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Communication

Chemically Programmed Fc Protein as Antibody Mimetic to Targeting Carbonic Anhydrase IX

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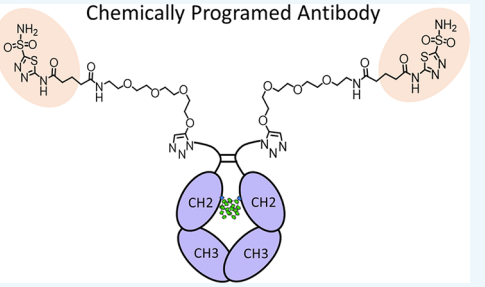
ABSTRACT: Chemically programmed antibody (cPAb) is a type of small molecule-antibody conjugate that relies on the conjugated chemical moiety for specific targeting. Although it is not as clinically successful as its comparator antibody-drug conjugate (ADC), cPAb is of great interest for rapidly developing economically attractive antibody mimetics to reduce the immunotherapeutic cost. While there are some reports on chemical approaches to develop cPAbs, the site-specific and bio-orthogonal conjugation of small-molecule ligands to an antibody including its fragment remains an ongoing challenge. Here, we describe a generalizable method for the construction of Fc-based cPAb by site-specifically conjugating the acetazolamide (AAZ) ligands (with high affinity to the tumor-associated antigen carbonic anhydrase IX) to a human IgG1-derived Fc protein in a two-step bio-orthogonal conjugation: sortase A-mediated terminal azidation and click reaction. The resulting cPAb exhibits high specificity and avidity to the cell-surface antigen carbonic anhydrase IX of renal cancer SK-RC-52 cells. In addition, the cPAb retains the IgG recycling function, reflected by a prolonged circulation half-life (∼20 h) in mice. Cell-based bioassays also reveal that cPAb can mediate the antibody-dependent cellular cytotoxicity (ADCC) to tumor cells in a fashion dependent on glycosylation status. Our results demonstrate that cPAb can mimic antibodies in aspects of both targeting capability and effector functions, showing potential as a class of immuno-therapeutics.



1. INTRODUCTION

With the development of targeted therapy, monoclonal antibody (mAb) is emerging as one of the largest drug classes, providing impressive therapeutic benefits for patients. Nearly 200 antibody-based therapeutics have been approved for the treatment of major diseases including cancers, immune disorders, musculoskeletal diseases, Alzheimer's diseases, infectious diseases, and others.[1,2] The majority of approved antibodies are in IgG format that elicits therapeutic effects by antigen neutralization, antibody-dependent cell-mediated cytotoxic (ADCC) activity, antibody-dependent cellular phagocytosis (ADCP) or complement-dependent cytotoxic (CDC) activity.[3] With the development of antibody technology, the innovation of antibody design, such as antibody-drug conjugate (ADC), can bring more therapeutic benefits. Its chemical moiety offers bonus effects such as extraordinary tumor-killing capability, by-standing effect, and others.[4,5] By appropriate control of conjugation sites and numbers, it has been demonstrated that the chemical moieties have a neglectable impact on the stability, in vivo pharmacokinetics, immunogenicity, and binding affinity of antibodies.[6] The feasibility of this concept has been proved by the statistical fact that 14 ADCs got the approval from the U.S. Food and Drug Administration (FDA) and more than 100 are in clinical development.[7]

The chemically programmed antibody (cPAb) is another type of chemically modified antibody that develops parallelly with ADC.[8,9] Instead of utilizing the attached chemicals as cytotoxic payloads in the ADC, cPAb features the chemical moiety as an antigen-binding component to modulate the targeting property of the parent antibody.[10] In cPAb, a small-molecule ligand is conjugated with an antibody or antibody fragment to adapt its pharmacological properties while retaining the mode of antigen recognition by small molecules.[10] The cPAb blends the favorable features of antibody and small molecule. Meanwhile, it provides an economically attractive approach to reduce the developing and production cost of antibody therapeutics, which has been extensively discussed in several insightful reviews.[8,11] Within the known cPAbs architected from the whole antibody or antibody fragments, the crystallizable fragment (Fc)-based cPAb represents an optimal molecular assembly as it can closely resemble the canonical antibody with respect of both shapes and functions.[11,12] This type of cPAb directly conjugates the already validated small-molecule ligands to an Fc domain, eliminates the unnecessary Fab sequence, and

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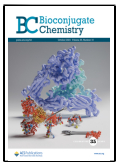

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enables the rapid development of mAb-like therapeutics against diverse targets, which is gaining growing interest.[13−16]

Acetazolamide (AAZ) is one type of such small-molecule ligand that has been validated to recognize the tumor-relevant antigen carbonic anhydrase IX (CAIX).[17] CAIX is a cell-surface glycoprotein expressed upon the activation of the hypoxia-inducible factor-1α (HIF-1α). Because of this, it is significantly upregulated by the hypoxia in tumors of solid type.[18] In contrast, the expression of CAIX on normal tissues is low and only restricted in the epithelial cells of the stomach, small intestine, and bile duct mucosa.[19,20] The cell-surface location and restricted expression in normal cells implicate CAIX as an attractive target for tumor imaging.[21] Several CAIX inhibitors including AAZ have been explored as small-molecule ligands to image the mouse xenograft models using positron emission tomography (PET) or fluorescence.[22,23] These imaging modalities, together with the AAZ-based small-molecule drug conjugates previously reported, have validated that AAZ is a useful ligand to target CAIX in cancers.[24−27] In this study, we site-specifically conjugated the AAZ ligand to the N-termini of a recombinant human IgG1-derived Fc protein and generated an Fc-based cPAb to target the CAIX for renal cancer therapy (Figure 1). To ensure the bio-orthogonal

