MICROBIAL CELL FACTORIES

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Efficient production of single-chain fragment variable-based N-terminal trimerbodies in *Pichia pastoris*

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Abstract

Background: Recombinant antibodies are highly successful in many different pathological conditions and currently enjoy overwhelming recognition of their potential. There are a wide variety of protein expression systems available, but almost all therapeutic antibodies are produced in mammalian cell lines, which mimic human glycosylation. The production of clinical-grade antibodies in mammalian cells is, however, extremely expensive. Compared to mammalian systems, protein production in yeast strains such as *Pichia pastoris*, is simpler, faster and usually results in higher yields.

Results: In this work, a trivalent single-chain fragment variable (scFv)-based N-terminal trimerbody, specific for the human carcinoembryonic antigen (CEA), was expressed in human embryonic kidney 293 cells and in *Pichia pastoris*. Mammalian- and yeast-produced anti-CEA trimerbody molecules display similar functional and structural properties, yet, the yield of trimerbody expressed in *P. pastoris* is about 20-fold higher than in human cells.

Conclusions: *P. pastoris* is an efficient expression system for multivalent trimerbody molecules, suitable for their commercial production.

Keywords: *P. Pastoris*, Monoclonal antibody, Recombinant antibody, Multivalent antibody, Trimerbody

Background

Over the past decades, there has been growing interest in the use of recombinant antibodies in bioanalytical and medical applications [1]. In an attempt to improve the therapeutic efficacy of antibodies, new recombinant formats with modified properties have been generated [2]. Multivalent and multispecific antibodies capable of simultaneously blocking multiple growth and survival pathways have the potential to meet the current and future therapeutic challenges, and indeed many of them are advancing in clinical development [3]. The most common strategy to create multivalent IgG-like formats has been the fusion of antibody fragments with homodimerization sequences (e.g., ZIP miniantibody [4], minibody [5] or single-chain fragment variable (scFv)-Fc antibody [6]). A different strategy to multimerize antibody fragments is based on the modification of the interdomain linker length to generate bivalent, trivalent or tetravalent molecules [7,8]. Other protein-protein interactions have been also used to create multivalent non-IgG-like formats, such as the streptavidin-biotin system, the C-terminal multimerization domain of the tumor-suppressor protein p53 [9], and the ribonuclease barnase with its inhibitor, barstar [10], among others [2].

A variety of expression systems ranging from bacterial cells to mammalian cells have been used to express recombinant antibodies [11,12]. *E. coli* is the most commonly used host for the expression of antibody fragments, whereas mammalian cells are used for the expression of large, multidomain antibodies such as full-length monoclonal antibodies or complex recombinant antibody fragments [13]. In fact, almost all approved therapeutic

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antibodies for human use are produced in mammalian cell culture systems [14].

In previous studies, we reported the *in vitro* and *in vivo* characterization of a multivalent antibody generated by fusing a trimerization (TIE) domain to the C-terminus of a scFv antibody [15-17]. TIE domains are composed of the N-terminal trimerization region of collagen XVIII NC1 (TIE^{XVIII}) or collagen XV NC1 (TIE^{XV}) flanked by flexible linkers. The new antibody format, termed trimerbody, is trimeric in solution and exhibited excellent antigen binding capacity and multivalency [15-17]. Furthermore, by fusing scFv antibodies with the same or different specificity to both ends of a TIE^{XVIII} domain, we have produced monospecific or bispecific hexavalent-binding molecules, expanding the scope of potential applications of trimerbody molecules [18].

To date, trivalent and hexavalent scFv-based trimerbodies have only been produced in mammalian cell cultures [15-18]. However, the generation of stable antibody-producing mammalian cell lines is an expensive and time-consuming procedure. Here, we evaluated the potential of the methylotrophic yeast *P. pastoris* [12,19,20] to produce with high yield a N-terminal trimerbody specific for the human carcinoembryonic antigen (CEA) [16]. The functional and biochemical properties of both mammalian- and yeast-derived trimerbodies were gauged demonstrating the functional equivalence of the two preparations. Our results demonstrate that *P. pastoris* is a viable alternative expression system for scFv-based N-terminal trimerbody molecules.

