Highly enhanced cytotoxicity of a dimeric bispecific diabody, the hEx3 tetrabody*

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Running head: Characterization of a bispecific EGFR x CD3 tetrabody
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We previously reported the utility for cancer immunotherapy of a humanized bispecific diabody (hEx3) that targets epidermal growth factor receptor and CD3. Here, we used dynamic and static light scattering measurements to show that the multimer fraction observed in hEx3 in solution is a monodisperse tetramer. The multimerization into tetramers increased the inhibition of cancer cell growth by the hEx3 diabody. Further, 1:2 stoichiometric binding for both antigens was observed in a thermodynamic analysis, indicating that the tetramer has bivalent binding activity for each target, and the structure may be in a circular configuration, as is the case for the single-chain Fv tetrabody. In addition to enhanced cytotoxicity, the functional affinity and stability of the hEx3 tetrabody were superior to those of the hEx3 diabody. The increase in molecular weight is also expected to improve the pharmacokinetics of the bispecific diabody, making the hEx3 tetrabody attractive as a therapeutic antibody fragment for cancer immunotherapy.

Bispecific antibodies (BsAbs) are recombinant antibodies that can bind to two different antigenic epitopes. Bispecificity can be used in cancer immunotherapy to cross-link tumor cells to immune cells such as cytotoxic T cells, natural killer cells, and macrophages. This crosslinking accelerates the destruction of the tumor cells by the immune cells, which may translate into improved antitumor therapy and lower production costs by decreasing the doses needed (1,2). However, the use of BsAbs in clinical studies has been hampered by difficulties in producing them on a large scale. Conventional chemical conjugation has been used, but the quality of the antibody produced is inconsistent (3). The production of BsAbs by somatic fusion of two hybridomas to form a quadroma yields BsAbs of more consistent quality but results in the formation of various chain-shuffled antibodies; for instance, 10 different antibodies can be generated after random association of two heavy and two light chains (4,5).

Advances in recombinant technology have made it feasible to generate small recombinant BsAbs constructed from two different variable antibody fragments. Bispecific diabodies are the smallest available BsAbs, and the distance between the two antigen binding sites is sufficient to link two cells (6,7). The effectiveness of bispecific diabodies in cancer therapy has been extensively shown in in vitro and in vivo models (8-10). We have also constructed functional bispecific diabodies (11,12). In particular, the humanized bispecific diabody hEx3 has marked antitumor activity and can retarget lymphokine-activated killer cells with the T-cell phenotype (T-LAK cells) against epidermal growth factor receptor (EGFR)-positive cell lines.
The compact structure of bispecific diabodies contributes to low immunogenicity, high tumor penetration, and the potential for large-scale preparation through bacterial expression systems; however, the downsizing results in rapid clearance from blood. In addition, the structure contains only one binding domain for each target, which results in low functional affinity (15, 16).

Multimerization of small recombinant antibodies is one available strategy for improving their pharmacokinetic and binding affinity. In single-chain Fvs (scFvs), the length and composition of the polypeptide linker between the variable heavy (VH) and light (VL) domains strongly influence the formation of the multimeric structure. A linker of 15 amino acid residues leads to the formation of an scFv, but reducing the linker length to 8–12 residues causes the scFvs to assemble into dimers, so that diabodies are formed. A further reduction to less than 5 residues leads to the formation of scFv trimers or tetramers (known as triabodies or tetrabodies; (17-21). These scFv multimers are larger and have higher valency than the monomeric form; consequently, their clearance from circulation and accumulation on tumors are improved (22, 23).