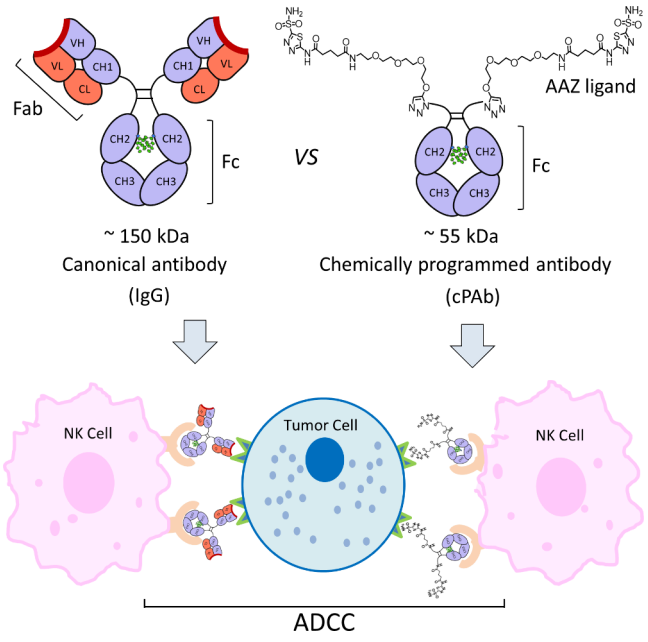

Figure 1. Structural illustration of the chemically programmed Fc protein (cPAb) in comparison to the canonical antibody. The cPAb utilizes the small-molecule AAZ ligand to replace Fab for the CAIX targeting, which reduces the size to ~55 kDa but retains the effector functions including ADCC.

conjugation, the azido reactive sites were introduced to N-termini of the Fc protein by sortase A ligation, followed by the installation of tumor-homing AAZ ligands via click chemistry, which eventually yielded the cPAb. The subsequent evaluation by flow cytometry, confocal imaging and in vitro ADCC assay on the CAIX positive cell line SK-RC-52 demonstrated that the cPAb we developed is functional as an antibody mimetic to target CAIX.

2. RESULTS AND DISCUSSION

Sortase A is a well-known transpeptidase enzyme that enables the site-specific incorporation of functional groups into protein termini with high efficiency and specificity.[28,29] In this study, we work with a heptamutant (7M) variant of *Staphylococcus aureus* sortase A reported previously.[30,31] This sortase A variant is Ca^{2+}-independent and exhibits increased catalytic activity, thus allowing the calcium-sensitive Fc protein to be ligated in the absence of Ca^{2+} at room temperature in a couple hours.[32,33] After azidation by sortase A ligation, the Fc protein is then programmed with the small-molecule ligand AAZ to generate the cPAb. AAZ (acetazolamide) is an antiepileptic drug used in clinic since the 1960s.[34] It is a potent carbonic anhydrase inhibitor with 1,3,4-thiadiazole-2-sulfonamide (zolamide) as the pharmacophore. Using zolamide as the scaffold, we synthesized an AAZ derivative for CAIX targeting (Figure 2A). We started by hydrolyzing the commercially available API acetazolamide (AAZ) into zolamide using a previously established method.[35] This step unmasks the thiadiazole amine and allows it amidated with glutaric anhydride in the presence of the acid scavenger diisopropylethylamine (DIPEA). The resulting compound 1 was then coupled with alkyne-PEG3-amine (2) to obtain the AAZ-alkyne, which can be chemically programmed on Fc protein by the copper-catalyzed azide−alkyne cycloaddition (CuAAC) reaction. Next, we synthesized the ligation substrate peptide. It is comprised by a LEPTGG sequence recognizable by sortase A but decorated with a N-terminal azido group for CuAAC (Figure 2B). To increase the ligation efficiency, a depsipeptide azide-LPET*GG was designed and used for N-terminal labeling reaction according to Theile et al.'s reports.[36] This depsipeptide features an ester linkage (annotated as *) between amino acids threonine (T) and glycine (G). It was more easily recognized by sortase A and able to achieve high ligation turnover even at a low substrate concentration. The key intermediate 4 for depsipeptide synthesis was obtained using the standard solid phase peptide synthesis (SPPS), briefly by coupling T, E, P, L, and azidoacetic acid in order, followed by cleaving from the 2-Cl-Trt resin in the weak acid (10% acetic acid in DCM). This method yielded a side-chain protected azido-LPET but left the carboxylic terminal free for esterification with t-butyl 2-bromoacetyl−glycine (5) in the presence of tetrabutylammonium iodide (TBAI). After deprotecting compound 6 in 20% TFA, the depsipeptide was obtained in moderate yield (55%, two-step). During ligation, sortase A recognizes the LPET*GG motif and cleaves the threonine-glycine ester bond by transferring the N-terminal fragment (azide-LPET) to the active site of sortase A (cysteine residue). The resulting acyl intermediate is quite active and ready to ligate the N-terminal diglycine of a protein of interest (Figure 2C). In our case, the N-termini of Fc protein were engineered with two glycine residues to resolve the acyl intermediate, yielding an azido-functionalized Fc (azido Fc) and at the same time regenerating the active sortase A (Figure 2D). Note that the acyl intermediate may undergo hydrolysis to break up the catalytic peptide transfer, which defines the prerequisite of an excess amount of substrate azide-LPET*GG to push the ligation complete in the reaction. The obtained azido Fc protein was conjugated with the AAZ-alkyne via a standard CuAAC protocol, yielding the cPAb with AAZ ligands on the N-termini.

