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Construction of bispecific antibodies by specific pairing between the heavy chain and the light chain using removable SpyCatcher/SnoopCatcher units

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Abstract

During the production of bispecific antibodies (bsAbs), nonspecific pairing results in low yields of target bsAb molecules, an issue known as the "mispairing problem." Several antibody engineering techniques have been developed to overcome mispairing issues. Here, we introduce "bsAb by external pairing and excision" (BAPE), a novel chain pairing method that induces specific chain pairing by fusing external SpyCatcher/Tag and SnoopCatcher/Tag units. These tags are then removed via protease cleavage. In this study, we applied this method to force the correct pairings of heavy and light chains while the heavy-chain pairing was achieved by the Knobs-into-Holes mutation. We then confirmed the formation of interchain bridges with covalent isopeptide bonds. Both anti-CD3/anti-Her2 and anti-CD3/anti-EGFR bsAbs displayed satisfactory target binding activities and in vitro cell-killing activity with activated T-cells.

Keywords Bispecific antibody, Cancer, Chain pairing, SpyCatcher, SnoopCatcher

Introduction

Bispecific antibodies (bsAbs) are genetically/chemically modified antibodies that can bind to two different antigens simultaneously, i.e., within one molecule [1–6]. This bispecificity makes bsAbs a promising medicinal tool to treat several diseases. For example, a bsAb against a cancer antigen and an immune cell surface antigen can bridge two cells, thereby effectively inducing anticancer immune responses. Although various structural formats for bsAbs have been proposed, IgG-type bsAbs are the most popular and widely investigated. However, the production of IgG-type bsAbs can be complicated by the "mispairing problem," which is used to describe incorrect interchain pairing occurring during recombinant cell culture. To produce IgG-type bsAbs from antibodies A

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and B, all chains, both heavy and light, should be paired with correct counterpart chains. However, the similarity between heavy chains A and B, or between light chains A and B, can induce between-chain mispairing. As a result, the theoretical yield of the desired recombinantly-produced bsAb molecule is only 12.5%. To overcome the mispairing problem, several antibody engineering techniques have been proposed [7]. Of these, the Knobs-into-Holes (KiH) mutation and CrossMab are two popular methods of achieving desired interchain pairings [8]. KiH enables the correct heavy chain pairing by introducing a concave/convex surface on the CH3 dimerization interface, while CrossMab enables correct pairing between the heavy and light chains by swapping the CH1/CL or VH/VL domains of one side of the Fab arm [9]. Although these methods are widely used, both require the introduction of new mutations or domain rearrangements.

In this study, we report a novel chain pairing method for bsAb production that does not require artificial modification, and instead fuses external interchain pairing units. Here, we first show how specific pairings of heavy and light chains can be achieved via the external pairing. We then use SpyCatcher/Tag and SnoopCatcher/Tag protein pairs to forcibly pair heavy and light chain pairs at their N-termini via a process named as "BsAb by external pairing and excision" (BAPE; Fig. 1). The SpyCatcher/Tag [10] and SnoopCatcher/Tag [11] systems are pairs of protein molecules that spontaneously form an intermolecular isopeptide bond between specific Catcher and tag sequences. These two units are orthogonal and therefore a specific one-pot reaction can be designed. Moreover, due to its simplicity and robustness, the Catcher/Tag system can be used for a variety of protein engineering applications [12, 13]. Here we use this system to form correct heavy/light chain pairings by fusing the Catcher/Tag proteins at the N-terminus of each chain. We used the KiH mutations for the heavy-chain pairing. After expression, the external Catcher/Tag proteins can be removed via protease digestion. Since the external units induce the correct pairings, the resulting bsAb is constructed without artificial sequence modifications on either Fab arm.

Methods

Sample preparation

All protein samples used in this study were expressed using the Expi293 mammalian cell expression system (Thermo Fisher Scientific, MA USA) in HE400 medium (GMEP, Fukuoka, Japan). Mammalian expression vectors for all chains (i.e., the genes for both heavy chains and both light chains) were simultaneously transfected into cells using PEI MAX reagent (Polyscience Inc., PA USA). The mixing ratios of the four expression vectors were set to their respective molar concentrations, 1:1:1:1. After seven days of culturing, supernatants were collected and applied to a Protein A column (UNOsphere SUPrA, Bio-rad, CA USA). Finally, to remove N-terminus Catcher/Tag units, Thrombin was added at a concentration of 0.2 U/1 mg protein. The final purification was performed by applying the cleaved sample to the Protein A column again.