Results

Generation of anti-CEA scFv-based N-terminal trimerbody expression vectors

In this study we have generated a pPICZαA-based vector for the expression of the MFE-23 scFv-based N-terminal trimerbody ($MFE\text{-}23^{N}$) in *P. pastoris* (Figure 1), and we demonstrated that $MFE\text{-}23^{N}$ molecules are efficiently secreted as soluble proteins by transformed *P. pastoris* cells. Western blot analysis shows that under reducing conditions, a single polypeptide chain with mass around 37 kDa was seen (Additional file 1: Figure S1B). As previously shown [16], the $MFE\text{-}23^{N}$ trimerbody is efficiently secreted as soluble functional protein by HEK-293 cells transfected (Additional file 1: Figure S1A) with the expression vector $pCEP4\text{-}MFE\text{-}23\text{-}NC1^{ES\text{-}}$ (Figure 1). Secreted $MFE\text{-}23^{N}$ trimerbodies from both sources are able to recognize immobilized human CEA with high affinity and specificity (Additional file 2: Figure S2).

Purification and functional characterization of yeast- and mammalian- produced anti-CEA scFv-based N-terminal trimerbodies

For purification, the extracellular medium of *P. pastoris* cells after 72 hours of methanol induction, and the serum-free conditioned media from stably transfected HEK-293 cells were independently collected. Both $MFE\text{-}23^{N}$ trimerbodies were purified by immobilized metal affinity chromatography, which yielded >95% pure 37 kDa proteins as assessed by reducing SDS-PAGE (Figure 2A). Both systems produced soluble and functional $MFE\text{-}23^{N}$ molecules, but with significant differences in antibody yields from *Pichia* and HEK-293 cells, 6 and 0.35 mg/l, respectively. Importantly, the yeast-produced $MFE\text{-}23^{N}$ trimerbody was functional and recognized, as efficiently as the mammalian-produced $MFE\text{-}23^{N}$ trimerbody, human CEA either plastic immobilized (Figure 2B) or expressed on the tumor cell surface (Figure 2C).

Structural characterization of yeast and mammalian produced anti-CEA scFv-based N-terminal trimerbodies

Both mammalian- and yeast-produced trimerbodies elute from the analytical gel-filtration columns as major peaks at 13 ml with molar masses of 110 or 108 kDa, respectively. These masses are consistent with the calculated values for the trimeric molecules (110 and 113 kDa, respectively) (Figure 3A, B). A minor peak eluting at 11 ml is also seen in the chromatograms of both molecules, with molar mass of 214 and 210 kDa (yeast and mammalian

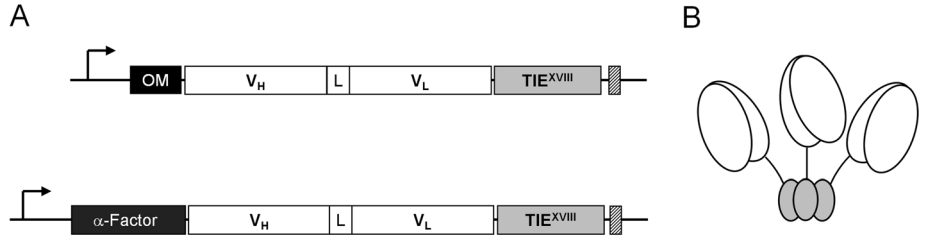

Figure 1 Schematic diagrams showing the genetic and domain structure of scFv-based N-terminal trimerbodies. (A) Diagrammatic representation of gene constructs. Both constructs bear the anti-CEA MFE-23 scFv gene (VH-linker-VL), a TIE^{XVIII} domain and c-myc and His tags (hatched box), for subsequent purification and immunodetection. Signal peptides were from oncostatin M (OM) and α-factor for expression in HEK-293 cells (upper) and *P. pastoris* (lower) respectively. **(B)** Schematic representation of the domain structure of the scFv-based N-terminal trimerbody.

