

Development of a novel engineered antibody targeting *Neisseria* species

Chih-Yueh Liu · Chang-Ching Weng · Chih-Hsiang Lin ·
Chiou-Ying Yang · Kwok-Kong Tony Mong · Yaw-Kuen Li

Received: 7 October 2016 / Accepted: 10 November 2016 / Published online: 25 November 2016
© Springer Science+Business Media Dordrecht 2016

Abstract

Objectives A *Neisseria* bacterial pilus sugar, bacillosamine, was synthesized and, for the first time, used as a probe to screen a single-chain variable fragment (scFv). **Results** Four *Neisseria*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Neisseria sicca* and *Neisseria subflava*, and two negative controls, *Streptococcus pneumoniae* and *Escherichia coli*, were tested through ELISA, immunostaining and gold nanoparticle immunological assay. All results indicated that the selected scFv is feasible for the specific detection of *Neisseria* species via the recognition of bacillosamine. **Conclusions** The recombinant scFv could detect *Neisseria* strains at 10^6 CFU/ml.

Keywords Bacillosamine · *Neisseria meningitidis* · *Neisseria gonorrhoeae* · Single-chain variable fragment

Introduction

Neisseria meningitidis and *Neisseria gonorrhoeae* are human-specific pathogens. The latter colonizes the genito-urinary tract and is a common cause of sexually transmitted infections (Aral et al. 1991). *N. meningitidis* is often found in the upper respiratory tract of healthy individuals, where it can cross the mucosal epithelium and cause sepsis or meningitis (Carbonnelle et al. 2009). The commonly-used clinical testing of *Neisseria* with Gram staining and culture is often imprecise and/or time-consuming. During the past decade, nucleic acid amplification tests have rapidly replaced culturing for the detection of *Neisseria*. However, people living in sub-standard areas or with uncommon sexual activity need a tool that is portable and efficient to help control the risk of transmitted infection. In light of this need, an scFv with specific recognition for the *Neisseria* strain is essential for the development of a rapid, efficient and non-cultural detection that can act as an alarm for the threat of infection (Ahmad et al. 2012).

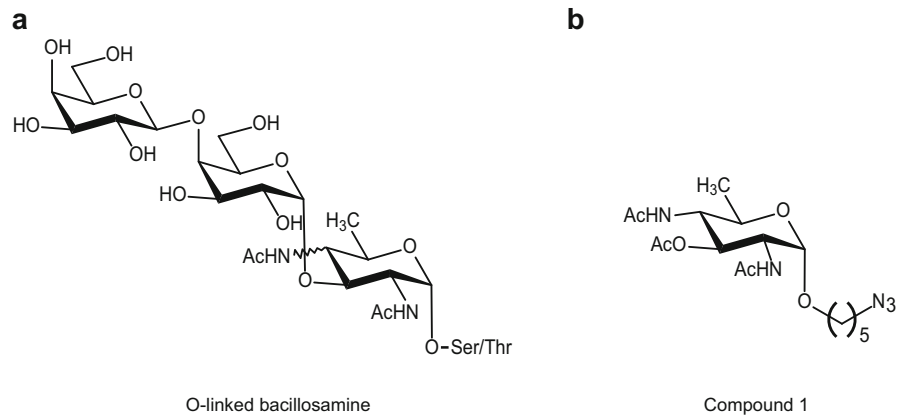
Rather than DNAs or membrane proteins, we chose neisserial pilus glycan as the target for bio-sensing development. *Neisseria* species express the type IV-A class of bacterial pili, which are covalently modified with an *O*-linked galactose-(β -1-4)-galactose-(α -1-3)-2,4-diacetimido-2,4,6-trideoxyhexose (Gal-Gal-DATDH) trisaccharide (Power et al. 2007) (Fig. 1a). The unusual deoxy amino sugar portion, DATDH, was further identified as bacillosamine (Hartley et al.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-016-2258-1) contains supplementary material, which is available to authorized users.

C.-Y. Liu · C.-C. Weng · C.-H. Lin ·
K.-K. T. Mong · Y.-K. Li (✉)
Department of Applied Chemistry, National Chiao Tung
University, Hsinchu 30010, Taiwan
e-mail: ykl@cc.nctu.edu.tw

C.-Y. Yang
Institute of Molecular Biology, National Chung Hsing
University, Taichung 40227, Taiwan

Fig. 1 **a** Structure of pilus sugar on *Neisseria* species. **b** The synthetic bacillosamine derivative (compound 1) used as a probe for scFv screening of a phage display library



2011), which has not been identified on human cell surfaces to date. This rare *O*-linked bacillosamine structure is a good candidate for developing a bio-recognition strategy against *Neisseria* species, including pathogenic strains.

