSA in a Western blot.

Information Figure S1). Coomassie stained SDS-PAGE gels and Westerns blot of samples analyzed under reducing conditions showed that the amine-directed conjugates, despite being coupled at low molar ratios to obtain as close to 1:1 coupling as possible, had higher ratios and were much more dispersed in terms of molecular weight. The longer smear suggests high multiples of PSA conjugates even though the mean ratio was 1.4–1.5:1. This was in contrast to the 1:1:1 ratio seen with the site-specific coupled scFv and more importantly, the lower level of heterogeneity.

The reduced immunoreactivity upon amine coupling was likely to account for these observations, and this was confirmed by analyzing the uptake and biodistribution of the fully active MFE-23 cys-PSA immunoconjugate (Figure 5a, Figure 6, Table 2). Once again, polysialylation led to an increased blood half-life but lower than that of the multiply substituted amine conjugates. The t_{1/2}β increased 3.6-fold to 15.6 h and the bioavailability increased 9.8-fold Figure 6a. This increase led to increased tumor uptake compared to the faster-clearing unmodified MFE-23 scFv. Greater than 2-fold increase in tumor exposure was seen (Figures 5e and 5b) with the percentage uptake per gram of tumor increasing approximately 2-fold at 6 h (4% to 8.2%) and at 24 h (4.5% to 9.6%). There was 30-fold more scFv in the tumor by 48 h (0.1% to 3.3%). The higher tumor uptake caused by increased blood half-life was at the expense of lower tumor:tissue ratios (Figure 6c). However, there was still significant selectivity, with tumor:blood ratios of 4.4:1 at 24 h and 10.3:1 at 48 h. Throughout, it was observed that all tissue:blood ratios of polysialylated antibodies <1 indicating that polysialylation did not impart nonspecific binding.

**DISCUSSION**

We have employed chemical conjugation of PSA to scFv antibodies in order to extend their biological half-life. We have tested the immunoreactivity of the conjugates and investigated their biodistribution and ability to localize specifically to human tumors in murine models. All the scFvs showed an increase in apparent molecular weight as a result of conjugation. Although there was some heterogeneity of the immunoconjugates due to the polydisperse nature of naturally occurring PSA, this was consistent with previous observations (14, 16, 17).

Many researchers have previously used surface-exposed (30, 31) or engineered (32, 33) thiol groups for nondisruptive chemical conjugations. The use of mammalian systems often circumvents expression and yield problems (32). Furthermore, the incorporation of thiol sites for specific PSA conjugation high negative charge) for either to be detected by antibodies. This confirms that the PSA association was covalent and insignificant amounts of free PSA were present in the preparations.

**Biodistribution and Pharmacokinetic Studies of MFE-cysPSA11 scFv in a Tumor-Bearing Murine Model.** In vivo experiments showed that polysialylation conjugation of scFv led successfully to an increased blood half-life in all cases. We first assessed the effects of polysialylation on the F1 scFv which had no specificity for CEA-expressing LS174T murine xenografts (Figure 5a). The blood half-life (beta elimination phase, t_{1/2}β) for F1-PSA increased almost 3.5-fold (Figure 5a and 5b, Figure 6, Table 2) from 5.6 h to 19.3 h with a 10-fold increase in blood exposure (bioavailability area under the blood clearance curve, Figure 6a, Table 2). Not unexpectedly, polysialylation conjugation also resulted in higher concentrations of scFv in normal tissues. However, this was observed in all tissues, and there was no specific effect on tumor uptake (Figure 5b). We next assessed the effect of polysialylation on biodistribution of MFE-23 (Figure 5c and 5d, Figure 6, Table 2). A greater increase in t_{1/2}β was seen for the amine-coupled MFE-23 scFv (a 4.8-fold increase to 20.9 h) and bioavailability (15.2-fold increase). However, compared to the unmodified MFE-23 scFv which localized well and specifically (Figure 5c), the MFE-23-Lys-PSA conjugate was less effective in specifically targeting to the LS174T tumors (Figure 5d, Figure 6b). Consistent with previously published work, the MFE-23 scFv accumulates in this tumor model with a tumor:blood ratio of around 14:1 by 24 h, but the amine-coupled immunonconjugate had a ratio of 1.4 at this point. There was, however, some specificity for MFE-23-Lys-PSA for tumors compared to organs such as kidney, liver, spleen, and muscle.