Bispecific diabodies are generally produced from heterodimerization of two different hetero scFvs (e.g. VHₐ-VLₐ and VHₐ - VLₐ) with a glycine-rich linker (GGGS; (8, 24). The hetero scFvs can also form higher multimeric structures (25), and the multimeric bispecific diabodies formed are expected to have multivalent bispecificity and appropriate molecular weight. Here, we examined the multimerization of hEx3 by preparing monodisperse tetramers (hEx3 tetrabodies). These bispecific tetrabodies had much higher affinity for each antigen than normal diabodies due to an avidity effect, which led to strong inhibition of cancer cell growth. To our knowledge, this is the first detailed quantitative characterization of functional bispecific tetrabodies.

**EXPERIMENTAL PROCEDURES**

**Preparation of recombinant BsAbs-** For the expression and preparation of hEx3, we used three different methods in accordance with previous reports: preparation using a bacterial expression and in vitro refolding system (13), preparation using a mammalian expression system (14), and preparation using Fc fusion format and restriction protease digestion (26, 27). Size-exclusion chromatography with a HiLoad Superdex 200-(pg column (26/60; GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) was used to fractionate each prepared hEx3 solution. The column was equilibrated with phosphate-buffered saline (PBS), and then 5 mL of purified recombinant antibodies was applied to the column at a flow rate of 2.5 mL/min.

Dynamic light scattering and static light scattering measurements- Dynamic light scattering (DLS) and static light scattering (SLS) measurements were carried out at 20 °C on a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, U.K.) using a He-Ne laser (λ = 633 nm). All the antibody solutions were filtered through a PTFE (polytetrafluoroethylene) filter. For DLS, the antibody solutions at 15 μM were measured using a non-invasive back-scatter optical system, and the correlation curve was fitted using the default exponential g2(τ) fit function to estimate the hydrodynamic diameters of the antibodies. For analyzing molecular weight, SLS of the antibody solutions at 0.3–1.0 mg/mL was measured, and a Debye plot was made using the scattering intensity.

**In vitro growth inhibition assay-** T-LAK cells were induced as previously reported (28). In brief, peripheral blood mononuclear cells were cultured for 48 h at a density of 1 × 10⁶ cells/mL in a medium supplemented with 100 IU/mL of recombinant human IL-2 (kindly supplied by Shionogi Pharmaceutical Co., Osaka, Japan) in a culture flask (A/S Nunc, Roskilde, Denmark) that was precoated with anti-CD3 mAb (10 μg/mL).

In vitro growth inhibition of TFK-1 (human bile duct carcinoma) cells was assayed with a 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethox
yphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay kit (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA) as reported previously (28).

Isothermal titration calorimetry (ITC)-Thermodynamic analyses for the interactions of recombinant antibodies for soluble EGFR (sEGFR) and CD3 were performed by microtitration calorimetry using a VP-ITC from MicroCal Inc. (Northampton, MA, USA; (29). The method for expression and purification of sEGFR has been described previously (30). The expression vector for CD3 was kindly provided by Dr. Katsumi Maenaka (Kyushu University) and preparation of CD3 was performed according to the previous report (31). Each sample (1.5 μM in PBS, pH 7.2, containing 0.005% Tween 20) was placed in a calorimeter cell and titrated with 30 μM sEGFR in the same buffer; for CD3, 1.25 μM hEx3 was titrated with 50 μM CD3. The ligand solution was injected 25 times in 10-μL portions over a period of 15 s. Data acquisition and subsequent nonlinear regression analysis were done in terms of a simple binding model, using the MicroCal ORIGIN 5.0 software package.

Surface plasmon resonance (SPR)-The interactions between sEGFR and bispecific antibodies were analyzed by SPR spectroscopy with BIACORE 2000 (GE Healthcare). sEGFR was immobilized onto the cells in a CM5 sensor chip up to 2716 RU. Various concentrations of bispecific antibodies in 0.005% PBS with Tween 20 (PBS-T) were flowed over the sEGFR. The data were normalized by subtracting the response of a blank cell only with blocking. BIAevaluation software (GE Healthcare) was used to analyze the data. Kinetic parameters were calculated by a global fitting analysis with the assumptions of the 1:1 Langmuir binding model.