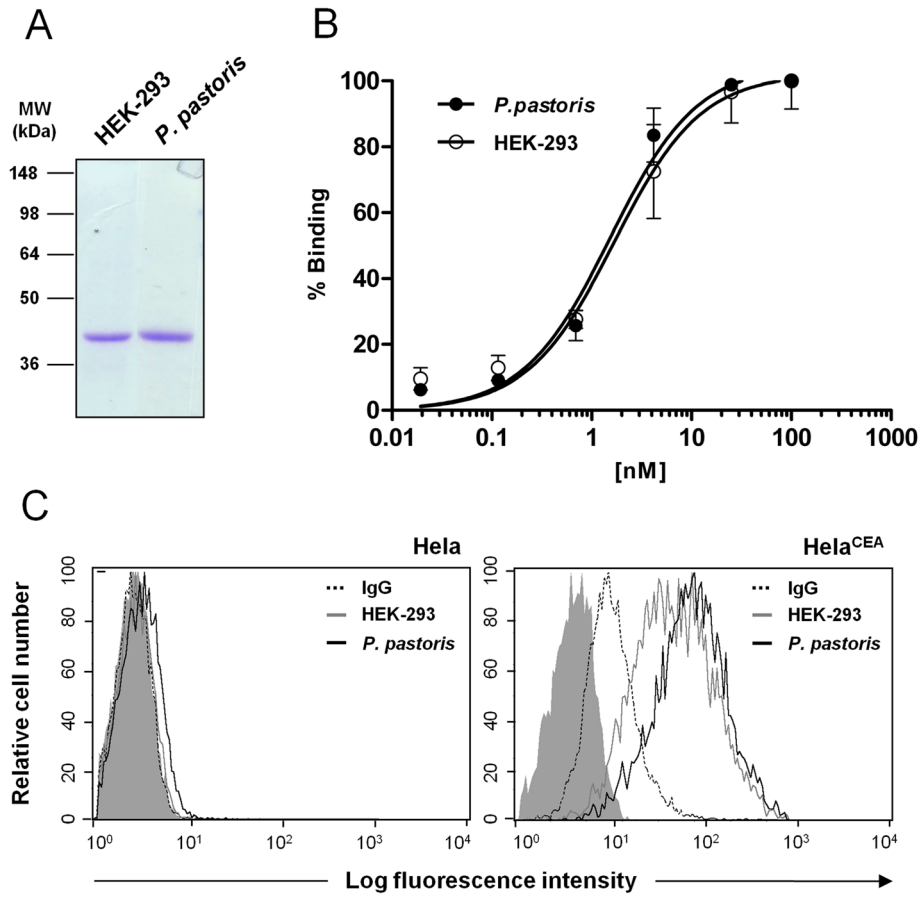

Figure 2 Characterization of purified trimerbodies. (A) Reducing SDS-PAGE of anti-CEA scFv-based N-terminal trimerbody (MFE-23^{N}) purified from HEK-293 cells or *P. pastoris*. The functionality of purified MFE-23^{N} trimerbodies is demonstrated by ELISA against plastic immobilized CEA **(B)** and by FACS on CEA^{-} and CEA^{+} tumor cells **(C)**. The anti-CEA C6G9 mAb (IgG) was used as control.

cells, respectively) (Figure 3A, B). These minor peaks contain about 10% of the protein (relative to the major ones as estimated from absorbance at the corresponding maxima), and their masses are consistent with hexamers (possibly dimers of the corresponding trimmers). SDS-PAGE analysis of the two species separated in the gel filtration column showed a single band at the same position (Additional file 3: Figure S3), and at the expected position relative to the molecular weight markers (between the 45 kDa and 35 kDa markers, in agreement with the calculated values of 37 and 38 kDa for the yeast- and mammalian-produced MFE-23^{N}, respectively). These results demonstrate that the purified MFE-23^{N} trimerbodies behave predominantly as trimers with a small proportion of hexamers, independently of the producer organism.

The CD spectra of both trimerbodies were very similar, with minima at 217 nm and less negative minima at 228–230 nm (Figure 3C). This is consistent with the secondary structures of the scFv domain, mainly β-sheet and irregular loops, plus the contribution of the helical structures of the trimerization domains of collagen XVIII NC1 domain and the linker sequences (which probably are flexible random coils). The MFE-23^{N} molecules produced in *P. pastoris* and in HEK-293 cells showed a major cooperative thermal transition, with essentially the same mid-point denaturation temperature of 48–49°C. At high temperatures another minor transition is observed, possibly due to aggregation phenomena of the denatured polypeptide chains. The same behavior was observed in experiments recorded at 210 nm with the trimeric molecules separated from hexameric ones by gel filtration (Figure 3D). These results show that the scFv-based N-terminal trimerbodies produced in *P. pastoris* and in HEK-293 cells have very similar structures and thermal stabilities.