SDS-polyacrylamide gel electrophoresis (PAGE)

10% polyacrylamide gels were used for the SDS-PAGE measurements. Samples were concentrated using a TCA-DOC (trichloroacetic acid-deoxycholic acid) precipitation method with 7% TCA and 0.02% DOC to apply 5 μg sample in each well. Samples were prepared in a reduced condition using β-mercaptoethanol.

Analytical size exclusion chromatography (SEC) measurements

For chromatography, we used a superdex75 10/300 GL column with PBS to evaluate the IgG-size bispecific

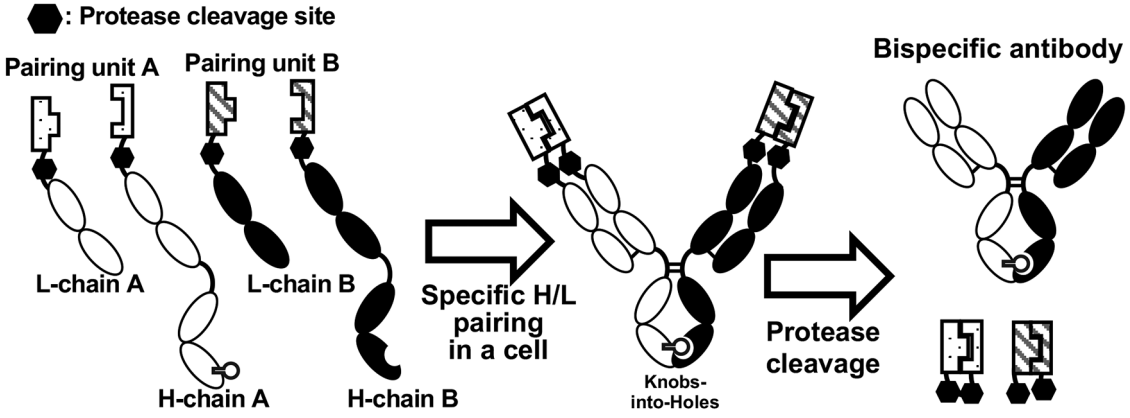

Fig. 1 Overview of the BAPE (bsAb by external pairing and excision) procedure. Two different antibody clones are shown in black and white. Two external pairing units and protease cleavage sites are also indicated

molecules after cleavage. All procedures were performed at room temperature. Briefly, 100 µL samples with a concentration of 200 nM were loaded onto the column and detection was performed at 280 nm absorption using a Biorad NGC chromatography system (Biorad Inc.). To minimize the fluctuation of the elution volume of the sample, we measured control experiment in every measurement.

Differential scanning fluorometry (DSF)

Protein thermal stability measurements were then determined via DSF using a StepOne real-time PCR system (Applied Biosystems). Briefly, protein concentrations were first set to 0.1 mg/mL in PBS buffer. Samples were then mixed with 250X diluted SYPRO Orange dye (Sigma Aldrich). 25 µL aliquots were then loaded onto 48-well sample plates, and samples were then heated from 25 °C to 99 °C.

Flow cytometry

We performed flow cytometry analyses using 10^6 cells of HPB-ALL ($CD3^+$), A431 ($EGFR^+$), and SK-BR-3 ($Her2^+$) cells. All cell lines were obtained from the Cell Resource Center for Biomedical Research at Tohoku University. Prior to flow cytometry, cells were incubated for 10 min with 30 nM of bsAb in PBS. After washing cells with PBS twice, PBS-suspended cells were then incubated with anti-human-Fc FITC conjugated goat antibody (SIGMA, F9512, RRID: AB_259808) and washed with PBS twice. PBS-suspended cells were analyzed using an RF-500 flow cytometer (Sysmex, Kobe, Japan). All analyses used 100 µL cell suspension for each measurement. FCSalyzer software (Sourceforge; https://sourceforge.net/projects/fcsalyzer/) was used to analyze all obtained data. Finally, we also obtained measurements for positive controls, anti-CD3 IgG FITC (Proteintech Group, Inc., IL, USA, RRID: AB_2883794), Cetuximab (anti-EGFR), and Herceptin (anti-Her2).