A single-chain antibody generated from fused variable domains (scFv) is a versatile tool (Raag and Whitlow 1995). Its low immunogenicity due to a lack of the Fc region makes it an alternative therapeutic agent in comparison to full-length monoclonal antibodies for numerous applications. In addition, an scFv can be cloned and expressed in bacteria (e.g., *Escherichia coli*) and can easily be produced in large quantities in a cost-effective manner. To minimize possible cross-interactions due to galactosyl moieties of the pilus sugar, we propose to use only the bacillosamine portion (compound 1, Fig. 1b) as the probe for scFv screening.

Materials and methods

Plasmids, strains and reagents

All reagents were commercially available and were of reagent grade or better. All restriction enzymes and DNA modification enzymes were of molecular biology grade. XL1-Blue, BL21 (DE3) and T4 ligase were purchased from Yeastern Biotech Company, Taiwan. The pET-22b vector was purchased from Novagen. A phage display vector (pIT2), helper phage and phage library were obtained as a gift from Prof. Hiroshi Ueda (Chemical Resources Laboratory, Tokyo Institute of Technology, Japan). *Neisseria meningitidis*, *N. sicca*,

N. subflava and *Streptococcus pneumoniae* were purchased from the Bioresource Collection and Research Center, Taiwan. *Neisseria gonorrhoeae* was obtained as a gift from the lab of Prof. Chiou-Ying Yang (Institute of Molecular Biology, National Chung Hsing University, Taiwan). Mouse anti-M13 HRP-conjugated monoclonal antibody was purchased from GE Healthcare Life Science.

Preparation of the screening target: bacillosamine moiety

To mimic the structure of the bacillosamine moiety on pilus sugar, we blocked its C-3 hydroxyl group and integrated a linker containing the azido group to form a bacillosamine derivative, namely compound 1 (Fig. 1b), for further screening. Our synthesis began with *tert*-butyldiphenylsilyl 2,4-diazido-2,4,6-trideoxy-3-*O*-naphthylmethyl- β -D-glucopyranoside, which is derived from D-galactosamine (Amin et al. 2006). The sugar portion of compound 1 was then synthesized and further incorporated with an azido group for a click reaction with alkyne-modified bovine serum albumin (BSA). Details of the procedure are presented in the Supporting Information.

Biopanning

Six rounds of panning were performed on compound 1-BSA. Panning of our phage scFv library was performed against compound 1-BSA, which was coated on microtitre plates at 5 μ g/well for 1 h at 37 °C. After five rinses with PBS, the immune tube was blocked with 3% (w/v) BSA in PBS for 1 h at

37 °C. The phage library was then added and incubated for 2 h at 37 °C. Unbound phage was removed by rinsing five times with PBST (PBS containing 0.1% v/v Tween 20). The compound 1-binding phage was eluted from the immune tube with 100 µl trypsin. The eluted phage was then used to infect *E. coli* DH5α for 1 h at 37 °C. Subsequently, the enriched phagemids were incubated with KM13 helper phage for 1 h at 37 °C, followed by centrifuging and resuspending in fresh 2xYT-AK medium (2xYT medium containing 100 mg ampicillin/ml, 50 mg kanamycin/ml and 1% glucose). Amplified phage was used for the next round of panning.

Screening of compound 1-specific clones by monoclonal phage ELISA (see also Supporting Information)

After six rounds of panning, 96 individual clones from round 6 of panning were randomly selected to assay the specificity by ELISA. Single colonies were cultured in 200 µl 2xYT-A medium (containing 100 mg ampicillin/ml and 1% glucose) overnight at 37 °C using 96-microwell plates. Individual phage clones were produced by the addition of KM13 helper phages. Microtitre plate wells were coated with 100 µl 10 µg compound 1-BSA/ml in PBS. After 1 h incubation at 37 °C, each well was blocked with 200 µl 3% BSA and incubated at 37 °C. After 1 h, wells were washed five times with PBST (0.1% Tween 20), and then the individual phage solution was added and incubated for 1 h at 37 °C. After five washes with PBST, HRP-conjugated anti-M13 antibody (1:3000 v/v) (GE Healthcare, USA) was added and incubated for 1 h at 37 °C. Following washing, TMB (Thermo Fisher Scientific, Germany) was added. The developing reaction was stopped by the addition of 1 M H₂SO₄, and the absorbance was measured at 450 nm.