The reduced immunoreactivity upon amine coupling was likely to account for these observations, and this was confirmed by analyzing the uptake and biodistribution of the fully active MFE-23 cys-PSA immunoconjugate (Figure 5e, Figure 6, Table 2). Once again, polysialylation led to an increased blood half-life but lower than that of the multiply substituted amine conjugates. The t_{1/2}β increased 3.6-fold to 15.6 h and the bioavailability increased 9.8-fold Figure 6a. This increase led to increased tumor uptake compared to the faster-clearing unmodified MFE-23 scFv. Greater than 2-fold increase in tumor exposure was seen (Figures 5e and 6b) with the percentage uptake per gram of tumor increasing approximately 2-fold at 6 h (4% to 8.2%) and at 24 h (4.5% to 9.6%). There was 30-fold more scFv in the tumor by 48 h (0.1% to 3.3%). The higher tumor uptake caused by increased blood half-life was at the expense of lower tumor:tissue ratios (Figure 6c). However, there was still significant selectivity, with tumor:blood ratios of 4.4:1 at 24 h and 10.3:1 at 48 h. Throughout, it was observed that all tissue:blood ratios of polysialylated antibodies <1 indicating that polysialylation did not impart nonspecific binding.

**DISCUSSION**

We have employed chemical conjugation of PSA to scFv antibodies in order to extend their biological half-life. We have tested the immunoreactivity of the conjugates and investigated their biodistribution and ability to localize specifically to human tumors in murine models. All the scFvs showed an increase in apparent molecular weight as a result of conjugation. Although there was some heterogeneity of the immunoconjugates due to the polydisperse nature of naturally occurring PSA, this was consistent with previous observations (14, 16, 17).

Many researchers have previously used surface-exposed (30, 31) or engineered (32, 33) thiol groups for nondisruptive chemical conjugations. The use of mammalian systems often circumvents expression and yield problems (32). Furthermore, the incorporation of thiol sites for specific PSA conjugation high negative charge) for either to be detected by antibodies. This confirms that the PSA association was covalent and insignificant amounts of free PSA were present in the preparations.
should result in a more reproducible, homogeneous product that can be more definitively characterized as has been shown for scFv–PEG immunoconjugates (33). This is of particular importance for any subsequent biopharmaceutical interest which requires definitive characterization to be determined for the purpose of therapeutic applications. By applying this methodology for PSA conjugation, it is believed that the longevity of antibodies in circulation can be improved without compromising its bioactivity or structural integrity.

Our immunoreactivity experiments with the PSA conjugations revealed that the thiol-specific conjugation method was superior to amine conjugation. The retention of protein bioactivity observed after thiol-specific PSA conjugation of the scFv MFE-23 has mirrored the success of other groups that have used thiols for chemical attachments. PEGylation of proteins, which represents the biggest contribution of polymer conjugates to the biopharmaceutical industry, has recently shown various successes in thiol-specific conjugation such as in the C-terminal

Figure 5. Biodistribution of scFvs and immunoconjugates in LS174T tumor model at 6 and 24 h time points. Percentage injected dose of radiolabeled protein per gram of tissue was determined for F1 scFv (A), amine-coupled F1 scFv-PSA (B), MFE-23 scFv (C), amine-coupled MFE-23 scFv-PSA (D), and thiol-coupled MFE-23-cys-PSA (E).