Cytotoxicity assay

We performed cytotoxicity assays. To do so, T-LAK cells, a type of activated T-cell, were first induced from peripheral blood mononuclear cells. The reason for the use of T-LAK instead of naïve PBMC is to obtain a sufficient number of cells for the experiments. These included Normal Human PBMCs, Purified and Non-Characterized (Precision for Medicine) for Her2/CD3 bsAb measurements and PBMCs from Cellular Technology Limited for EGFR/CD3 bsAb measurements. Briefly, PBMCs were stimulated with IL-2 and Dynabeads Human T-Activator CD3/CD28 beads (Thermo Fisher Scientific) for Her2/CD3 bsAb measurements, or in a culture flask (A/S Nunc) pre-coated with anti-CD3 monoclonal antibody for EGFR/CD3 measurements. In vitro growth inhibition measurements of cancer cells using HER2 positive SK-BR-3 cells or EGFR positive TFK-1 cells were performed using MTS assay kits (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay; Promega). For these assays, the E/T ratio was set to five and the incubation time was 48 h for Her2/CD3 bsAb assays, and the E/T ratio was set to two and the incubation time was 20 h for the EGFR/CD3 bsAb assays. PBS-incubated wells were used for a 100%-cytotoxicity reference because this treatment kills all the cancer cells during the incubation time and medium-incubated wells were used for a 0%-cytotoxicity reference.

Results

Design of an anti-CD3/anti-Her2 bsAb via BAPE (CD3-Her2(Spy-Snoop))

Figure 2A shows the molecular design of an anti-CD3/anti-Her2 bsAb using the BAPE protocol. First, we selected two clones for the anti-CD3/anti-Her2 bsAb that would be suitable for pharmaceutical applications. The anti-CD3 antibody we chose was a humanized M291 antibody clone, HuM291; this has been widely used in bsAb studies to engage T-cells to tumor sites [14]. We also selected Herceptin for the anti-Her2 antibody [15]. Herceptin is an FDA-approved antibody clone used for breast cancer treatment and has also been used for the antibody part of approved ADCs Kadcyla and Enhertu, and several others in clinical testing, an antibody-drug conjugate cancer treatment [16]. We then fused SpyCatcher/Tag and SnoopCatcher/Tag pairs to the N-termini of the bsAb from these clones as an external pairing unit for the heavy/light chain pairings (Fig. 2A). A KiH mutation (knobs T366W and holes T366S, L368A, and Y407V) [17] with a disulfide link (knobs side: S354C and holes side: Y349C) [18] was introduced in the CH3 domains of the two heavy chains to form the correct pairing. The Knobs mutations were introduced in the anti-cancer antibodies and the holes mutations were introduced in the anti-CD3 antibody. SpyCatcher and SpyTag were fused to the anti-CD3 heavy and light chains, respectively, while SnoopCatcher and SnoopTag were fused to the anti-Her2 heavy and light chains, respectively, followed by a thrombin cleavage site. In addition, VHH domains (anti-EGFR Ia1 for the heavy chain and anti-EGFR cAbGFP4 for the light chain) was fused to the N-terminus of all four bsAb chains to enhance the recombinant expression. The resulting BAPE construct was called CD3-Her2(Spy-Snoop). CD3-Her2(Spy-Snoop) was then expressed and purified using a mammalian expression system and a protein A column. Figure 2B lane 1 shows SDS-PAGE results of the purified sample. A band near 200 kDa was present, which corresponds to the size of the two heavy/light chains once covalently connected via Catcher/Tag proteins (i.e., 113 kDa for anti-CD3 and 118 kDa for

