NRC Publications Archive Archives des publications du CNRC

Single-Domain Antibody Bioconjugated Near-IR Quantum Dots for Targeted Cellular Imaging of Pancreatic Cancer

Zaman, Md. Badruz; Baral, Toya Nath; Jakubek, Zygmunt J.; Zhang, Jianbing; Wu, Xiaohua; Lai, Edward; Whitfield, Dennis; Yu, Kui

This publication could be one of several versions: author's original, accepted manuscript or the publisher's version. / La version de cette publication peut être l'une des suivantes : la version prépublication de l'auteur, la version acceptée du manuscrit ou la version de l'éditeur.

For the publisher's version, please access the DOI link below. / Pour consulter la version de l'éditeur, utilisez le lien DOI ci-dessous.

Publisher's version / Version de l'éditeur:

<https://doi.org/10.1166/jnn.2011.4167>

Journal of Nanoscience and Nanotechnology, 11, 5, pp. 3757-3763, 2011-01-01

NRC Publications Record / Notice d'Archives des publications de CNRC:

<https://nrc-publications.canada.ca/eng/view/object/?id=bc8a1ff6-0b8b-4268-a243-69ff2cef5943>

<https://publications-cnrc.canada.ca/fra/voir/objet/?id=bc8a1ff6-0b8b-4268-a243-69ff2cef5943>

Access and use of this website and the material on it are subject to the Terms and Conditions set forth at

<https://nrc-publications.canada.ca/eng/copyright>

READ THESE TERMS AND CONDITIONS CAREFULLY BEFORE USING THIS WEBSITE.

L'accès à ce site Web et l'utilisation de son contenu sont assujettis aux conditions présentées dans le site

<https://publications-cnrc.canada.ca/fra/droits>

LISEZ CES CONDITIONS ATTENTIVEMENT AVANT D'UTILISER CE SITE WEB.

Questions? Contact the NRC Publications Archive team at

PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca. If you wish to email the authors directly, please see the first page of the publication for their contact information.

Vous avez des questions? Nous pouvons vous aider. Pour communiquer directement avec un auteur, consultez la première page de la revue dans laquelle son article a été publié afin de trouver ses coordonnées. Si vous n'arrivez pas à les repérer, communiquez avec nous à PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca.

Single-Domain Antibody Bioconjugated Near-IR Quantum Dots for Targeted Cellular Imaging of Pancreatic Cancer

Md. Badruz Zaman^{1,2}, Toya Nath Baral², Zygmunt J. Jakubek¹, Jianbing Zhang²,
Xiaohua Wu³, Edward Lai⁴, Dennis Whitfield^{2,*}, and Kui Yu^{1,*}

¹Steacie Institute for Molecular Sciences, ²Institute for Biological Sciences, ³Institute for Microstructural Sciences,
National Research Council of Canada, 100 Sussex Drive, Ottawa, Ontario K1A 0R6, Canada

⁴Department of Chemistry, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada

Successful targeted imaging of BxPC3 human pancreatic cancer cells is feasible with near-IR CdTeSe/CdS quantum dots (QDs) functionalized with single-domain antibody (sdAb) 2A3. For specific targeting, sdAbs are superior to conventional antibodies, especially in terms of stability, aggregation, and production cost. The bright CdTeSe/CdS QDs were synthesized to emit in the diagnostic window of 650–900 nm with a narrow emission band. 2A3 was derived from llama and is small in size of ~13 kDa, but with fully-functional recognition to the target carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), a possible biomarker as a therapeutic target of pancreatic cancer. For compelling imaging, optical may be the most sensible among the various imaging modalities, regarding the sensitivity and cost. This first report on sdAb-conjugated near-IR QDs with high signal to background sensitivity for targeted cellular imaging brings insights into the development of optical molecular imaging for early stage cancer diagnosis.

Keywords: Photoluminescent Near-IR Quantum Dots (QDs), Single-Domain Antibody, *In Vitro* Targeted Cellular Imaging, Colloidal Nanocrystals, Pancreatic and Breast Cancer.

1. INTRODUCTION

Photoluminescent (PL) colloidal semiconductor quantum dots (QDs) have attracted significant attention in medical applications involving imaging and diagnostics.^{1–4} As a novel class of contrast agents for optical molecular imaging in bio-oriented applications, there is an outstanding demand for the QDs to exhibit emission profiles in the near-IR diagnostic window of 650–900 nm, to avoid the absorption of water and bio-molecules including hemoglobin and proteins as well as to stay away from autofluorescence which is in the range of visible light (400–700 nm).^{5–7} Alloyed cadmium telluride selenium (CdTeSe) QDs are good candidates for such near-IR imaging probes.^{8–11} We have further developed such QDs for aqueous uses by further CdS shell coating and ligand exchange chemistries.

