ChemPubSoc Europe

DOI: 10.1002/cmdc.201900077

CHEMMEDCHEM Full Papers

Characterization of Disulfide Bond Rebridged Fab–Drug Conjugates Prepared Using a Dual Maleimide Pyrrolobenzodiazepine Cytotoxic Payload

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We describe the characterization of antigen binding fragments (Fab)–drug conjugates prepared using a dual maleimide pyrrolobenzodiazepine dimer cytotoxic payload (SG3710). Pyrrolobenzodiazepine dimers, which are DNA cross-linkers, are a class of payloads used in antibody–drug conjugates (ADCs). SG3710 was designed to rebridge two adjacent cysteines, such as those that form the canonical interchain disulfide bond between the light and heavy chain in Fab fragments. The rebridging generated homogenous Fab conjugates, with a drug-to-Fab ratio of one, as demonstrated by the preparation of rebridged Fabs derived from the anti-HER2 trastuzumab antibody and from a negative control antibody both prepared using recombinant expression and papain digestion. The resulting anti-HER2 trastuzumab Fab-rebridged conjugate retained antigen binding, was stable in rat serum, and demonstrated potent and antigen-dependent cancer cell-killing ability. Disulfide rebridging with SG3710 is a generic approach to prepare Fab–pyrrolobenzodiazepine dimer conjugates, which does not require the Fabs to be engineered for conjugation. Thus, SG3710 offers a flexible and straightforward platform for the controlled assembly of pyrrolobenzodiazepine dimer conjugates from any Fab for oncology applications.

Introduction

Antibody drug conjugates (ADCs) are a class of drugs designed to deliver cytotoxic payloads to tumors, thus potentially increasing the therapeutic index of small-molecule cytotoxic drugs.[1] Four ADCs are approved for clinical use by the US Food and Drug Administration (FDA): brentuximab vedotin (Adcetris),[2] approved for Hodgkin lymphoma and systemic anaplastic large cell lymphoma; inotuzumab ozogamicin (Besponsa),[3] approved for relapsed or refractory B-cell precursor acute lymphoblastic leukemia; ado-trastuzumab emtansine (Kadcyla),[4] approved for human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer; and gentuzumab ozogamicin (Mylotarg),[5] approved for CD33-positive acute myeloid leukemia. More than 65 ADCs are in various stages of clinical development, and numerous other ADCs are in late-stage preclinical characterization.[6]

Most of the ADCs in preclinical and clinical development contain cytotoxic payloads that inhibit cell division, such as auristatin and maytansine.[7] Another class of ADCs contain DNA-damaging agents such as pyrrolobenzodiazepine dimers (PBD)[8] and duocarmycin analogues.[9] These ADCs are based on full-length antibodies. The molecular weight of these ADCs (≈150 kDa) could pose multiple problems including poor tumor penetration and uptake, increased systemic accumulation and slow clearance.[10,11] In addition, the presence of the Fc region, while beneficial for effector functions and half-life in naked therapeutic antibodies, can cause Fc-mediated side effects in ADCs.[12] Adcetris was shown to have no detectable complement-dependent cytotoxicity (CDC) and minimal antibody-dependent cellular cytotoxicity (ADCC).[13] McDonagh et al. reported that comparable ADCs with or without Fc effector functions could not conclusively demonstrate any benefit for Fc-enabled ADCs.[14] Gentuzumab ozogamicin (Mylotarg),[5] is based on IgG4, which has decreased Fc receptor binding.[15] More recently, IgG1 engineered for lack of Fc binding are being used as ADC scaffolds.[16,17]