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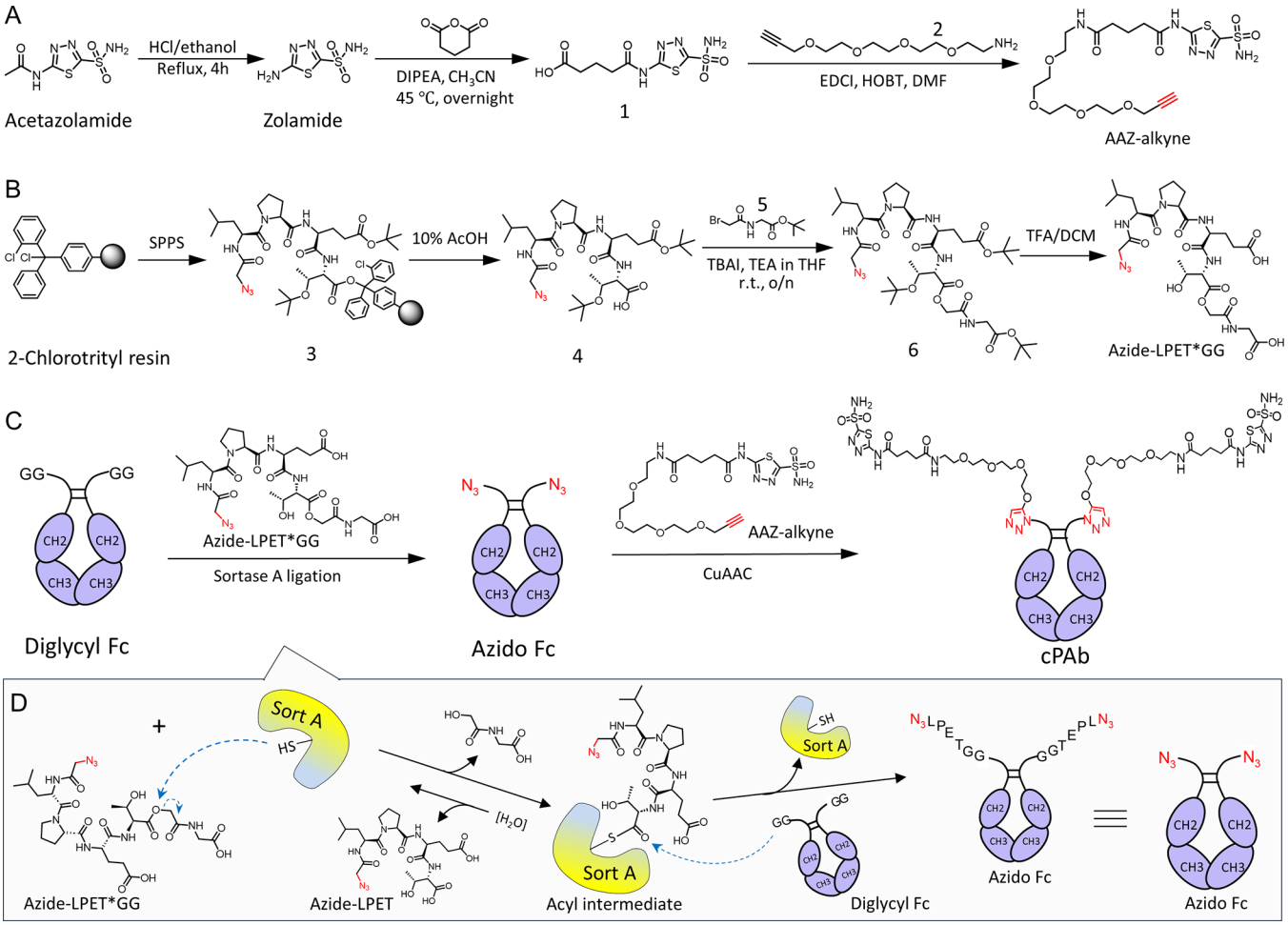

Figure 2. Chemical programming of Fc protein to yield the cPAb by sortase A ligation and bio-orthogonal click chemistry. (A) Synthesis of AAZ-alkyne from acetazolamide (AAZ). (B) Synthetic route to the sortase A substrate depsipeptide azide-LPET*GG. TBAI, tetrabutylammonium iodide. (C) Construction of cPAb from diglycyl Fc protein by two-step conjugations. The diglycyl Fc was first converted to azido Fc by sortase A mediated transpeptidation of azide-LPET*GG, followed by copper-assisted click reaction with ligand AAZ-alkyne in the biophysical conditions. (D) A gray box depicting the catalytic mechanism of sortase A to label the Fc protein at N-termini with azide group.