Serum stability study of yeast and mammalian produced N-terminal trimerbodies

Both MFE-23^{N} trimerbodies were further analyzed to evaluate their long-term stability in serum, an important feature of recombinant antibodies for potential diagnostic or therapeutic applications. For this purpose, purified

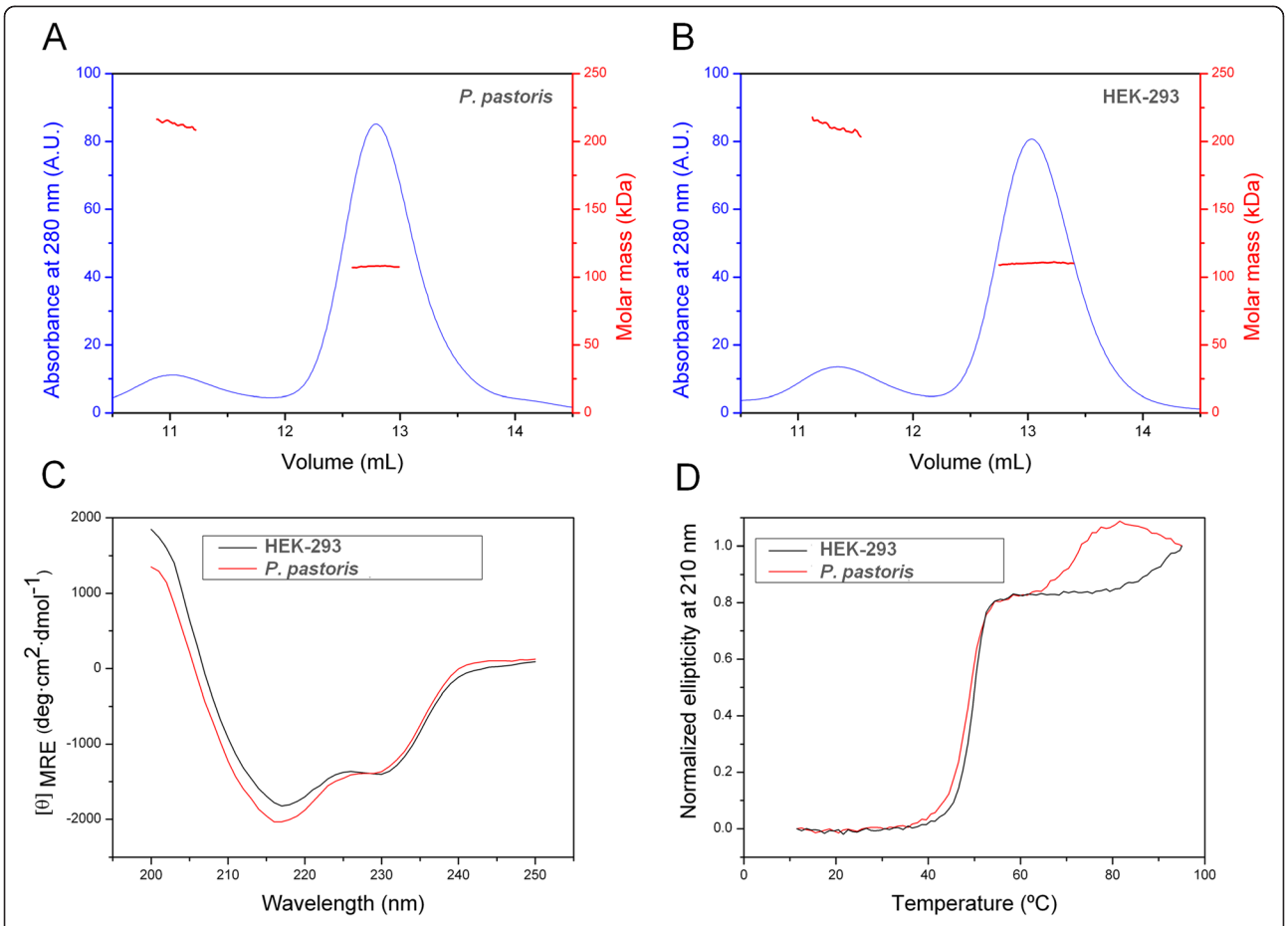

Figure 3 Structural characterization of purified trimerbodies. Oligomeric analysis of MFE-23^{N} purified from *P. pastoris* **(A)** or HEK-293 cells **(B)**. Circular dichroism spectrum **(C)** and thermal denaturation **(D)** for MFE-23^{N} molecules purified from *P. pastoris* (red line) and HEK-293 cells (black line).