Small fragments derived from IgGs or from other highly structured scaffolds derived from natural proteins have found use as ADCs.[10,11,18] Antibody fragments may be ideal formats as ADC cancer therapeutics because they could have higher tumor uptake, a better tumor-to-blood ratio, and faster clearance than full-length antibodies.[10,11,18] The former may be a desirable property in cases where toxicities to healthy tissues can increase with prolonged exposures. Furthermore, fragments do not have the Fc region, which could be beneficial to overcome potential effector function related toxicities.[12] Amongst the plethora of antibody formats, Fab fragments, which comprise the constant and variable domains of immunoglobulins, have advantages over other antibody fragments, including: 1) high levels of recombinant expression, 2) straightforward of preparation via papain digestion of full-length antibodies, 3) standard affinity chromatography purification from culture supernatant using anti-lambda, anti-kappa, or anti-CH1/CL media, 4) high thermostability, and 5) possession of

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The ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/cmdc.201900077.

native thiols forming a solvent-accessible disulfide bond, which upon reduction can be used as attachment points for conjugation.[19]

Rebridging of the native interchain disulfide bond has been used as a strategy to prepare Fab–drug conjugates (FDCs) and ADCs.[20–27] For example, Godwin and colleagues[20,21] developed a rebridging conjugation approach using a bis-sulfone linker–drug, in which interchain disulfide bonds in either full-length antibodies or Fab fragments were first partially reduced, followed by bis-alkylation to conjugate both thiols of the two native cysteines. The bis-sulfone linker carried a cytotoxic payload (monomethyl auristatin E), and the rebridging approach resulted in FDCs and ADCs that were more stable in human serum than conjugates prepared using non-rebridging maleimide chemistry. Similarly, Behrens et al.[22] rebridged reduced interchain cysteines in antibodies using a dibromomaleimide monomethyl auristatin F payload, which resulted in the conjugation of four cytotoxic drug–linkers per antibody. The resulting ADCs were highly stable and had potent cytotoxicity against tumor cells. Caddick and colleagues contributed significantly over the past few years to the development of several rebridging approaches to prepare well-defined FDCs and ADCs.[23–26] Finally, rebridging of disulfide bonds through a thiol–yne reaction with a cyclooctyne has also been successfully applied to an antibody Fab.[27]

Recently, we reported a novel dual-maleimide pyrrolobenzodiazepine dimer cytotoxic payload (SG3710) that rebridged the cysteines at position 220 of the hinge domain of an engineered antibody, to produce a rebridged ADC with a drug-to-antibody ratio (DAR) of one.[28] The rebridged ADCs were highly resistant to payload loss in serum, had potent and selective cytotoxicity, and importantly were tolerated in rats at twice the dose of equivalent non-rebridged ADCs but with DAR of two prepared using a mono-maleimide PBD (SG3249). Our data suggested that SG3710, which offers the opportunity to prepare IgG1-based ADCs with DAR of one, could be a strategy to improve the therapeutic index of PBD-based ADCs.[28]

Here, we investigated whether SG3710 can be used to rebridge the native cysteines that form the interchain disulfide bond between the light and heavy chain in Fabs. We demonstrated that SG3710 efficiently rebridged a Fab derived from the anti-HER2 trastuzumab antibody and a Fab from an isotype control antibody, and the resulting trastuzumab rebridged FDC was highly cytotoxic and specific against cancer cells in vitro. Furthermore, the rebridged Fabs were highly stable in rat serum. We also demonstrated that the SG3710 rebridging approach is broadly applicable to Fabs produced either recombinantly or by papain digestion of full-length antibodies. The rebridged FDC platform described here may offer an alternative to full-length ADCs for oncology therapeutics that use PBDs.

Results and Discussion

Structural characteristics of SG3710

The dual-maleimide PBD SG3710[28] was synthesized using starting material from tesirine (SG3249, Figure 1 a),[29,30] a PBD