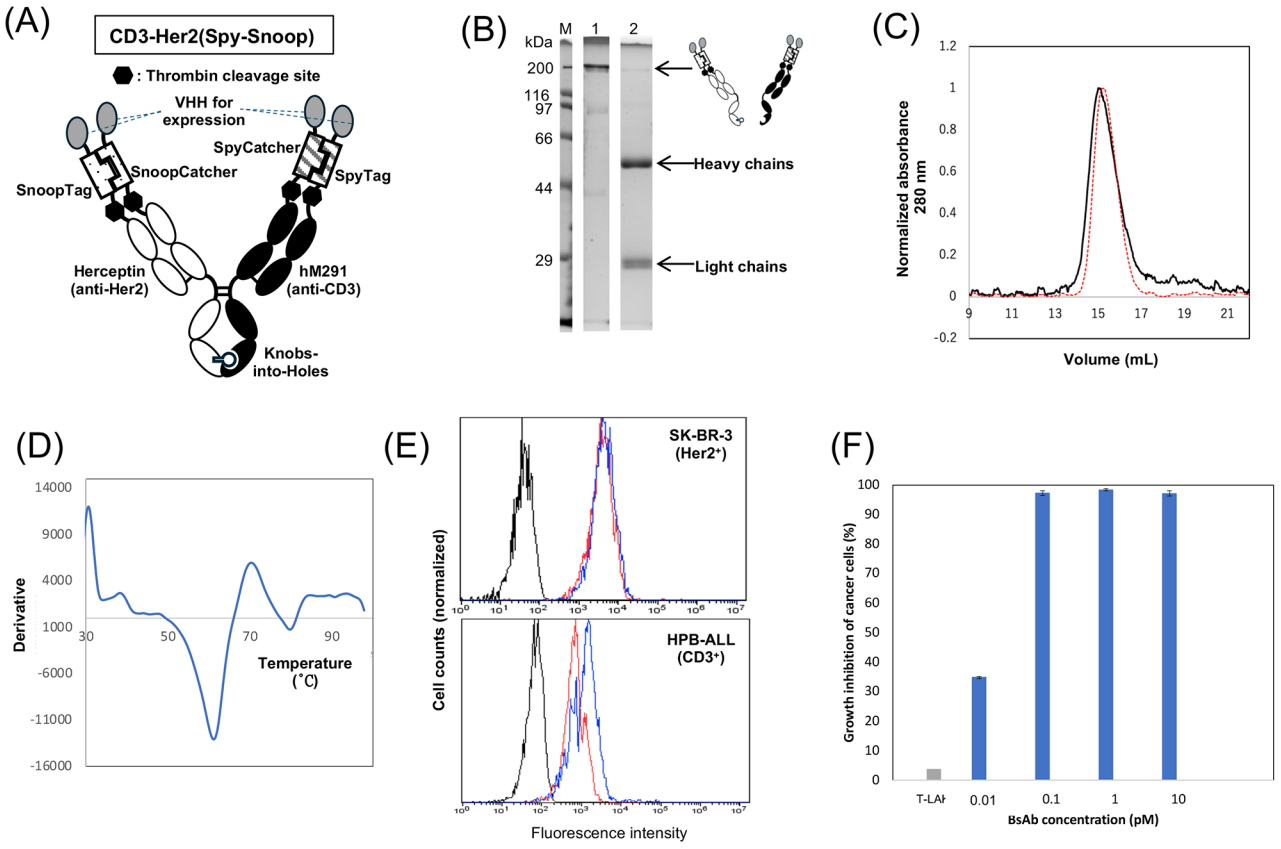

Fig. 2 (**A**) Construction of CD3-Her2(Spy-Snoop) for BAPE. (**B**) SDS-PAGE of purified CD3-Her2(Spy-Snoop) with a reduced condition (Lane 1) and CD3-Her2 bsAb molecules following cleavage and purification (Lane 2). M: molecular weight marker. Arrows indicate labeled proteins. (**C**) Analytical size-exclusion chromatography after the removal of the pairing tags (black line). Red dashed line indicates the IgG elution profile under the same experimental settings. (**D**) Differential scanning fluorometry. (**E**) Flow cytometry results for Her2-positive SK-BR-3 cells (top) and CD3-positive HPB-ALL cells (bottom). Black: without antibody, red: constructed bsAb, blue: positive control antibody. (**F**) In vitro cytotoxicity assay. Tumor cell: SK-BR-3, Effector cell: Activated T-cell (T-LAK), E/T = 5, incubation time 48 h. Error bars indicate standard deviation

anti-Her2). This result therefore indicates that the inter-chain pairing occurred during expression. Marker size mismatches may reflect branched polypeptide structures at the isopeptide bonds between the chains of the pairing units. The SDS-PAGE measurements were performed in reduced conditions, and thus the linked structure by disulfide bridges cannot be detected. The expression yield of CD3-Her2(Spy-Snoop) was 1.0 mg/1L culture.

CD3-Her2 bsAb construction via BAPE

Next, we performed thrombin digestion and protein L purification to construct the CD3-Her2 bsAb by removing the two Catcher/Tag portions from CD3-Her2(Spy-Snoop), (Fig. 2B Lane 2). The digestion reaction was conducted at 25 °C overnight. Following digestion, the covalently connected heavy/light chain complex disappeared and bands for the heavy and light chains appeared, indicating that the Catcher/Tag portions were successfully removed. After the thrombin digestion, a spacing GSHMHM sequence remained at the N-terminus of both chains (Supplemental_material_1). The yield of the final CD3-Her2 bsAb after thrombin digestion and purification was 0.31 mg/1L culture.