Stability tests- To examine in vitro stability, hEx3s were pre-incubated at 37 °C for 1 h in human plasma. Growth inhibition relative to untreated hEx3s was then evaluated with the MTS assay.

Gel filtration analysis with a HiLoad Superdex 200-μg column (10/300; GE Healthcare) was used to evaluate the long-term stability of the hEx3 tetramer in storage. After storage for one month at 4 °C, 250 μL of fractionated hEx3 tetramers was applied to a column equilibrated with PBS at a flow rate of 0.5 mL/min.

RESULTS

Structural analysis of prepared hEx3. We prepared the small recombinant bispecific antibody hEx3 using three different methods: refolding from insoluble aggregates expressed in E. coli, secretory expression by Chinese hamster ovary (CHO) cells, and Fc fusion expression by CHO cells. Size-exclusion chromatography of each hEx3 preparation showed the predominant formation of dimers, but multimeric forms were also observed. The proportion of multimers varied with the method of preparation: refolded hEx3 produced only a small amount of multimers (Fig. 1A), whereas the secretory preparation using CHO cells promoted the formation of multimeric forms, which corresponded to the fraction position of tetramers (Fig. 1B). Thus, hEx3 predominantly formed dimers but has the potential to form tetramers. hEx3 prepared from the Fc fusion format via restriction protease digestion also formed tetramers (Fig. 1C), and this method enabled the preparation of sufficient amounts of dimers and tetramers for further evaluation. The final yields of dimers and tetramers are 5 mg and 1 mg/L culture, respectively. An SDS-PAGE analysis of the fractionated hEx3 showed that both the anti-EGFR VH — linker — anti-CD3 VL (h5HhOL) and anti-CD3 VH — linker — anti-EGFR VL (hOHh5L) hetero scFv fragments formed equal proportions of dimers and tetramers with all the expression methods (SDS-PAGE for hEx3 from the Fc fusion is shown in Fig. 1D as a representative example).

To confirm the formation of tetramers, we employed DLS and SLS spectroscopy to quantify the size and molecular weights of the dimers and tetramers fractionated from hEx3 prepared with the Fc fusion format. An IgG-type mouse anti-CD3 antibody, OKT3, was used as a control.
for comparison. Both the dimer and tetramer had narrow distributions, centered at 3.4 nm and 6.6 nm, respectively; the size of tetramer was about twice that of dimer and two-thirds that of IgG (Fig. 2, Table 1). SLS measurement supported the molecular weight estimated from size-exclusion chromatography. Therefore, the multimer at the 190-mL fraction formed a monodisperse tetramer with equal amounts of h5HhOL and hOHh5L scFvs.

**Growth inhibition effect of each fraction in hEx3 solution.** To analyze the influence of the tetramerization on the inhibition of human carcinoma cell growth, we analyzed prepared dimeric and tetrameric hEx3s with MTS. In the presence of T-LAK cells, both hEx3 forms strongly inhibited the growth of TFK-1 cells, but the tetramer was effective at a much lower concentration, 10 fmol/mL (Fig. 3A). When PBMCs were applied as effector cells, although high concentrations of hEx3s were required, the tetramer also inhibited more effectively than the dimer (Fig. 3B). Thus, the multimerization into tetraramers increased the function of hEx3.

**Thermodynamic analysis of each fraction of hEx3.** To investigate the binding stoichiometry of each fraction in hEx3 for EGFR and CD3, we performed thermodynamic analyses by means of ITC. IgG and Fab were used as control molecules with bivalent and monovalent binding, respectively. The binding constants and stoichiometry are summarized in Table 2. Dimeric hEx3 showed 1:1 stoichiometric binding for EGFR (Fig. 4C) and CD3 (Fig. 4D), similar to Fab, indicating that the anti-EGFR Fv and anti-CD3 Fv portions in the dimeric hEx3 had formed correctly; that is, the prepared dimers were monomorphous diabodies without inactive homodimers. This result is consistent with our previous results supporting the formation of monomorphous hEx3 diabodies (14). In the case of tetraramers, the integration plots for EGFR and CD3 showed the same binding stoichiometry as IgG but not Fab, that is, a 1:2 stoichiometric binding for both antigens (Fig. 4C, D). The tetramer therefore formed monomorphous hEx3 tetrabodies with bivalency for two individual targets.