Recombinant antibody engineering, prokaryotic cell expression and purification

The highest affinity phage clones were selected for further analysis. The scFv fragment was digested from the pIT2 vector, ligated into the *NcoI/NotI* expression vector pET22b (Progen Biotechnik GmbH, Heidelberg, Germany), and fused to the C-terminal His-tag sequence. *Escherichia coli* BL21 (DE3) was transformed with vector pET22b and grown in 5 ml 2xYT-

A medium (containing 100 mg ampicillin/ml and 1% glucose) at 37 °C to an OD₆₀₀ of 0.4–0.6, followed by induction with 1 mM IPTG and incubation for 16 h at 16 °C. Due to the *pelB* leader signal sequence in the vector, soluble scFv was expressed as a periplasmic protein. After collection according to Sockolosky and Szoka (2013), functional scFv produced in the supernatant was purified using Ni–NTA metal affinity chromatography. The scFv was eluted with 300 mM imidazole and dialyzed against PBS for 24 h at 4 °C.

Immunofluorescence staining and confocal laser scanning microscopy

To investigate the identification of the selected scFv antibody with *Neisseria* species, confocal microscopy was performed on *N. gonorrhoeae*, *N. meningitidis*, *N. sicca* and *N. subflava* and two negative controls (*S. pneumoniae* and *E. coli* XL1B). Bacteria were dried on a glass coverslip and then incubated with FITC-labelled D7 scFv antibodies diluted 1:50 in PBS. After a 15 min incubation, the slides were washed five times in PBST (PBS containing 0.1% Tween 20) and mounted on microscope slides with ProLong Gold Antifade Reagent (Thermo Fisher Scientific, Germany). The distribution of FITC-labelled scFv antibodies was analysed using a confocal scanning laser Leica TCS SP5 II (Leica, Germany). Fluorophores were excited with an argon laser (488 nm) for FITC.

Preparation of nano-gold-labelled scFv solution

Colloidal nano-gold solutions were prepared by citrate reduction of HAuCl₄ (Storhoff et al. 1998) and were then filtered through a 0.45 µm nylon filter. After incubation with D7 scFv at 0.5 mg/ml for 16 h, the solution was incubated with 10% NaCl for 1 h, followed by centrifugation for 30 min at ~10,000×g to remove excess reagents. Following removal of the supernatant, the red precipitate was washed with 5 ml PBS buffer, recentrifuged, and resuspended in 5 ml PBS buffer.

Results and discussion

To generate the scFv, we used the compound 1-coated BSA as a probe for scFv screening from the phage display library. After six rounds of panning, 96

individual clones were randomly selected to test the specificity with ELISA. The highest affinity phage clone D7 was selected; the corresponding DNA of the scFv was further inserted into pET22b and overexpressed in *E. coli* BL21 (DE3). The His-tagged scFv was purified from a periplasmic extract using Ni-NTA column chromatography. SDS-PAGE analysis of D7 scFv revealed a band at ~29 kDa (Fig. 2a), which is consistent with the expected protein size.

The binding affinities of the purified D7 scFv toward *Neisseria* pilus sugar and compound 1 were confirmed by ELISA. As shown in Fig. 2b, scFv binds to compound 1-coated BSA and *Neisseria* pili with a significantly higher affinity (~fourfold) compared to that of BSA only. A competitive ELISA was further performed. The binding of D7 scFv (10 µg/ml in PBS) on immobilized *Neisseria* pili (30 µg/ml in PBS) isolated from *N. meningitidis* (Huttunen et al. 2016) was measured in the presence and absence of compound 1 (10 µg/ml in PBS). The signal significantly decreased by ~sixfold (after subtracting the value for BSA only) in the presence of compound 1. This finding indicates that D7 scFv can specifically recognize the neisserial pili through the bacillosamine moiety.