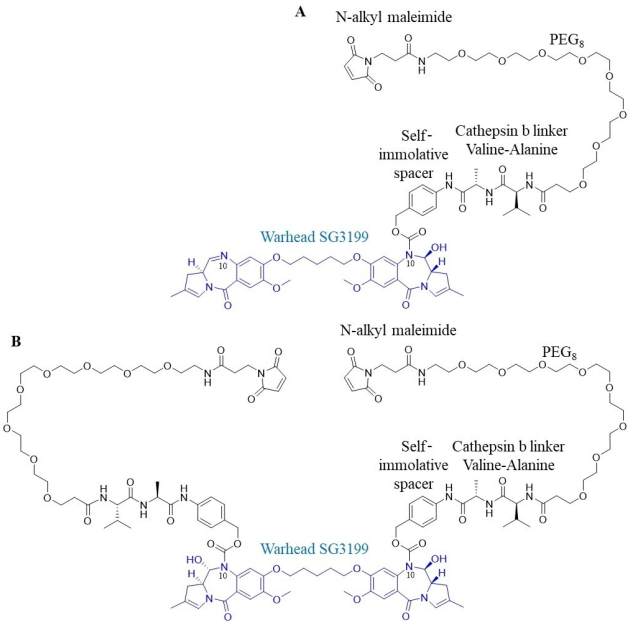

Figure 1. Structures of A) SG3249 and B) SG3710. Blue coding indicates the SG3199 warhead. Other key features of both payloads are shown. The N10 position of the warhead is labeled in both SG3249 and SG3710. The molecular weights are 1496 and 2408 Da for SG3249 and SG3710, respectively, and the respective log*D* values are −0.6 and −2.64.

payload used in several ADCs in clinical development.[31–34] SG3710 contains two maleimide-$(polyethylene\ glycol)_8$-valine-alanine-*para*-aminobenzoic acid linkers at each of the two symmetrical N10 positions of the PBD (Figure 1 b). The warhead (i.e., the sequence-selective DNA minor-groove cross-linker) of SG3710 is SG3199 (depicted in blue in Figure 1),[35] which is also the warhead of tesirine (SG3249). SG3199 is released from SG3710, and from SG3249, in the lysosomal compartment upon cleavage by cathepsin B. After translocation to the nucleus and insertion in the DNA minor groove, an aminal bond is formed through nucleophilic attack of the N2 of a guanine base at the electrophilic C11 position on the SG3199.[35]

The symmetrical structure of SG3710 offers two distinct and beneficial structural properties compared to SG3249. First, the additional $(polyethylene\ glycol)_8$ linker decreases the hydrophobicity of SG3710 versus SG3249. The SG3710 calculated log*D* value at pH 7.4 is −2.64, which is lower than the calculated log*D* value of SG3249 (−0.6). This can have a significant impact in improving the in vivo performance of ADCs[28] and FDCs prepared with SG3710, as it has been reported that ADCs with high hydrophobicity have poor pharmacokinetics and biodistribution.[36,37] Second, the release of the SG3199 warhead from SG3710 requires two cathepsin B cleavages,[38] whereas SG3249 requires only one cleavage.

Preparation of Fabs from trastuzumab and from an isotype control antibody

Fabs from the anti-HER2 antibody trastuzumab and from the isotype control antibody NIP228 were prepared by papain digestion using full-length antibodies and by transient expression. Trastuzumab and NIP228 antibodies were transiently expressed in Chinese hamster ovary (CHO) cells with yields of 300 mg L^{-1} after 14 days of transient expression. Expression levels were quantified by using a protein A quantification method.[39,40] Purification was carried out by protein A affinity chromatography, which resulted in 99% monomeric content for both antibodies (Figure 2a). Fabs were prepared by papain digestion of antibodies. Digestion was monitored by size-exclusion chromatography (SEC, data not shown). After approximately 80% digestion, both trastuzumab and NIP228 Fabs were purified by anti-kappa affinity chromatography. SEC of the purified Fabs showed monomeric content for both Fabs of approximately 80%, with the presence of high-molecular-weight aggregates, undigested antibodies and fragments (Figure 2b). For the transient expression, the Fabs were cloned into a MedImmune mammalian expression vector,[39,40] and expression was carried out in CHO cells for 14 days, resulting in 250 and 150 mg L^{-1} for trastuzumab Fab and NIP228 Fab, respectively. The Fabs were purified from the culture medium by anti-kappa affinity chromatography. Both recombinant Fabs had a monomeric content of 95%, with minor amounts of aggregates and fragments (Figure 2c). The Fabs prepared by papain digestion and by transient expression were used directly for rebridging without further purification.