**Comparison of binding kinetics with surface plasmon resonance.** To confirm the effect of the multivalency of tetrameric hEx3, we evaluated the binding kinetics for immobilized sEGFR by SPR. The binding kinetics for CD3 were not determined, because the CD3 receptors were inactivated when immobilized on a sensor chip. The sensogram for tetrameric hEx3 against EGFR showed an association curve similar to that of the dimer, but the dissociation of the tetramer was slower than that of the dimer (Fig. 5). A global fitting to a 1:1 interactional model with a mass transport term indicated that the tetraramers had an association rate similar to that of the dimers, but the dissociation rate was one-seventh of the dimer rate (Table 3); consequently, the affinity constant of the tetramer was 17-fold that of the dimer. The multimerization into a tetramer influenced the dissociation process of diabodies, resulting in increased affinity for the antigen.

**Stability test under physiological conditions.** Physiologic stability is a critical factor for potential therapeutic recombinant proteins. Therefore, we examined the cytotoxicity of dimeric and tetrameric hEx3 after preincubation at 37 °C in human plasma. The activity of the dimers was slightly reduced; however, the intense cytotoxicity of the tetramer was retained (Fig. 6A). The stability of assembled structure was also evaluated by size-exclusion chromatography of the tetramer after storage for a month (Fig. 6B). Although a few tetraramers were converted into dimers, we confirmed that the tetrameric structure was sufficiently stable for use after 1 month in storage.

**DISCUSSION**

Bispecific diabodies show several advantages over BsAbs produced from hybrid hybridomas or chemical conjugation; however, Bispecific diabodies are also cleared rapidly, and their decrease in valence generally causes low functional affinity (15,16). Shortening the middle linker in scFv leads to self-multimerization, and the multimerization can improve the
pharmacokinetics and increase functional affinity due to an avidity effect (17-20). Multimerization of bispecific diabodies has previously been observed (25), but the effectiveness of bispecific diabodies has not been studied to date.

In the present study, we found that hEx3 formed multimers. We purified these multimers, and kinetic and thermodynamic analyses of each hEx3 fraction quantitatively demonstrated that the tetramer had two functional binding sites for each antigen (Fig. 4, Table 2). This increase in binding sites provided strong growth inhibition activity. The multimerization was effective even for bispecific diabodies.

Engineering of linkers in single-chain diabodies (scDbs), in which two hetero scFvs are tandemly conjugated, can provide tetravalent bispecific dimers called tandem scDbs (tanDbs). The tanDbs exhibit not only higher functional affinity and stability under physiological conditions in vitro than scDbs, but also longer blood retention and higher therapeutic effects in vivo (15,32,33). In this study, we prepared highly functional bispecific tetrabodies from hetero scFv fragments with molecular sizes approximately half those of scDbs. Although the bispecific tetrabodies formed as a byproduct of bispecific diabodies, the formation from smaller fragments might be an advantage in protein expression. In contrast to the structure of tanDbs, in which all four variable domains of one chain interact with the variable domains of the second chain (33,34), the structure of the hEx3 tetramer is probably a circular structure, similar to that of the scFv tetramer (known as a tetrabody; (18,35)). We previously reported a strong interdomain interaction between the cognate VH and VL domains of hEx3 (14); this strong interaction probably contributes to the formation of a stable circular structure for the hEx3 tetramer with four active binding sites. To date, several different small BsAb formats have been proposed to increase efficacy and availability, including not only scDb and tanDb but also tandem scFv (36) and minibodies (37). Bispecific tetrabodies like the hEx3 tetramer should also be considered small BsAb formats for the development of effective cancer therapeutic antibodies. Although in vivo experiments with the hEx3 tetramer are now under way, we did confirm their stability in physiologic conditions and in long-term storage (Fig. 6).