The molecular size of the resulting CD3-Her2 bsAb after the removal of the pairing tags and purification with a protein A column was evaluated using analytical size exclusion chromatography (SEC) (Fig. 2C). We found that CD3-Her2 bsAb was eluted at an elution volume close to Herceptin IgG, suggesting that IgG-like molecules had formed. BsAb stability was then measured using differential scanning fluorometry (DSF) (Fig. 2D), and two peaks were observed at 61.7 °C and 79.7 °C. We then evaluated bsAb binding activity by flow cytometry analysis (Fig. 2E); a CD3 binding activity was evaluated using the HPB-ALL cell line, and a Her2 binding activity was evaluated using the SK-BR-3 cell line. Overall, CD3-Her2 bsAb showed significant binding activity for both cell lines, confirming the dual specificity of the bsAb. Next, we evaluated T-cell mediated cytotoxicity using CD3-Her2 bsAb (Fig. 2F). To do so, Her2-positive SK-BR-3 cells were incubated with activated T-cells (T-LAK). We observed an increase in growth-inhibition activity in a

dose-dependent manner according to the concentration of the added CD3-Her2 bsAb. Near 100% growth-inhibition was observed at concentrations beyond 0.1 pM bsAbs. The observed anticancer cell activity was indicative of bispecific activity by bridging between cancer and activated immune cells.

Design of an anti-CD3/anti-EGFR bsAb via BAPE (CD3-EGFR(Spy-Snoop))

Based on the success of the CD3-Her2 bsAb construction using the BAPE protocol, we then evaluated different bsAb combinations. We therefore combined the anti-EGFR antibody Cetuximab [19, 20] with the anti-CD3 antibody HuM291 to construct a CD3-EGFR bsAb by BAPE. In a manner similar to CD3-Her2(Spy-Snoop), SnoopCatcher and SnoopTag were fused to the N-termini of the heavy and light chains of the Cetuximab antibody, respectively. In addition, the VHH domain was used for expression (Fig. 3A; CD3-EGFR(Spy-Snoop)). Next, expression vectors of the two heavy and two light chains were transfected to mammalian cells. Figure 3B shows the purified bsAb molecule before the removal of the two pairing units (Lane 1) and after removal and purification (Lane 2). The expression yield of CD3-EGFR(Spy-Snoop) was 0.81 mg/1L culture. Before digestion, the bands of the covalently connected heavy and light chains were observed, thereby indicating the formation of isopeptide bonds (i.e., 113 kDa for the anti-CD3 part and 118 kDa for the anti-EGFR part). After thrombin digestion, the bands corresponding to the native heavy and light chains again demonstrated the success of the BAPE preparation method. A faint band near the 97-kDa marker in lane 1 and 2 could be an unpaired heavy chain with the tag (75 kDa). The yield of the final CD3-EGFR bsAb after thrombin digestion and purification was 0.39 mg/1L culture. The molecular size of CD3-EGFR bsAb was then evaluated by analytical SEC (Fig. 3C). A major peak was eluted