Figure 6. Pharmacokinetic profiles derived from full biodistribution study in LS174T tumor model over 48 h. (A) Blood clearance profile, (B) tumor uptake profile, and (C) tumor to blood ratio profile of F1 scFv (●), amine-coupled F1 scFv-PSA (○), MFE-23 scFv (●), amine-coupled MFE-23 scFv-PSA (●), and thiol-coupled MFE-23-cys-PSA (●).
The blood clearance profile was fitted to a biexponential decay model by calculating the area under the blood clearance curve. The main reason for chemically modifying scFvs with PSA is to modulate their half-lives so that they clear more slowly, accumulate to higher levels in the target tissues (tumors in this case), and still retain some high degree of selectivity (e.g., high tumor:blood ratios). Whole immunoglobulins characteristically clear slowly with half-lives in the region of 3–5 days, resulting in tumor:blood ratios >1 after about 48 h (20, 39). Single-chain Fv's clear in a few hours but have tumor:blood ratios of >20:1 at this time. This research has shown that site-specific polysialylation can impart a blood clearance half-life (in murine models) slower than scFvs (4–5 h) or Fab (around 10 h), better blood bioavailability (2–3 fold better than some Fabs and only 2–3 lower than some whole IgGs), and specificity ratios much more favorable than whole immunoglobulins. Polysialylated scFvs, easily and inexpensively prepared, could represent a viable alternative to whole IgG without the concerns associated with the Fc-domain (39, 40). In vivo analyses confirmed the pharmacokinetic benefit of chemical polysialylation, but unlike the nonspecific approach shown to be successful with the H17E2 Fab (17), site-specific coupling was required to maintain scFv function. In all three examples shown here, polysialylation resulted in around 3- to 5-fold increase in blood clearance and around 10-fold increase in bioavailability. There was a less than 2-fold increase in the alpha-phase (distribution) blood clearance, suggesting that the main pharmacokinetic enhancement was in the systemic elimination phase. The pharmacokinetic extension observed here is likely due to the increase in apparent size, stabilizing the protein and excluding it from renal clearance. This is consistent with the increased size observed by size-exclusion chromatography (>300 kDa), due to the hydrophilic polymer binding noncovalently to a network of water molecules as has been proposed for PEG conjugates (40).

Table 2. Summary of Pharmacokinetic Parameters

<table>
<thead>
<tr>
<th>ScFv species</th>
<th>t1/2α (h)</th>
<th>t1/2β (h)</th>
<th>Area under blood clearance curve (bioavailability or blood exposure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.43 ± 0.04</td>
<td>5.6 ± 0.12</td>
<td>13.03</td>
</tr>
<tr>
<td>F1-PSA</td>
<td>0.68 ± 0.19</td>
<td>19.3 ± 0.91</td>
<td>137.7</td>
</tr>
<tr>
<td>MFE-23</td>
<td>0.42 ± 0.01</td>
<td>4.3 ± 0.5</td>
<td>12.3</td>
</tr>
<tr>
<td>MFE-23-Lys-PSA</td>
<td>0.78 ± 0.03</td>
<td>20.9 ± 1.5</td>
<td>186.6</td>
</tr>
<tr>
<td>MFE-23-Cys-PSA</td>
<td>0.7 ± 0.07</td>
<td>15.6 ± 3.7</td>
<td>120.6</td>
</tr>
</tbody>
</table>

a The biodistribution experimental data are shown in Figures 4 and 5. The blood clearance profile was fitted to a biexponential decay model and t1/2α and t1/2β determined. The bioavailability or blood exposure was determined by calculating the area under the blood clearance curve.

ACKNOWLEDGMENT

This work was funded by a Lipoxen Technologies-BBSRC/CASE studentship to MPD and AC (ref. no. 01/A2/C/07249). We would like to thank Dr. Peter Laing and Prof. Gregory Gregoriadis for useful discussions and members of the Imperial College CBS Unit for advice and technical help with the in vivo experiments.

LITERATURE CITED