The initial construct of cPAb was performed on an aglycosylated Fc protein. It can be expressed in the bacterial system and serves as an affordable option for pilot studies. We evaluated three *Escherichia coli* strains looking for a host with the maximal yield and correct folding of Fc protein. Comparing the target band intensity of the whole lysate in SDS-PAGE revealed that all three hosts can express the Fc protein well, but BL21(DE3) showed a slightly higher production of this heterologous protein (Figure 3A). However, Shuffle T7 outperformed the other two competitors, BL21-(DE3) and Rosetta 2(DE3), with an isolated yield of~100 mg/L; meanwhile, a significant portion of products was already dimerized. As a result, Shuffle T7 was selected as the host strain. Fc protein was expressed, purified and oxidized into the dimer using dehydroascorbic acid (DHA).[37] The diglycyl Fc is the cleaved product of the Fc protein and prepared as per our previous reports.[38] The azido Fc was obtained by coincubation of the diglycyl Fc with substrate depsipeptide azide-LPET*GG (10 equiv) in the presence of sortase A (200 nM). The ligation process can be monitored by fluorescent agent sulfo Cy3-DBCO, which can light up the product band on SDS-PAGE by a fast copper-free click reaction (Figure 3B). Once complete, the azido Fc can be purified from the ligation reaction by a simple desalting step and subsequently conjugated with the AAZ ligand by CuAAC chemistry. The molecular weight and purity of all the Fc variants were analyzed by SDS-PAGE, showing the band shift after each labeling reaction (Figure 3C). Despite the bands of minor impurities migrating at higher molecular mass present in Fc variants, they can be removed by Protein A-based chromatography at the final step, yielding a pure construct of cPAb.

Chemically programmed Fc protein represents a compact form of cPAb that not only mimics the biological function of antibody but may also exhibit higher physical and chemical stabilities. Antibody is a typical heterotetramer composed of 2 heavy and 2 light chains. The four chains fold into multiple domains, some of which are enriched in the form of beta-sheet secondary structures, a region empirically observed to form aggregates.[39] Structural informatic analysis of commercial therapeutic antibodies suggested that these regions (for example CH2), together with the variable region (CDRs) in Fab domains, may be susceptible to unfolding and reveal the aggregation-prone "hot spots".[40,41] The Fc-based cPAb exhibits as a compact structure by omitting the unnecessary Fab domains, which may have an enhanced thermostability. To confirm the hypothesis, an accelerated experiment was performed to compare the stability of antibody and cPAb in the acidic buffer at elevated temperature.[42] The size exclusion

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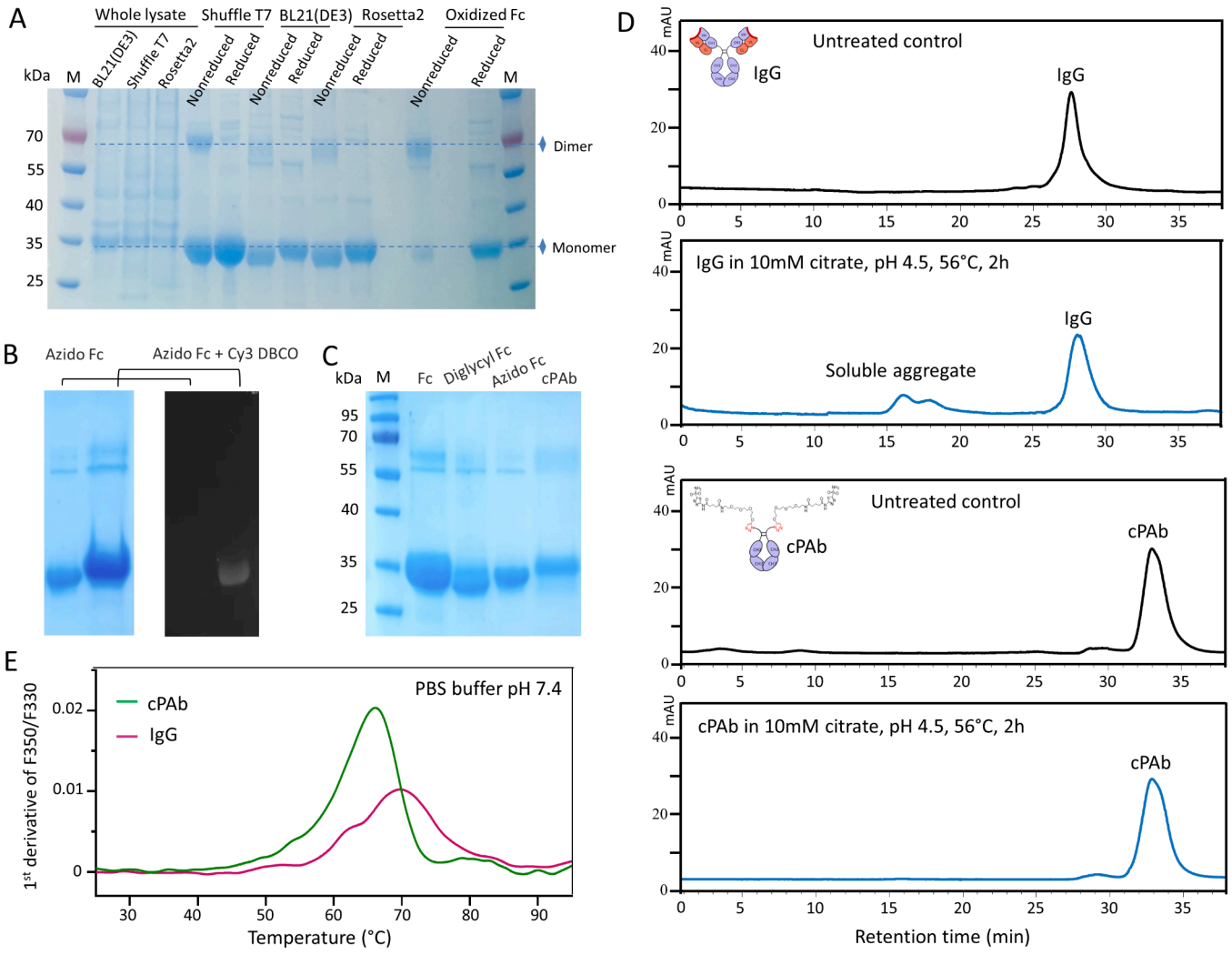