MFE-23 scFv N-terminal trimerbodies were incubated in human serum for 0 (control) to 4 days at 37°C (Figure 4). MFE-23^{N} molecules purified from *P. pastoris* were more stable with 60% CEA binding activity after 4 days of incubation, whereas mammalian-produced MFE-23^{N} molecules retained around 40% CEA binding activity. The stability was also analyzed by western blot (Figure 4B), and we found that after 4 days at 37°C 60% of the MFE-23^{N} trimerbody produced in *P. pastoris* was structurally intact, while around 40% of the mammalian-produced MFE-23^{N} trimerbody was functional at the end of the assay.

Discussion

In the present study we demonstrate that methylotrophic yeast *P. pastoris* secreted functional CEA-specific MFE-23 scFv-based N-terminal trimerbody at significant levels. Moreover, we demonstrate that both yeast- and mammalian-produced MFE-23^{N} trimerbodies have similar functional and structural properties. Purified MFE-23^{N} molecules were trimeric in solution, as unambiguously shown by the light scattering measurements. The anti-CEA scFv-based N-terminal trimerbodies produced in *P. pastoris* and in HEK-293 cells are highly efficient at recognizing antigen either immobilized in plastic, or associated to the cell surface. The dose-dependent binding curves of purified MFE-23^{N} molecules to plastic immobilized human CEA were comparable. Furthermore, both scFv-based N-terminal trimerbodies specifically recognize CEA cancer cells. Additionally, we demonstrated that MFE-23^{N} molecules produced in yeast are slightly more stable in human serum than MFE-23^{N} molecules produced in mammalian cells.

P. pastoris is widely used for the secretion of properly folded proteins with high yields in a cost-efficient and rapid way [21]. It offers complex posttranslational modification pathways avoiding pyrogenic contamination. In this sense, *P. pastoris* holds a generally recognized as safe (GRAS) status [22]. The yield of anti-CEA scFv-based N-terminal trimerbody expressed in *P. pastoris* was of 6 mg of pure protein per liter of culture, which is about 20-fold higher than in mammalian cells. This is

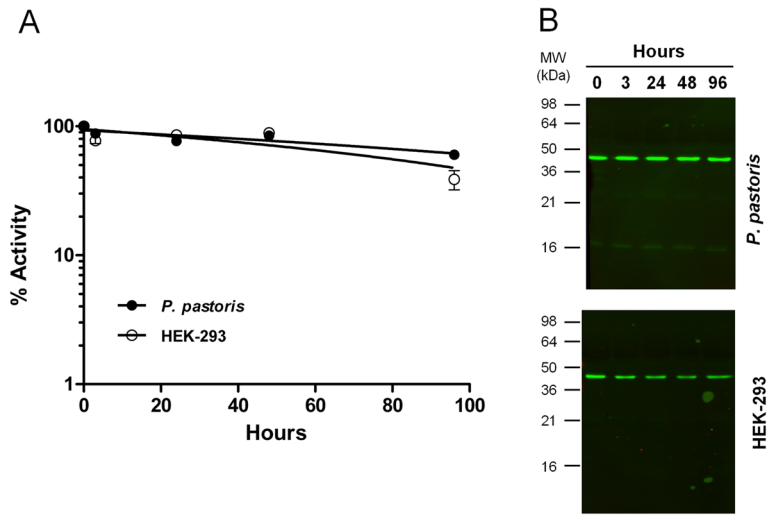

Figure 4 Serum stability of MFE-23^N purified from *P. pastoris or* HEK-293 cells. ELISA against plastic immobilized CEA **(A)** and western blot **(B)** were performed after incubation at 37°C for different time periods in human serum, as indicated in material and methods.