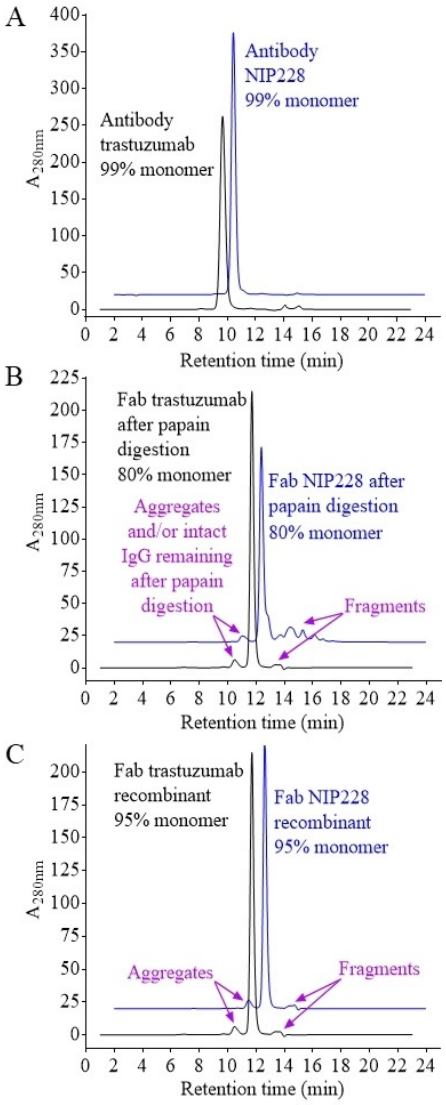

Figure 2. SEC traces for A) antibodies, B) Fabs prepared using papain digestion from antibodies, and C) Fabs prepared using transient expression. The percentage of monomeric content is shown. The arrows show fragments, aggregates and/or full-length antibodies remaining after papain digestion.

Conjugation process for preparing rebridged FDCs

A schematic representation of the rebridging process used in this study is shown in Figure 3. Conjugation of SG3710 to the cysteines forming the interchain disulfide bond between the light and heavy chains in Fabs occurs via thiol-Michael addition.[41] The Fabs prepared using papain digestion contain four additional residues, DKTH, at the C terminus of the CH1 domain. Those residues come after the cysteine at position

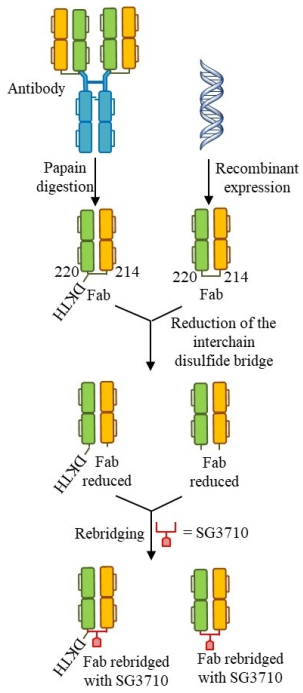

Figure 3. Experimental procedure used to rebridge the cysteines that form the interchain disulfide bonds in Fabs. The Fabs prepared using papain digestion contain four additional residues (DKTH) after the CH1 C-terminal cysteine at position 220. The interchain disulfide bond in Fabs is formed by the CH1 cysteine at position 220 and the light-chain cysteine at position 214.

220 that forms the interchain disulfide bond with the C-terminal cysteine at position 214 of the light chain. The Fabs prepared using recombinant expression do not have the additional four residues after the CH1 C-terminal cysteine at position 220.