In conclusion, we showed that the multimeric molecules in hEx3 solution were homogenous tetrabodies with high cytotoxicity. The multimerization of small antibody fragments can lead to improved pharmacokinetics and binding affinity, resulting in an enhancement of the therapeutic effect. To increase the population of hEx3 tetrabodies for therapeutic application, we are working to modify the middle linker in hetero scFvs, to change the orientation of VH and VL, and to create mutations to minimize the steric interference in the tetrameric form, similar to how the scFv multimer has been modified (35,38,39).

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FOOTNOTES

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The abbreviations used are: BsAbs, bispecific antibodies; CHO, Chinese hamster ovary; DLS, dynamic light scattering; EGFR, epidermal growth factor receptor; hShHOL, anti-EGFR VH — linker — anti-CD3 VL; hOHh5L, anti-CD3 VH — linker — anti-EGFR VL; ITC, isothermal titration calorimetry; MTS, 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; PBS-T, PBS with Tween 20; PTFE, polytetrafluoroethylene; RU, resonance unit; scDb, single-chain diabody; scFv, single chain Fv; sEGFR, soluble EGFR; SLS, static light scattering; T-LAK, lymphokine-activated killer cells with the T-cell phenotype; tanDb, tandem single-chain diabody.

FIGURE LEGENDS

Fig. 1. Gel filtration of hEx3 prepared with three different methods. The elution volume is noted on the x-axis. A, hEx3 from a refolding system (13); B, hEx3 from a mammalian expression system (14); and C, hEx3 from an Fc fusion format (27). D, SDS-PAGE analysis of the eluted fractions under reducing conditions. The tetramer (T) and dimer (D) fractions of hEx3 from the Fc fusion format are shown.

Fig. 2. Distributions of hydrodynamic diameters determined with the DLS histogram method.

Fig. 3. Growth inhibition of EGFR-positive TFK-1 cells by the dimeric and tetrameric fractions of hEx3. A, hEx3s and T-LAK cells were added to TFK-1 cells at a ratio of 5 to 1. B, hEx3s and PBMCs were added to TFK-1 cells at a ratio of 10 to 1. Data are presented as the mean value ± SD and are
representative of at least three independent experiments with similar results.

Fig. 4. Isothermal titration calorimetry of the interactions of antibodies with sEGFR and CD3. Representative graphs of calorimetric titration of hEx3 tetramers at pH 7.2 and 25 °C for sEGFR (A) and CD3 (B) are shown. (C,D) Overlaid integration plots calculated from the raw data for Fab, IgG, hEx3 dimers, and hEx3 tetramers. The solid lines correspond to the best fit curves obtained by least-squares deconvolution.

Fig. 5. SPR sensorgrams for hEx3 dimers and tetramers. The results from the indicated analyte concentrations are shown as colored lines, and global fitting kinetic analyses are shown as black lines.

Fig. 6. A, Stability test of hEx3 dimers and tetramers under physiologic conditions by assay of growth inhibition of EGFR-positive TFK-1 cells. Each bispecific antibody and T-LAK cells (effectors) were added to TFK-1 cells (targets) at a ratio of 5 to 1. Data are presented as the mean value ± SD and are representative of at least three independent experiments with similar results. B, Gel filtration of hEx3 tetramer for confirmation of stability in storage. The elution volume is noted on the x-axis, and the kDa values are shown above the figure. Fractionated hEx3 tetramers were applied to the column after storage for one month at 4 °C.
### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Calculated MW [kDa]</th>
<th>Diameter from DLS (distribution) [nm]</th>
<th>Mw from SLS [kDa]</th>
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<tr>
<td>dimer</td>
<td>53</td>
<td>3.43 (2.7-4.19)</td>
<td>n.d.</td>
</tr>
<tr>
<td>tetramer</td>
<td>106</td>
<td>6.57 (4.85-10.1)</td>
<td>111</td>
</tr>
<tr>
<td>mOKT3 IgG</td>
<td>150</td>
<td>9.07 (5.61-15.7)</td>
<td>156</td>
</tr>
</tbody>
</table>