Figure 3. (A) SDS-PAGE assessment of Fc expression efficiency in three bacteria strains and the subsequent disulfide rebridging by oxidation. (B) The presence of azide group in azido Fc was confirmed by sulfo Cy3-DBCO. (C) The band shifts on SDS-PAGE revealed the molecular weight changes of Fc variants after each processing step. (D) The accelerated stability test of cPAb and IgG antibody by the SEC chromatography. (E) The DSF plot showing the melting temperatures of cPAb (Tm ~ 66 °C) and IgG (Tm ~ 69.5 °C).

chromatographs (SEC) in Figure 3D showed that a soluble aggregate peak was monitored in the human IgG treated under accelerated condition; whereas it was almost indiscernible in the cPAb group of the same condition. The result may indicate that cPAb is more stable than the canonical IgG that contains two Fab fragments. However, differential scanning fluorimetry (DSF) in Figure 3E revealed that IgG has a higher melting temperature (Tm ~ 69.5 °C) than cPAb (Tm ~ 66 °C). Since SEC can only monitor the soluble aggregates, we speculated that not all of the cPAb was eluted out in the SEC and there were protein aggregates retained in the column.

Our scaffold utilizes small-molecule ligand AAZ as the source of affinity to tumor cells. Due to the prominent hypoxia in the microenvironments of solid tumors, overexpression of CAIX has been reported in carcinomas of many types including cervical cancer, colorectal cancer, nonsmall cell lung cancer (NSCLC), breast cancer and others.[21,22] In particular, a strikingly high proportion of renal cell carcinomas can upregulate CAIX 100-fold times over basal levels because of the VHL gene mutation and subsequent HIF-1 α activation.[43,44] Accordingly, we assessed the specificity of cPAb to the renal cancer cell line SK-RC-52 by flow cytometry (Figure 4A). The result revealed that cPAb can bind to the cell line in a concentration-dependent manner. A significant shift of median fluorescence intensity (MFI) was observed at a concentration as low as 5 nM. Meanwhile, the avidity of cPAb to tumor cells can be competed off by free AAZ ligands (20 μM, >200-fold molar excess). Further analysis of cPAb on CAIX-negative HEK-293 cells by flow cytometry showed a weak and nonspecific binding profile, confirming the specificity of cPAb to CAIX receptors. The binding of cPAb to SK-RC-52 cells was also visualized by confocal microscopy (Figure 4B). The results showed that both sulfo-Cy5 labeled AAZ and cPAb were associated with the cell membrane, suggesting that AAZ-appended Fc (cPAb) can recognize the CAIX-positive tumor cells as effectively as the small-molecule ligand.

Compared to the Covx-body using a whole antibody as the scaffold, our Fc-based cPAb presents an optimized construct that truncates unnecessary protein sequences but retains the Fc domain for the desired effector function. It is well-known that the Fc domain of antibodies takes part in the IgG recycling by binding to the neonatal Fc (FcRn) receptor in a pH-favorable manner, thus rendering a long circulation half-life.[45] To investigate whether the Fc domain of cPAb remains functional,