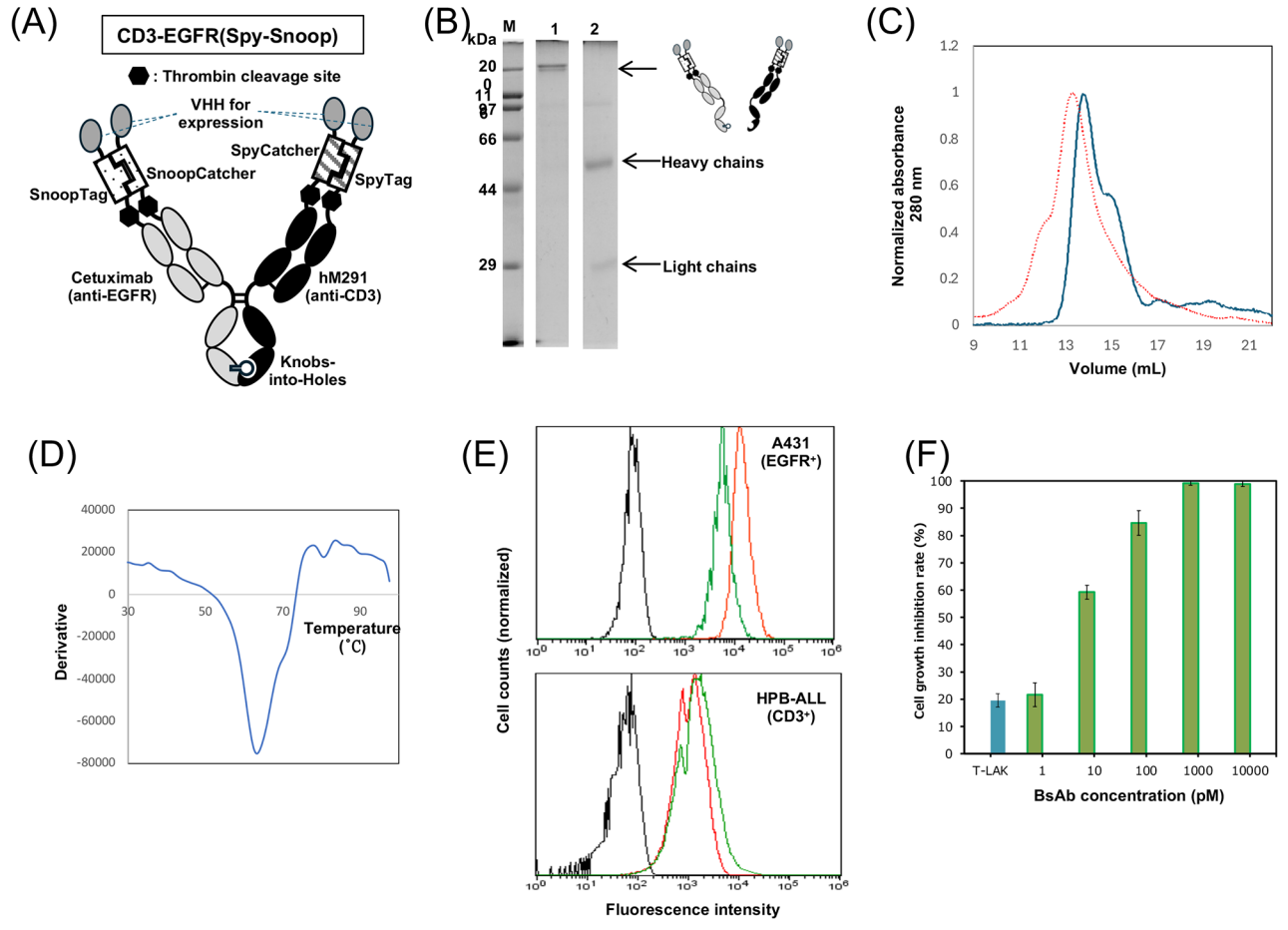

Fig. 3 (**A**) Construction of CD3-EGFR(Spy-Snoop) via BAPE. (**B**) SDS-PAGE of the purified precursor bsAb with a reduced condition (Lane 1) and the final bsAb molecule after cleavage and purification (Lane 2). M: molecular weight marker. Arrows indicate labeled proteins. (**C**) Analytical size exclusion chromatography. The elution profile for CD3-EGFR(Spy-Snoop) is shown with a blue line. The Red dashed line indicates the IgG elution profile in the same experimental settings. (**D**) Differential scanning fluorometry. (**E**) Flow cytometry results for EGFR-positive A431 cells (top) and CD3-positive HPB-ALL cells (bottom). Black: without antibody, green: constructed bsAb, red: positive control antibody. (**F**) In vitro cytotoxicity assay. Tumor cell: TFK-1, Effector cell: Activated T-cells (T-LAK). E/T = 2, incubation time = 20 h. Error bars indicate standard deviation

near the elution position of the IgG control, which suggests that the formation of the target bsAb molecule was successful. The control IgG has a shoulder at a higher molecular weight position possibly due to the aggregation of the sample. A chromatogram shows a shoulder after the main elution peak, which may be attributed to impurities in the final product. An additional band around 100 kDa is observed in Fig. 3B (Lane 2). This impurity may appear in the SEC result. Next, we examined the thermal stability of CD3-EGFR bsAb and its Tm value was determined to be 63.3 °C. Subsequently, the binding activity of CD3-EGFR bsAb was evaluated by flow cytometry (Fig. 3E), and cytograms indicated that the constructed molecule showed binding activity against both EGFR-positive and CD3-positive cells. Finally, we measured the cytotoxic activity against EGFR-positive cells by co-culturing with activated T-cells (T-LAK). Figure 3F shows that growth inhibition activity increased in a concentration-dependent manner with respect to bsAb concentration. Overall cytotoxicity assay results showed antitumor activity via cellular bridging induced by CD3-EGFR bsAb molecules.