Rebridging of trastuzumab and NIP228 Fabs prepared using papain digestion

The trastuzumab and NIP228 Fabs prepared using papain digestion were rebridged by reducing the interchain disulfide bridge between the light and heavy chains, followed by conjugation with SG3710 (Figure 3). The reducing conjugation reactions were optimized to be carried out sequentially without the need to remove excess reducing reagent. Reduced reversed-phase liquid chromatography mass spectrometry (rRPLC–MS) showed that rebridging produced nearly homogenous conjugates for both trastuzumab and NIP228 Fabs, as demonstrated by a very minor amount of non-rebridged light and heavy chains (Figure 4). The deconvoluted mass spectrometry data show the presence of unidentified molecular weight species and the minor presence of non-rebridged light and heavy chains (Figure 4, arrows). These Fabs used for the rebridging after papain digestion were about 85% monomer, which could explain the presence of non-rebridged light and heavy chains and the unidentified mass species in the rRPLC–MS data (Figure 4 arrows).

Rebridging of trastuzumab and NIP228 Fabs prepared by transient expression

The Fabs prepared by recombinant expression were rebridged by using a process similar to that of the papain-digested Fabs. The rebridging reaction analyzed by rRPLC–MS revealed high

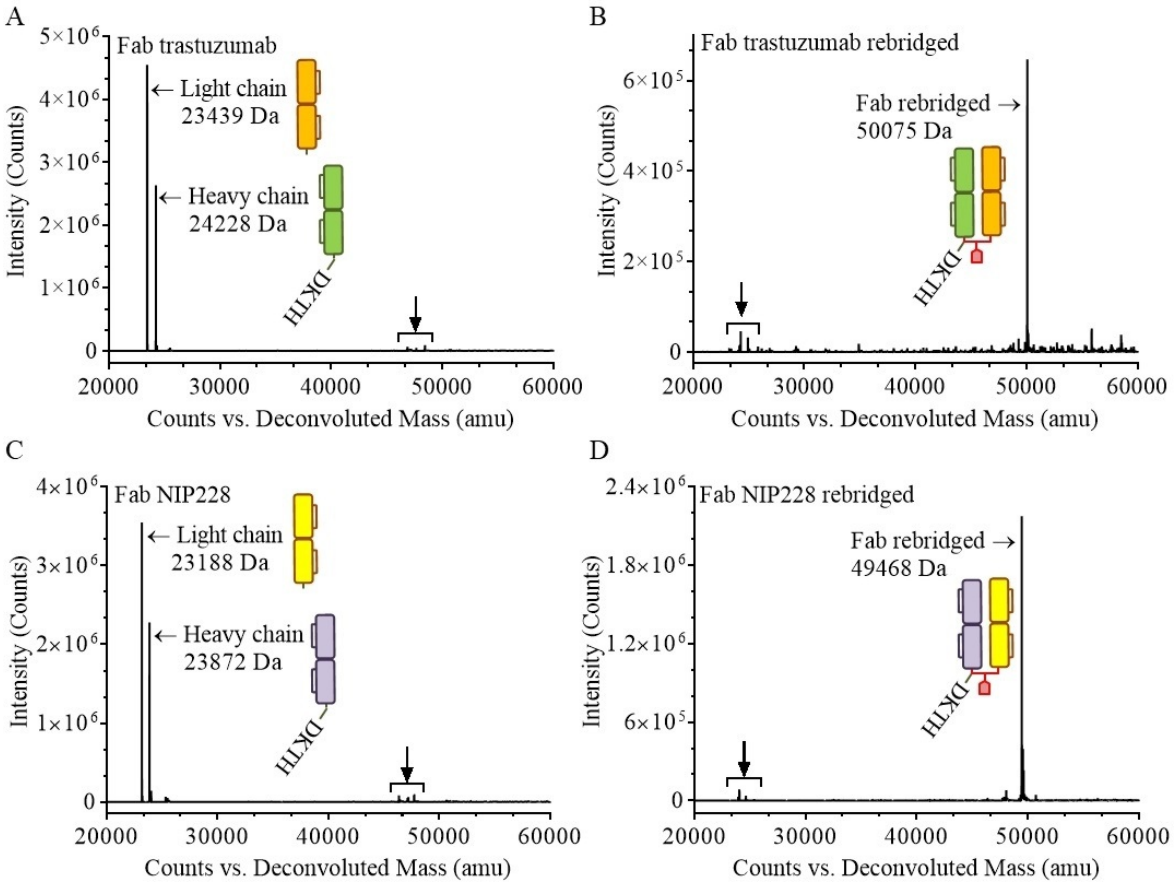

Figure 4. Rebridging of Fabs prepared using papain digestion analyzed by rRPLC–MS. Shown are the deconvoluted mass spectra of A) trastuzumab Fab, B) rebridged trastuzumab Fab, C) NIP228 Fab, and D) rebridged NIP228 Fab. The molecular weights of the light and heavy chains, and of the rebridged Fabs are shown. The cartoons represent the reduced light and heavy chain (A, C) and the rebridged Fabs (B, D). The calculated molecular weight of the papain-digested trastuzumab Fab is 47667 Da (23439 Da molecular weight light chain + 24228 Da molecular weight heavy chain). The determined molecular weight of the rebridged trastuzumab Fab is 50075, which is 2408 Da more than the non-rebridged Fab, which correspond to the molecular weight of SG3710 (Figure 1). The molecular weight of the papain-digested NIP228 Fab is 47060 Da (23188 Da molecular weight light chain + 23872 Da molecular weight heavy chain). The determined molecular weight of the rebridged NIP228 Fab is 49468, which is 2408 Da more than the non-rebridged Fab, which correspond to the molecular weight of SG3710 (Figure 1). The arrows in A and C indicate unidentified molecular weight species. The arrows in B and D indicate reduced light and heavy chains and unidentified mass species.