consistent with *P. pastoris* ability to reach very high cell densities, up to 100 OD_{600}, allowing significantly increased amounts of secreted protein. *P. pastoris* has been widely used in the expression of recombinant antibodies, such as scFv [23,20], tandem scFvs, also known as $(scFv)_2$ [24], diabodies [25], Fab fragments [26-28], tribodies [29], scFv-Fc [30], scFv-immunotoxins [31,32] and full-length IgG [33,34]. Moreover, favorable protein folding by *P. pastoris* seems to play a fundamental role in the stability and activity of a single domain antibody fragment against botulinum neurotoxin in comparison to the same produced in E. coli [35]. Another relevant issue is that *P. pastoris* displays both O- and N-linked glycosylation, but glycosylation patterns are different from those found in higher eukaryotes and may lead to reduction in activity and antigenic response. Moreover, *P. pastoris* is known to glycosylate proteins that are non-glycosylated in mammalian cells [14]. Therefore, although the prediction of potential glycosylation sites using the GlycoEP server [36] showed that the MFE-23^N trimerbody does not contain putative *N*- and *O*-glycosylation sites, we can not rule out that yeast-produced trimerbodies may be "decorated" with some extra sugars, and this could be the explanation for the subtle difference in size observed between yeast- and mammalian-produced MFE-23^N molecules. Importantly, we have demonstrated that even if trimerbody glycosylation takes place, it does not affect antigen binding. A current alternative is the use of the *P. pastoris* genetically engineered to produce humanized glycosylation patterns. In fact, anti-Her2 mAb produced in glyco-engineered *P. pastoris* exhibit features comparable to those of trastuzumab in preclinical assays [34]. New marketed therapeutic proteins produced in *Pichia* evidence the rise of *P. pastoris* as a producer organism. In 2009 the FDA approved ecallantide, a small recombinant protein acting as a potent, specific and reversible inhibitor of plasma kallikrein for the treatment of acute hereditary angioedema [37,38].

Conclusions

Wave demonstrated that scFv-based N-terminal trimerbodies can be efficiently produced in *P. pastoris* in a trimeric fully functional active form. These results illustrate the potential of *Pichia pastoris* for the secretion of multivalent antibodies.

Methods

Reagents and antibodies

The mAb used include: C6G9 (Sigma-Aldrich, St. Louis, MO, USA) anti-human CEA (CD66e) and Tetra-His (Qiagen, GmbH, Hilden, Germany). The polyclonal antibodies included: phycoerytrin (PE)-conjugated goat $F(ab')_2$ fragment anti-mouse IgG (Fc fragment specific, Jackson Immuno Research, Newmarket, UK), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Fc specific) (Sigma-Aldrich), and IRDye800-conjugated donkey anti-mouse IgG (H&L) (Rockland Immunochemicals, Gilbertsville, PA, USA). Human CEA was obtained from Calbiochem (Merck, Darmstadt, Germany) and bovine serum albumin (BSA) was from Sigma-Aldrich.

Cells and culture conditions

HEK-293 (CRL-1573) and HeLa (CCL-2) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). They were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD, USA) supplemented with 10% (vol/vol) heat inactivated fetal calf serum (FCS) (Thermo Fisher, MA, USA).

The HeLa^{CEA} cell line [39] was cultured in medium containing 750 μg/ml G418 (Promega, Madison, WI; USA). The methylotrophic yeast *P. pastoris* strain KM71 was obtained from Invitrogen (Life Technologies, Carlsbad, CA, USA). Cells were grown on yeast extract peptone dextrose (YPD) plates or YPD medium at 30°C. When harbouring an expression vector the cells were grown on YPD plates with zeocin.

Construction of expression vectors

The mammalian expression vector pCEP4-MFE-23-NC1^{ES-}encoding the CEA-specific MFE-23 scFv-based N-terminal trimerbody, containing a murine TIE^{XVIII} domain, has been previously reported [16]. To generate the *P. pastoris* expression vector the DNA fragment encoding the MFE-23 scFv was PCR amplified from pCEP4-MFE-23-NC1^{ES-} with primers *Eco*RI FW and *Not*I RV (Table 1). The *Eco*RI /*Not*I-digested PCR fragment was ligated into the *Eco*RI/*Not*I-digested backbone of plasmid pPICZαA (Life Technologies) to generate the intermediate plasmid pPICZαA-MFE-23. The DNA encoding human TIE^{XVIII} was PCR amplified from plasmid pCR3.1-L36-hNC1 [18] with primers *Not*I FW and *Sal*I RV (Table 1). The *Not*I/*Sal*I-digested PCR fragment was ligated into the *Not*I/*Sal*I-digested backbone of plasmid pPICZαA-MFE-23 to obtain pPICZαA-MFE-23-TIE. The sequence was verified using primers 5' AOX1 and 3'AOX1 (Table 1).