Discussion

Here we report the development of BAPE, a novel chain pairing method used to construct bsAbs. In BAPE, external SpyCatcher/Tag and SnoopCatcher/Tag units induce target chain pairings, and since specific parings are enabled by external parts, target pairings can be achieved without modifying the bsAb part itself, which is an advantageous point compared with the existing pairing methods, such as KiH mutations and CrossMab. Yields of the bsAb preparation by the CrossMab method were reported to be 73% (CH1-CL exchange) and 85% (VH-VL exchange) determined by the homogeneity of the SEC measurements [9]. Our BAPE products show SEC profiles with monodisperse one peak for Her2/CD3 bsAb and one peak with a shoulder for EGFR/CD3 bsAb (Figs. 2C and 3C). Thus, in some cases, BAPE may show better yield in terms of homogeneity determined by SEC. The use of SpyCatcher/Tag for the bispecific antibody construction has been reported previously [21, 22]. Despite the promising results of constructing the bispecific molecules, residual SpyCacture/Tag unit would cause the immunogenicity problem for medical applications because SpyCatcher/Tag is constructed from bacterial protein. Similar to our external pairing method, a study reported a heavy chain pairing method termed LUZ-Y, in which C-terminus leucin zipper aligns chains and is then removed by cleavage [23]. LUZ-Y involves non-covalent interactions mediated by the leucin zipper, while BAPE uses covalent bond formation between two chains, and should therefore induce more stable pairings. Moreover, BAPE can be extended for inter-heavy chain pairings by introducing an additional heterodimerization unit at the C-termini of both heavy chains. In another respect, specific chain pairing without modification of the bsAb part can also be achieved via separate preparation and ligation. One method involves using a split-intein mediated ligation of one Fab arm to the rest of the bsAb IgG structure, a procedure termed Bispecific Antibody by protein Trans-splicing (BAPTS) [24, 25]. In BAPTS, the bsAb are separated into one Fab and the remainder, and are expressed and purified separately; thus each Fab part can form specific pairings. We recently reported a method to construct a bispecific antibody using an IgG-Fab^2 format that has two additional Fab arms at the N-termini of the IgG Fab arms [26]. For IgG-Fab^2 bsAb, the Fab portion is also expressed and purified separately and is ligated to the IgG portion via split-intein ligation. Thus, light chain mispairing does not occur. Compared to the split-intein-based method, BAPE is simpler, since it is performed using a single batch expression while separate expression cultures are necessary for split-intein-based ligation methods. Thus, BAPE may be advantageous given the importance of simplicity in facilitating large-scale production for industrial use. Although the recombinant expression level is still not enough, the use of a constitutive expression system, such as the CHO expression system instead of the transient expression system shown here, could resolve the problem.

It should be noted that cytotoxicity experiments shown in Figs. 2F and 3F did not include the measurements using antigen-negative cancer cells to evaluate the toxicity of the bsAb molecules. Therefore, the possibility that the observed cytotoxicity may derive from the toxicity of bsAbs themselves cannot be ruled out. Although there is no evidence that the anti-cancer bsAbs are toxic, further evaluation is needed in the future for the bsAb molecules generated by BAPE. We evaluated the final bsAb product using the SDS-PAGE analyses and the SEC measurements, and these evaluations do not reveal whether by-products with the incorrectly paired molecules are formed. Thus, an additional experiment using mass spectroscopy to confirm the formation of the target bsAb molecules may also be needed to evaluate this issue. In addition, the long-term stabilities of the resulting bsAbs by BAPE are not evaluated, and thus the stability tests, in which bsAbs are exposed to heated conditions for certain periods, may be needed for the clinical applications.

Abbreviations

BAPE bsAb by external pairing and excision
EGFR epidermal growth factor receptor

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13036-024-00454-z.

Supplementary Material 1

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Author contributions
J.Y., Y.K., A.I., Y.T., R.A., and T.N. performed experiments. I.K. and K.M. wrote the main manuscript text. All authors reviewed the manuscript.

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Data availability
Sequence data are provided within the supplemental materials. Other data are available on request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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