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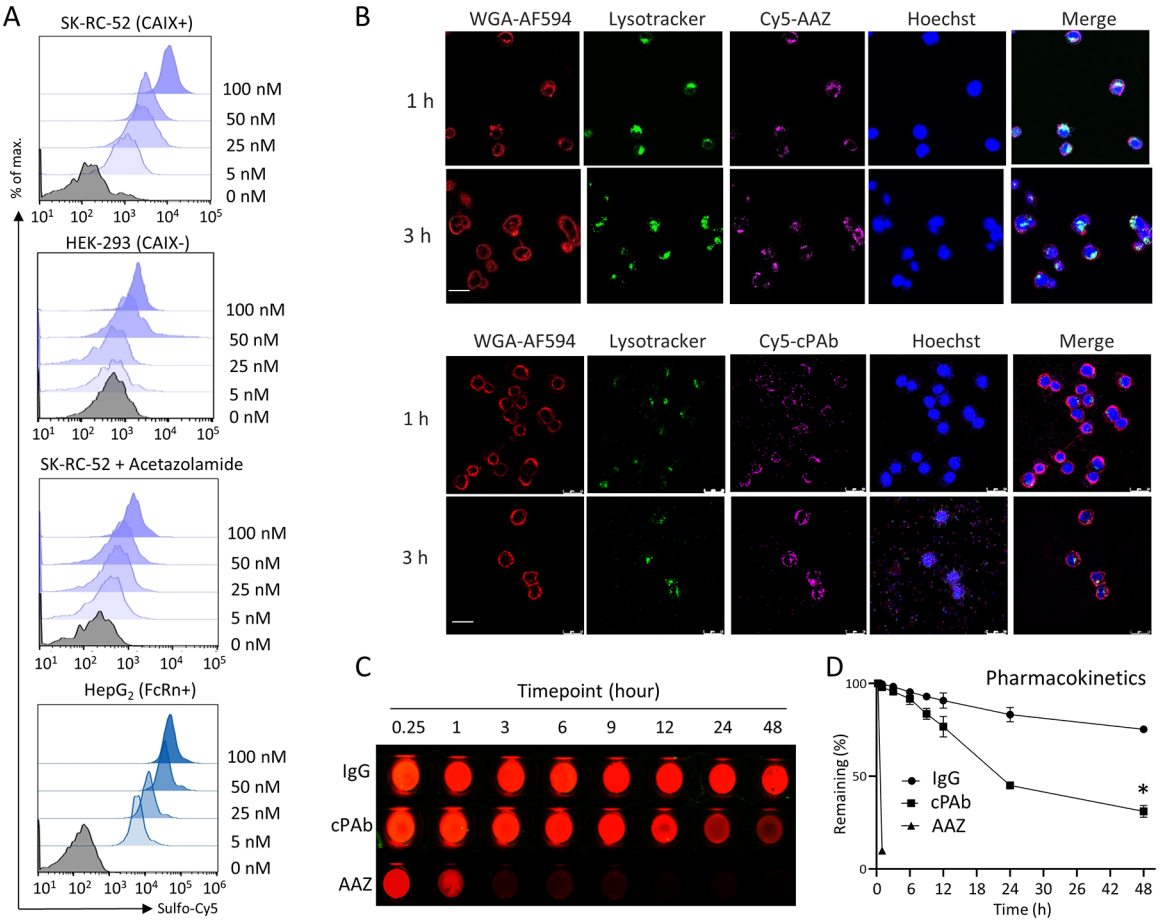

Figure 4. (A) Analysis of cPAb avidity to tumor cells by flow cytometry. (B) Visualization of the small-molecule AAZ ligand and cPAb binding to SK-RC-52 cells by confocal microscopy. The cell membrane, lysosome, and nucleus were counterstained by wheat germ agglutinin (WGA)-AF594, Lysotracker green, and Hoechst 33342, respectively. Scale bar, 30 μm. (C) Odyssey NIR imaging of blood samples to track the drug remained (sulfo-Cy5.5 labeled) in the bloodstream of mice after intravenous administration. (D) Pharmacokinetic profile of cPAb in comparison to IgG and AAZ in mice. * $P < 0.05$.

we incubated sulfo-Cy5 labeled cPAb with the FcRn-positive cell line HepG2 at an acidic pH mimicking endosomal conditions. The flow cytometric result demonstrated that it can dose-dependently bind with HepG2 cells (Figure 4A, bottom panel). The encouraging result prompted us to test the pharmacokinetic profile of cPAb in vivo. The balb/c mice were chosen as the animal model because human IgG1 Fc can be recognized by mouse FcRn receptors and remain functional in the recycling pathway of mouse species.[46,47] Human IgG antibody, cPAb and small molecule AAZ were labeled with near-infrared (NIR) fluorophore sulfo-Cy5.5 separately and injected into mice via tail vein at the same doses. The blood samples were collected at indicated time points and analyzed by the Odyssey infrared scanner. As shown in Figure 4C, the fluorescence was persistent in all the blood samples of both IgG and cPAb groups; whereas the fluorescent signal of small-molecule ligand AAZ decayed quickly in 1 h. Quantitative analysis revealed that cPAb exhibited a circulation half-life of around 20 h, which is shorter than IgG antibody but much longer than the small molecule AAZ (Figure 4D). These results collectively demonstrated that the Fc-based cPAb retains the antigen-targeting capability and effector function simutaneously, despite a faster clearance in mice than the full-length antibody.