Stable expression in mammalian cells

HEK-293 cells were transfected with pCEP4-MFE-23-NC1^{ES-} vector using calcium phosphate [40], and selected in DMEM with 150 μg/ml hygromycin B (Life Technologies) to generate stable cell lines. Supernatants from stably transfected cell populations were analyzed for protein expression by ELISA, SDS-PAGE and western blotting using Tetra-His mAb.

Stable expression in yeast cells

Electrocompetent *P. pastoris* KM71 cells were electroporated with linearized pPICZαA-MFE-23-TIE plasmid, as previously described [31,32], using a Bio-Rad Gene pulser apparatus (Bio-Rad, Hercules, CA, USA). Cells harboring the desired construct were selected after plating the transformation mixture on YPDS (Yeast Peptone Dextrose Sorbitol) media containing different amounts (100 to 750 μg/ml) of zeocin (Life Technologies), and three independent clones were tested by small-scale production. The colony that showed better results was selected for larger scale production, which was performed by inoculating 2 l baffled flasks containing 250 ml of buffered methanol-complex (BMMY) medium [1% yeast extract, 2% peptone, 100 m K_3PO_4 (pH 6.0), 1.34% yeast nitrogen base (NYD), $4.5x10^{-5}$% biotin, 0.5% methanol] for induction at 25°C and 250 rpm shaking for 72 h. Every 24 h, methanol was added to the medium, to afford a final methanol concentration of 0.5% (v/v).

Purification

Harvested serum-free conditioned mammalian medium was centrifuged, 0.22 μm filtered (Nalgene, Neerijse, Belgium), concentrated (10x) with a 10.000 MWCO Vivaflow 50 filter (Vivascience GmbH, Hannover, Germany), dialyzed against PBS (pH7.4) and loaded onto a HisTrap HP 1 ml column using and ÄKTA Prime plus system (GE Healthcare, Uppsala, Sweden). The purified trimerbody was dialyzed against PBS, analyzed by SDS-PAGE under reducing conditions, and stored at −80°C. Harvested yeast medium was dialyzed against 50 mM Na_3PO_4 buffer, containing 100 mM NaCl (pH 8.0), 0.22 μm filtered and loaded onto a HisTrap HP 1 ml column using and ÄKTA Prime plus system. The purified trimerbody, was dialyzed against Na_3PO_4 buffer, analyzed by SDS-PAGE under reducing conditions, and stored at −80°C. For lyophilization, samples were dialyzed with 50 mM $(NH_4)HCO_3$ (pH 8.0), and lyophilized protein was stored at −20°C.

Western blotting

Samples were separated under reducing conditions on 12% Tris-glycine gels and transferred to nitrocellulose membranes (Life Technologies) and reacted with Tetra-His mAb, followed by incubation with an IRDye800-conjugated donkey anti-mouse IgG. Visualization and quantitative analysis of protein bands were carried out with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Table 1 Oligonucleotide sequences of the various primers applied for the construction of the vectors, and subsequent verification of vector sequences

Name	Sequence (5′-3′)	Reference
*Eco*RI FW	ATTTCACAGAATTC CAGGTGCAGCTGCAGCAGTCT	This study
*Not*I RV	ATTTCACAGCGGCCGC CCGTTTCAGCTCCAGCTT	This study
*Not*I FW	ATTTCACAGCGGCCGC GAATTCAGGCGCC	This study
*Sal*I RV	ATTTCACAGTCGAC TTATTAATGGTGATGATGGTG	This study
5′ AOX1	GACTGGTTCCAATTGACAAGC	Life Technologies
3′ AOX1	GCAAATGGCATTCTGACATCC	Life Technologies

ELISA

The ability of scFv-based N-terminal trimerbodies to bind human CEA was studied by ELISA as previously described [16]. Briefly, Maxisorp plates (Nunc A/S, Roskilde, Denmark) were coated with CEA (0.25 μg/well) and after washing and blocking with 5% BSA in PBS, 100 μl with indicated amount of purified protein or supernatant were added for 1 hour at room temperature. After three washes, 100 μl of Tetra-His mAb (10 μg/ml) were added for 1 hour at room temperature. After three washes, 100 μl of HRP-conjugated goat anti-mouse IgG were added for 1 hour at room temperature, after which the plate was washed and developed. Antigen titration was performed with serial dilutions of the purified trimerbodies.