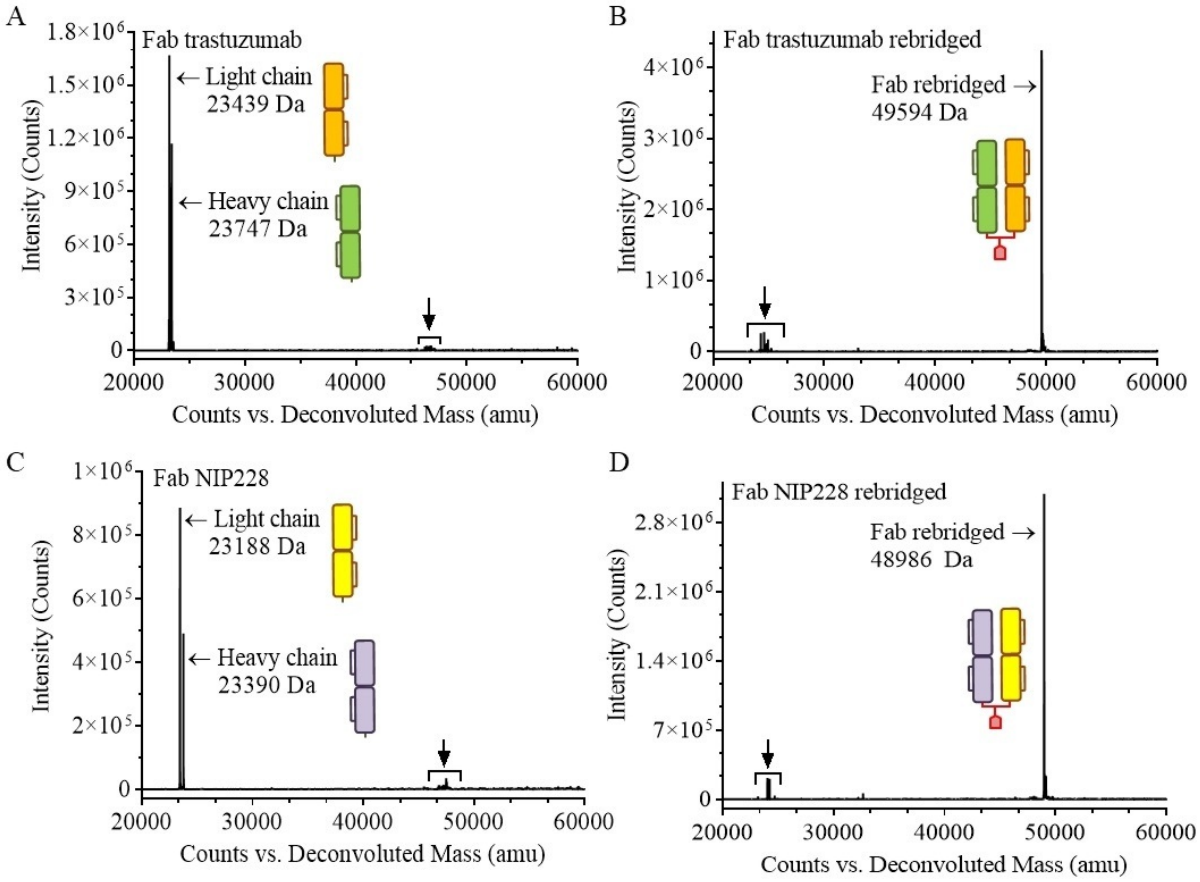

Figure 5. Rebridging of Fabs prepared using transient expression analyzed by rRPLC–MS: A) Trastuzumab Fab, B) rebridged trastuzumab Fab, C) NIP228 Fab, D) rebridged NIP228 Fab. The molecular weight of the respective light and heavy chains, and of the rebridged Fabs are shown. The cartoons represent the reduced light and heavy chain and the rebridged Fabs. The calculated molecular weight of the recombinantly produced trastuzumab Fab is 47186 Da (23439 Da molecular weight light chain + 23747 Da molecular weight heavy chain). The determined molecular weight of the rebridged trastuzumab Fab is 49594, which is 2408 Da more than the non-rebridged Fab, which correspond to the molecular weight of SG3710 (Figure 1). The calculated molecular weight of the recombinantly produced NIP228 Fab is 46578 Da (23188 Da molecular weight light chain + 23390 Da molecular weight heavy chain). The determined molecular weight of the rebridged NIP228 Fab is 48986, which is 2408 Da more than the non-rebridged Fab, which correspond to the molecular weight of SG3710 (Figure 1). The molecular weight difference between the Fabs prepared using papain digestion shown in Figure 4, and those prepared using transient expression shown here is due to the residues DKTH that remain on the heavy chains of the Fabs after papain digestion. As in Figure 4, the arrows in A and C indicate unidentified molecular weight species. The arrows in B and D indicate reduced light and heavy chains and unidentified mass species.