Our interest in cPAb was then aroused by its potential to elicit the ADCC effect, an Fc-dependent effector function of antibodies for immune-mediated antitumor responses. ADCC is triggered when Fcγ receptor (FcγR) IIIa-bearing effector cells recognize a target cell that has been opsonized by antibodies.[48] This receptor is expressed on the surfaces of immune effector cells such as NK cells, neutrophils and monocytes.[49] Upon being cross-linked by the Fc domain, they induce downstream cellular processes and trigger the lysis of the target tumor cells. The FcγR IIIa receptor recognizes an interface between the hinge region and the CH2 domain. It is distant from the binding site of FcRn, which is a region located between CH2 and CH3. The glycosylation of the binding interface (N297) on the Fc domain is critical for its interaction with the FcγR IIIa receptor and the subsequent induction of effector activities.[50] Our pilot cPAb is in an aglycoform, which is functional in recycling and transcytosis but unable to activate the ADCC (Figure 5A). We re-expressed the glycosylated Fc protein in the mammalian 293F suspension cell and constructed the cPAb of glycoform using the methods as before. The SDS-PAGE analysis revealed that the product bands migrated slower after each labeling step, which is in agreement with their theoretical molecular weight changes (Figure 5B). Furthermore, we noticed that the bands of glycoform always located slightly upper on the gel than the

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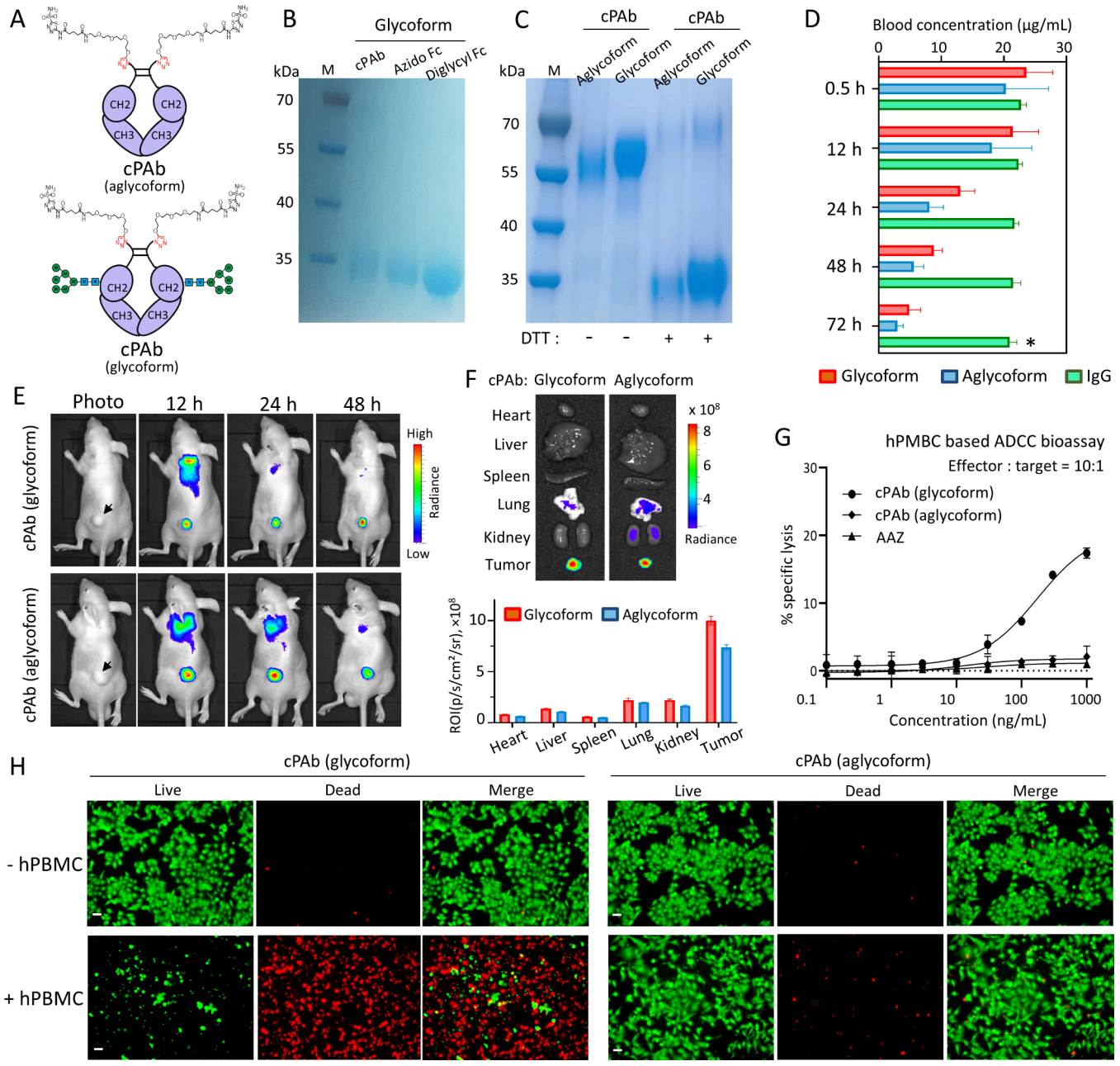