n.d., not determined
Table 2

$K_A$ value and stoichiometry ($n$) for sEGFR and CD3εγ evaluated by ITC

<table>
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<th></th>
<th>sEGFR</th>
<th>CD3εγ</th>
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<tr>
<td></td>
<td>$K_A \times 10^7 \text{M}^{-1}$</td>
<td>$n$</td>
</tr>
<tr>
<td>Fab</td>
<td>14.7</td>
<td>1.1</td>
</tr>
<tr>
<td>IgG</td>
<td>21.9</td>
<td>2.0</td>
</tr>
<tr>
<td>dimer</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>tetramer</td>
<td>15.7</td>
<td>1.8</td>
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</tbody>
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m528 IgG and Fab were used for sEGFR, and mOKT3 IgG and Fab were used for CD3εγ.
Table 3

Binding kinetics for sEGFR evaluated with surface plasmon resonance

<table>
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<th>$k_{on}$</th>
<th>$k_{off}$</th>
<th>$K_A$</th>
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</thead>
<tbody>
<tr>
<td>dimer</td>
<td>2.6</td>
<td>55.2</td>
<td>4.8</td>
</tr>
<tr>
<td>tetramer</td>
<td>6.7</td>
<td>8.1</td>
<td>81.9</td>
</tr>
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</table>

$* 10^5$ M$^{-1}$s$^{-1}$ $* 10^{-4}$ s$^{-1}$ $* 10^7$ M$^{-1}$
Figure 1

A. 10 mAU

B. 20 mAU

C. 80 mAU

D. T D

hOHH5L
h5HhOL
Figure 2

![Graph showing the distribution of dimer, tetramer, and mOKT3 IgG based on diameter (nm).](http://www.jbc.org/)

- **Dimer**
- **Tetramer**
- **mOKT3 IgG**
Figure 3

A

Growth inhibition of cancer cells (%)

Concentration of diabody (fmol/mL)

B

Growth inhibition of cancer cells (%)

Concentration of diabody (pmol/mL)
Figure 4

A

Time (min)

mcal/s

mcal/s

kcal/mol of sEGFR

kcal/mol of sEGFR

[sEGFR]/[tetramer]

B

Time (min)

mcal/s

mcal/s

kcal/mol of CD3ε

kcal/mol of CD3ε

[CD3ε]/[tetramer]

C

D

m528 Fab
m528 IgG
dimer
tetramer

mOKT3 Fab
mOKT3 IgG
dimer
tetramer

[kcal/mol of sEGFR]

[kcal/mol of CD3ε]

[sEGFR]/[Antibody]

[CD3ε]/[Antibody]
Figure 5

**dimer**

Response (RU) vs. Time (sec)

- 500 nM
- 250 nM
- 125 nM
- 63 nM
- 31 nM

**tetramer**

Response (RU) vs. Time (sec)

- 250 nM
- 125 nM
- 63 nM
- 31 nM
- 16 nM
Figure 6

A

Growth inhibition of cancer cells (%)

Concentration of hEx3 (pmol/mL)

(T-LAK)

- dimer
- dimer (preincubated with plasma)
- tetramer
- tetramer (preincubated with plasma)
Figure 6

B 67 43 25 [kDa]

↓ tetramer

40 mAU ↓ dimer

11 13 15 17

Elution volume (mL)
Highly enhanced cytotoxicity of a dimeric bispecific diabody, the hEx3 tetrabody
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