efficiency of rebridging, because there is very minimal measurable amounts of reduced light and heavy chain in the rebridging reaction (Figure 5). As for the rebridged Fabs prepared from papain digestion, the rRPLC–MS data showed the presence of unidentified molecular mass species and very minimal amounts of reduced light and heavy chains (Figure 5 arrow).

Analytical characterization of the rebridged FDCs

After the rebridging was demonstrated by rRPLC–MS (Figures 4 and 5), and the FDCs purified by preparative hydrophobic interaction chromatography (HIC, data not shown) to remove free SG3710 and other contaminants, the FDCs were further characterized by several analytical orthogonal methods.[16,17,28] SEC demonstrated that the purified rebridged FDCs were more than 97% monomeric (Figure 6A, B, G, H). rRPLC confirmed near homogeneity of the rebridged FDCs because there was very minimal presence of reduced light and heavy chains (Figure 6C, D, I, J). HIC further confirmed the high degree of homogeneity of the rebridged FDCs because it showed no detectable non-conjugated Fabs (Figure 6E, F, K, L). The analytical characterization demonstrated that rebridging of the cysteine forming the interchain disulfide bridge in Fabs results in homogenous FDCs for Fabs prepared using papain digestion and for Fabs prepared using recombinant expression.

In vitro rat serum stability of the rebridged FDCs

The stability of the rebridged FDCs was assessed by incubating the FDCs in rat serum for 15 days at 37 °C. The FDCs were affinity purified from the rat serum at the start of the incubation, which was used as time zero reference, and after 15 days incubation, and analyzed by rRPLC–MS (Figure 7). The stability in serum was determined by analyzing the mass peak signals of

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