Figure 5. (A) The schematic graphs illustrating the cPAb of aglycoform and glycoform, which were derived from the bacteria-expressed Fc protein and 293F suspension cell-expressed Fc protein, respectively. (B) SDS-PAGE analysis of the glycosylated Fc variants. (C) The band migration comparison of the glycosylated and aglycosylated cPAbs on SDS-PAGE. (D) ELISA data revealing blood retention of cPAbs (glycoform and aglycoform), IgG in mice after i.v. injection. * $P < 0.05$. (E) NIR fluorescence tracking of sulfo-Cy5 labeled cPAbs in mice bearing SK-RC-52 xenografts. Arrows indicate the tumor sites. (F) Ex vivo imaging of the dissected organs and tumors 48 h after administration. (G) The in vitro ADCC activities represented by percent specific target cell lysis after 7-h treatment of various concentrations of cPAbs in the presence of effector cells. The human PBMCs were used as effector cells at an effector:target ratio of 10:1. The small molecule AAZ was used as the negative control. (H) The LIVE/DEAD staining of SK-RC-52 cells after 30-h treatment of cPAbs in the absence or presence of human PBMCs. Scale bar, 30 μm.

aglycoform in both the reducing and nonreducing electrophoresis conditions (Figure 5C). This observation is consistent with its glycosylated status and the corresponding higher molecular weight. In order to evaluate the impact of glycosylation on IgG recycling function, we compared the blood retentions of two cPAb formats in mice. As the Fc ELISA (enzyme-linked immunosorbent assay) result shown in Figure 5D, more than 50% of the glycosylated cPAb retained in blood 24 h after i.v. administration, which is higher but not dramatically higher than the aglycoform. The long retention in blood is also beneficial to the tumor targeting capability of cPAbs as it can increase the exposure time of drugs to the tumors. Using the NIR in vivo imaging method (Figure 5E,F), we tracked the biodistribution of sulfo-Cy5 labeled cPAbs in SK-RC-52 tumor-bearing mice and revealed that both formats can well target the tumors.

We then investigated the specific target cell lysis capability of cPAb via an hPBMC ADCC bioassay. The in vitro cytotoxicity assay by lactate dehydrogenase (LDH) was performed after the 7 h co-culture of hPBMCs with SK-RC-52 (effector to target

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ratio = 10:1) in the presence of cPAb (a series dilution from 0.1 to 1000 ng/mL). The result in Figure 5G demonstrated that the significant lysis of target cell was present with the glycosylated cPAb, while there was no apparent cytotoxic effect observed in cells treated by either the cPAb of aglycoform or the small-molecule ligand AAZ. The absence of ADCC activity in the aglycoform is consistent with the previous reports that glycan-deficient antibody lacks the binding capability to the FcγR IIIa receptors.[50] To further profile the activity of glycosylated cPAb, we co-cultured the effector cells hPBMCs with the target cell SK-RC-52 over an elongated period (30 h), and then evaluated the target cell viability by LIVE/DEAD staining (Figure 5H). Near-complete cell death was found for cells treated with the glycosylated cPAb plus hPBMC, while no sign of toxicity was detected in cells treated with the aglycosylated cPAb or without the addition of effector cells. Collectively, the result demonstrated that the glycosylated cPAb is functional in ADCC and can participate in anti-tumor immunotherapy against renal cancer cells overexpressing CAIX.

3. CONCLUSION

In summary, we report a facile method to prepare the Fc-based antibody mimetic to target the CAIX receptor of cancer cells. In this study, the small-molecule ligand AAZ was site-specifically conjugated at the N-termini of Fc protein by click chemistry with the prerequisite of azidation via sortase A transpeptidation. Other approaches for generating the Fc-based cPAb construct have been reported previously,[51−53] but most were opted for the expressed protein ligation (EPL). A reactive site, such as thioester or terminal cysteine, has to be programmed intentionally by either the intein-mediated protein splicing or an endogenous yeast protease, which both require complicated molecular engineering and tedious bench work.[52,53] Netirojjanakul et al. applied a chemical approach, namely a chemical agent pyridoxal 5′-phosphate (PLP), to install a reactive ketone on the N-terminal alanine of Fc protein and utilized it as a chemical handle to graft small molecules (via oxime or hydrazone linker).[54,55] The process circumvents some drawbacks associated with EPL (e.g., complicated molecule design, single conjugation site); however, it may cause other potential problems such as toxic chemical residues and over-oxidization of antibodies.[54] As an example, we constructed the Fc-based cPAb by a sortase A-mediated N-terminal ligation approach. We envision it can be explored for scale-up by the on-resin enzyme immobilization and continuous-flow systems in the future.[56,57]

The cPAb molecule in the study is much more compact than a whole antibody but able to mimic antibody via its ligand AAZ for epitope binding. The results of flow cytometry and confocal microscopy showed that cPAb can bind specifically to the CAIX receptors of SK-RC-52 renal cancer cells. After chemical conjugation, the Fc domain of cPAb retains the desired effector functions, which is evidenced by the ADCC assay and elongation of blood circulation half-life in mice. Besides the small-molecule ligand AAZ, this technology can be applied to more targets by conjugating other high-affinity small-molecule ligands. Such ligands include but are not limited to folic acid for folate receptor targeting, DUPA ligand to recognize the prostate-specific membrane antigen (PSMA) for prostate cancer therapy, octreotide, cRGD and other cyclic peptide ligands that bind the overexpressed receptors of cancer cells. Overall, this work provides a new method for Fc protein grafting and expands the scope of chemically programmed antibodies, which may be used in potential therapeutically relevant applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.4c00259.

Synthesis procedure and experimental methods (PDF)

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Notes

The authors declare no competing financial interest.

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