The bio-recognition power of D7 scFv for *N. gonorrhoeae*, *N. meningitidis*, *N. sicca* and *N. subflava*, was tested through ELISA and immunostaining. Due to the unique O-linked bacillosamine structure present on the cell surface of *Neisseria* species, we hypothesized that our selected scFv antibody would be able to recognize all listed bacteria. Based on the

ELISA result (Fig. 3a), scFv binds to *Neisseria* species with significantly increased affinity (~sixfold) compared with *S. pneumoniae* and *E. coli* (after subtracting the value for BSA only) presumably due to the absence of bacillosamine on these control strains. Similar results were found in the immunostaining experiment. Staining with FITC-modified D7 scFv revealed colocalization of the scFv antibody against *Neisseria* species (Fig. 3b, four *Neisseria* species). In contrast, no immunoreactivity was observed for staining with FITC-modified D7 scFv against *S. pneumoniae* and *E. coli* (Fig. 3b, controls). These results indicate that D7 scFv is feasible for the specific detection of *Neisseria* species via the recognition of a bacillosamine moiety on pilus sugar.

A gold nanoparticle immunological assay was also applied to detect *Neisseria* species. After conjugation with D7 scFv, the colloidal gold nanoparticle-labelled scFv solution was incubated with *Neisseria* species, *S. pneumoniae* and *E. coli* (10^8 CFU/ml) to assess its specificity. In the presence of *Neisseria* species, the colloid turns blue and thus forms aggregates (Fig. 4a), resulting in a decreased absorbance at 520 nm. The effective detection was estimated by incubating gold nanoparticle-labelled scFv with various concentrations of *N. meningitidis*. As shown in Fig. 4b, *N. meningitidis* could be detected as low as 10^6 CFU/ml.

We report, herein, the production of *Neisseria* pilus sugar-specific scFv via the screening of a phage display library. The scFv screening was based on only the bacillosamine portion, a partial structure of pilus

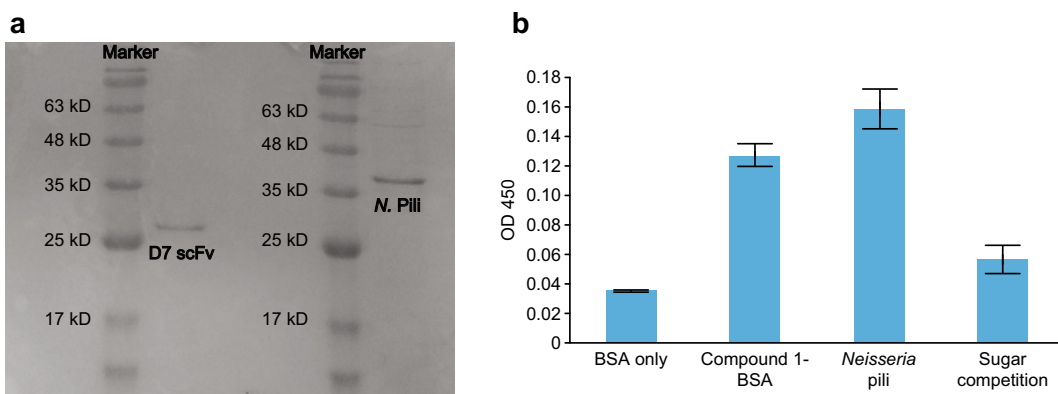


Fig. 2 a SDS-PAGE of purified D7 scFv and neisserial pili from *N. meningitidis*. b Interactions of the purified D7 scFv with compound 1 and neisserial pilus sugar analysed by ELISA (in triplicate)