Flow cytometry

The ability of purified antibodies to bind to cell surface CEA was studied by FACS as described previously [16]. Briefly, cells were incubated with anti-CEA mAb (10 μg/ml) or purified trimerbodies (10 μg/ml) and Tetra-His mAb for 30 min. After washing, the cells were treated with appropriate dilutions of PE-conjugated goat F(ab')2 anti-mouse IgG. All samples were analyzed with a Beckman-Coulter FC-500 Analyzer (Beckman-Coulter, Brea, CA, USA).

Size exclusion chromatography-multi-angle laser light scattering (SEC-MALLS)

Static light scattering experiments were performed at room temperature using a Superdex 200 10/300 GL column (GE HealthCare) connected to a DAWN-HELEOS light scattering detector and an Optilab rEX differential refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The column was equilibrated with running buffer (PBS pH 7.0 + 0.03% NaN_3, 0.1 μm filtered) and the SEC-MALLS system was calibrated with a sample of BSA at 1 g/l in the same buffer. Samples of 100 μl of the MFE-23^N molecules at 0.55 g/l were injected into the column at a flow rate of 0.5 mL/min. Data acquisition and analysis employed ASTRA software (WyattTechnology). Based on numerous measurements on BSA samples at 1 g/l under the same or similar conditions we estimate that the experimental error in molar mass is around 5%.

Circular dichroism and thermal denaturation studies

Circular dichroism (CD) measurements were performed with a Jasco J-810 spectropolarimeter equipped with Peltier thermal control unit (Jasco, MD, USA). The spectra were recorded at 25°C on protein samples at 0.05 g/l in PBS using a 0.2 cm path length stoppered quartz cuvette a response of 8 s and a band width of 2 nm. The spectra were baseline corrected by subtraction of the buffer spectrum recorded in same cuvette under identical conditions. The thermal denaturations were recorded on the same samples increasing the temperature from 10 to 95°C at a rate of 1°C/min and measuring the ellipticity at 210 nm every 1°C with a 32 second response and 4 nm bandwidth. For the graphical representation of the melting curves of both samples the ellipticity values were normalized from 0 (at 10°C) to 1 (at 95°C). The CD data were processed with the program Origin (OriginLab, MA, USA). We estimate that the uncertainty in the molar ellipticity is about 5% and the uncertainty in the mid-point denaturation temperature is 0.5°C.

Serum stability

One microgram of each purified scFv-based N-terminal trimerbody was incubated in 60% human serum at 37°C for up to 96 h. Samples were removed for analysis at 3, 24, 48 and 96 hours and frozen at −80°C until the entire study was completed. As a control, a second set of serum-exposed samples was frozen immediately to represent a zero time point. Aliquots were then subjected to western blot, using Tetra-His mAb, and tested for their capability to bind human CEA by ELISA.

Additional files

Additional file 1: Figure S1. MFE-23^N secretion in HEK-293 cells and *P. pastoris*. Western blot analysis of conditioned media from transfected HEK-293 cells **(A)**, and of culture supernatants after the induction of three different clones (1–3) in *P. pastoris* **(B)**.

Additional file 2: Figure S2. ELISA against plastic immobilized CEA with conditioned media (MFE-23^N SN) from transfected HEK-293 cells **(A)**, and culture supernatants after the induction of three different clones in *P. pastoris* **(B)**. In both cases MFE-23^N purified (10 μg/ml) from HEK-293 cells was used as a control.

Additional file 3: Figure S3. Reducing SDS-PAGE analysis of the samples used and separated in the SEC-MALLS experiments. Input corresponds to the sample injected in the column and the peaks correspond to fractions of the two peaks separated in the column. Peak1 and peak2 correspond to the peaks eluting at approximately volumes of 11 and 13 ml, respectively, as shown in Figures 3A and 3B. The total amount of protein loaded in each lane is different because for some of the samples eluted from the column a large volume was collected and the total protein was precipitated before loading in the gel.

Abbreviations

CEA: Carcinoembryonic antigen; mAb: Monoclonal antibody; scFv: Single-chain variable fragment; SEC-MALLS: Size exclusion chromatography-multi-angle laser light scattering; TIE: Trimerization domain; OM: Oncostatin M.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AB-T, JL, FJB and LA-V designed research; AB-T, JL, NN-P, AA-C, MV and MC performed research; AB-T, JL, LS, FJB and LA-V analyzed data; AB-T, JL, LS, FJB and LA-V wrote the manuscript. All authors read and approved the final manuscript.

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