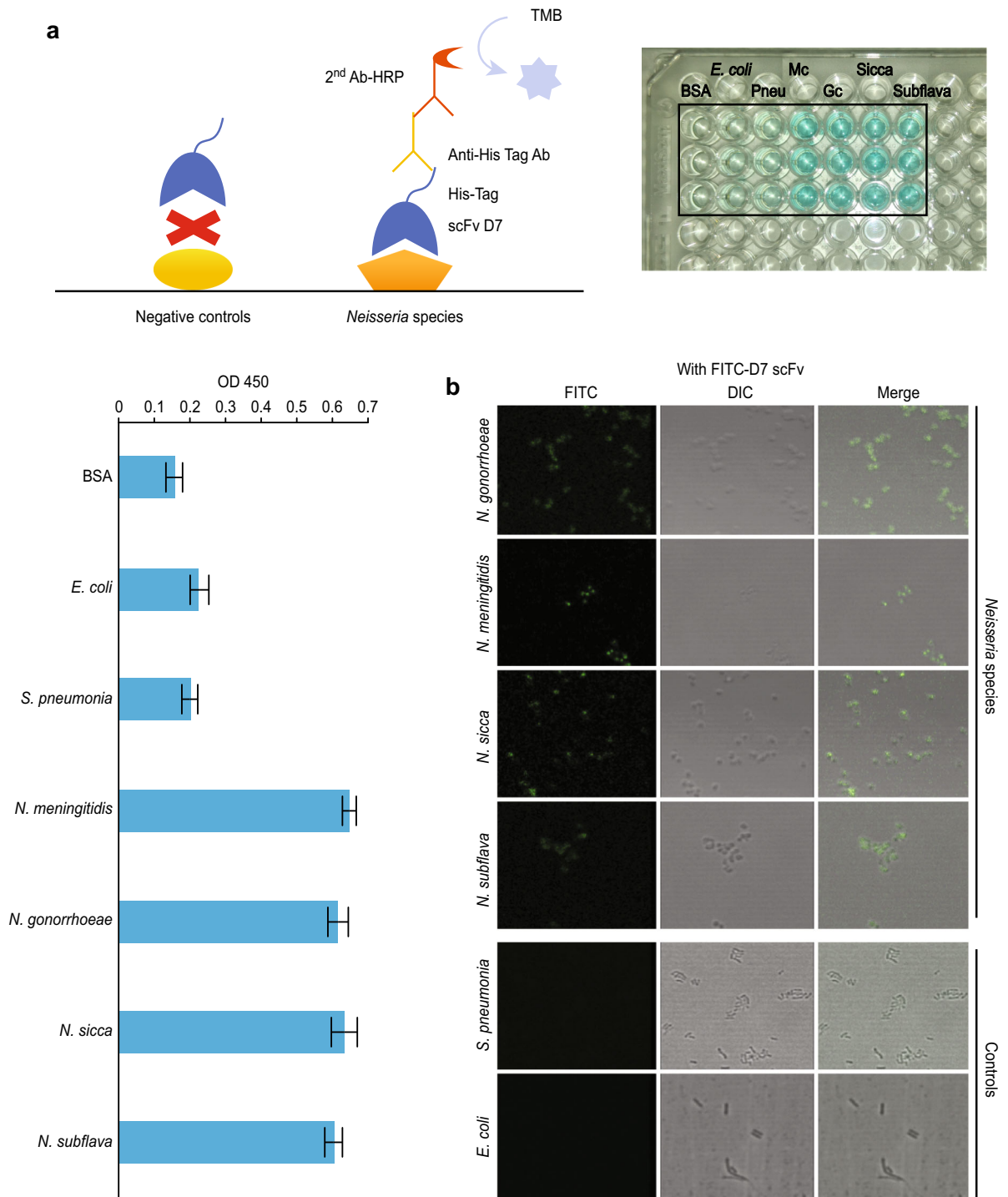


Fig. 3 a The specificity test of the D7 scFv against *Neisseria* species and two controls (*S. pneumoniae* and *E. coli*) as analysed by ELISA (in triplicate). **b** Immunostaining and imaging of four

Neisseria species and two controls; DIC differential interference contrast. Scale bar 10 μ m

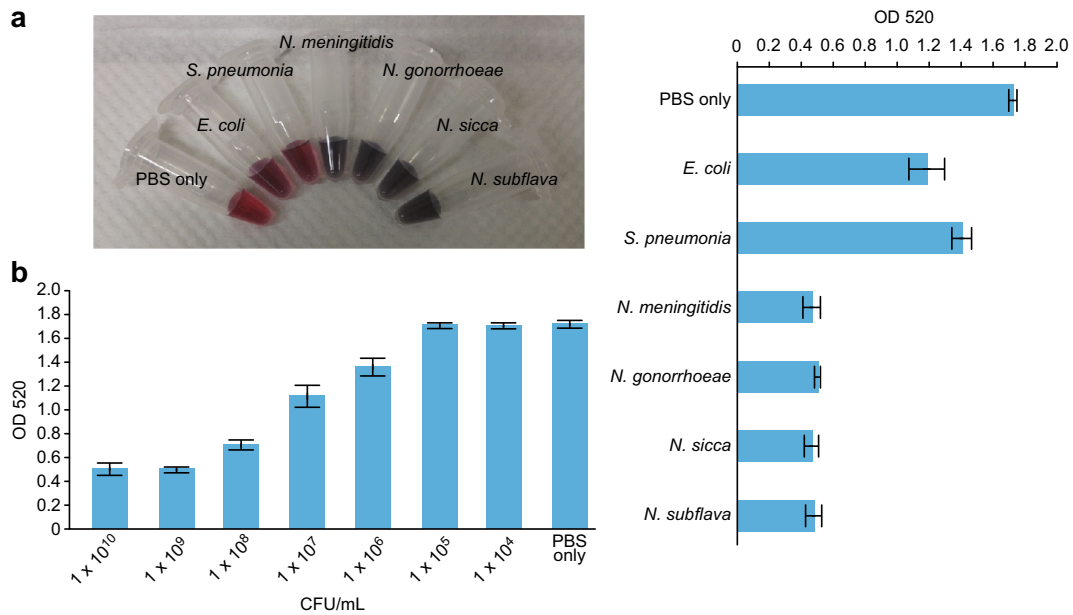


Fig. 4 **a** Images of gold nanoparticle-labelled scFv mixed with *Neisseria* species and controls and the corresponding absorbance at 520 nm. **b** The detection limit of gold nanoparticle-labelled scFv with *N. meningitidis*

sugar. The bio-recognition power of the screened scFv was confirmed via an ELISA test, immunostaining, imaging analysis, and detection via gold nanoparticles, with promising results. As an alternative to Gram staining, culture and nucleic acid amplification tests, the recombinant scFv is suitable for developing a convenient and rapid on-site detection kit for *Neisseria* infection.

Acknowledgements This work was financially supported by Grants from the Ministry of Science and Technology, Taiwan (MOST 105-2113-M-009-005), and the Center for interdisciplinary science, National Chiao Tung University.

Supporting information Supplementary Methods—Compound 1 synthesis and BSA modification. Screening of compound 1-specific clones by monoclonal phage ELISA. Bacterial strain and culture conditions. Isolation of *Neisseria* pili. Characterization of purified recombinant anti-compound 1 scFv antibody. Immunofluorescence staining and confocal laser scanning microscopy. Nano-gold particle immunological assay. Supplementary Scheme 1—Reagents and conditions of compound 1 synthesis. Supplementary Scheme 2—Installation of compound 1 to BSA. Supplementary Fig. 1—Procedure used to select compound 1-specific recombinant scFv antibodies. Supplementary Fig. 2—Approach used to determine scFv binding activity to compound 1. Supplementary Fig. 3—Immunofluorescence of *Neisseria* species. (Incubation with fluorescein were considered as the background.)

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

References

- Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NB, Hamid M (2012) scFv antibody: principles and clinical application. *Clin Dev Immunol* 2012:980250
- Amin MN, Ishiwata A, Ito Y (2006) Synthesis of asparagine-linked bacillosamine. *Carbohydr Res* 341:1922–1929
- Aral SO, Mosher WD, Cates W (1991) Self-reported pelvic inflammatory disease in the United States, 1988. *J Am Med Assoc* 266:2570–2573
- Carbonnelle E, Hill DJ, Morand P, Griffiths NJ, Bourdoulous S, Murillo I, Nassif X, Virji M (2009) Meningococcal interactions with the host. *Vaccine* 27:B78–B89
- Hartley MD, Morrison MJ, Aas FE, Borud B, Koomey M, Imperiali B (2011) Biochemical characterization of the O-linked glycosylation pathway in *Neisseria gonorrhoeae* responsible for biosynthesis of protein glycans containing *N,N'*-diacetyl bacillosamine. *Biochemistry* 50:4936–4948
- Huttunen S, Toivanen M, Liu C, Tikkanen-Kaukanen C (2016) Novel anti-infective potential of salvianolic acid B against human serious pathogen *Neisseria meningitidis*. *BMC Res Notes* 9:25–30
- Power PM, Ku SC, Rutter K, Warren MJ, Linnios EA, Tapsall JW, Jennings MP (2007) The phase-variable Allele of the

- pilus glycosylation Gene *pglA* is not strongly associated with strains of *Neisseria gonorrhoeae* isolated from patients with disseminated gonococcal infection. *Infect Immun* 75:3202–3204
- Raag R, Whitlow M (1995) Single-chain Fvs. *FASEB J* 9:73–80
- Sokolosky JT, Szoka FC (2013) Periplasmic production via the pET expression system of soluble, bioactive human growth hormone. *Prot Expr Purif* 87:129–135
- Storhoff JJ, Elghanian R, Mucic RC, Mirkin CA, Letsinger RL (1998) One-pot colorimetric differentiation of polynucleotides with single base imperfections using gold nanoparticle probes. *J Am Chem Soc* 120:1959–1964