The occurrence of pancreatic cancer has ascended over the years, and it has been documented as the fourth leading cause among cancer-related deaths in the United States,

for both men and women.^{12–14} It is extremely difficult to diagnose pancreatic cancer in its early stages. Current detection methods based on non-invasive imaging counts on the presence of morphological transformation (without targeting). Meanwhile, pancreatic tumors are resistant to radiotherapy and practically all chemotherapies except gemcitabine, which assists to extend the survival by only a few weeks.¹⁵ The mean survival is about six months, and the overall five-year survival rate is less than 5%. Accordingly, pancreatic cancer stands as one of the most difficult malignancies/diseases for diagnostics and therapeutics, while optical molecular imaging has the enormous potential to advance our capability for early detection.

The discovery of functional heavy-chain antibodies (HCAs) in camelidae led to the successful engineering of single-domain antibodies (sdAbs).^{16,17} These sdAbs of ~13 kDa are small proteins but exhibit high affinity,¹⁸ good refolding capabilities (after chemical-induced denaturation), and excellent biophysical stability (including their resistance to many unfavorable conditions such as chemical detergents, extreme pH, and to certain extent heat denaturation and proteolysis).¹⁹ Some sdAbs (and

*Authors to whom correspondence should be addressed.

their derivatives) have been scrutinized as powerful tumor imaging and therapeutic reagents.^{20,21} Accordingly, it is significant to develop a high-affinity sdAb targeting pancreatic tumors. 2A3, a sdAb with high affinity against carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) was isolated successfully from an immunized llama library.^{22,23} 2A3 was shown to bind strongly to the BxPC3 human pancreatic cell line cells. The detailed isolation and characterization of 2A3 can be found elsewhere.²² Thus, the combination of sdAb 2A3 for targeting pancreatic tumors that express CEACAM6 and the water dispersed CdTeSe/CdS near-IR QDs appeared to us to be an important target. This manuscript describes the preparation and properties of this combination.

2. EXPERIMENTAL DETAILS

2.1. Synthesis of CdTeSe and CdTeSe/CdS Quantum Dots

This manuscript addresses our targeted optical imaging upon the discovery of specific sdAb-antigen recognition which can lead to the development of optical molecular imaging aiming at the early-stage detection of pancreatic cancer. The contrast agent used was high-quality CdTeSe/CdS QDs exhibiting rich absorption substructures, high quantum yield and narrow emission bandwidth. For the synthesis carried out in the present study, all chemicals used were purchased from Sigma-Aldrich (or otherwise specified) and were used as received. They were cadmium Oxide CdO (99.99%), selenium powder (Se, 99%), tellurium powder (Te, 99%), trioctylphosphine (TOP, tech. 90%), tri-*n*-octylphosphine oxide (TOPO, tech. 90%), hexadecylamine (HDA, 90%), 1-octadecene (ODE, 90%), mercaptoundecanoic acid (MUA, 98%), DL-lysine (98%), (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide) (EDC), *N,N'*-dicyclohexylcarbodiimide (DCC, 99%), tetracyclphosphonic acid (TDPA, 98%, Alfa Aesar), and hexamethyldisilthiane ((TMS)₂S, Fluka). All reactions were conducted under inert atmosphere using Schlenk techniques.

For the synthesis of the CdTeSe QDs via a hot-injection approach, a three-neck round-bottom flask was equipped with a vacuum line and gas inlet, a thermometer and magnetic stirrer; 51.2 mg (0.4 mmol) CdO, 225.0 mg (0.8 mmol) TDPA, 930.0 mg TOPO, 920.0 mg HAD, and 1.4 g TOP were loaded. A SeTOP solution (with 7.4 mg (0.1 mmol) Se and 0.4 g TOP) was sonicated at ~40 °C for 30 min and a TeTOP solution (with 12.8 mg (0.1 mmol) Te and 0.7 g TOP) for 120 min. The two solutions were mixed and then added swiftly into the Cd precursor solution at 300 °C. The growth of the CdTeSe QDs was conducted at 270 °C for 30 min. The synthesis of the core/shell CdTeSe/CdS QDs was similar to that of our core/shell CdSe/ZnS QDs but with modification.⁴ Stock solutions of

0.1 M CdO/TOP and 0.1 M S/TOP were prepared separately and mixed at room temperature before the addition to the CdTeSe core at 140 °C. The growth was at ~155 °C for ~ three hours and then ~110 °C for ~16 hours.

2.2. Conjugation of sdAbs with CdTeSe/CdS QDs

The CdTeSe/CdS QDs were bio-conjugated with sdAbs 2A3 and EG2, with the same chemistry. Scheme 1 outlines the synthesis of the 2A3-QD bio-conjugate. Note that camelid sdAb EG2 was engineered to bind to a tumor marker protein, epidermal growth factor receptor (EGFR).⁴ For a typical synthesis, a 500 μ l QD stock solution ($\sim 10^{-4}$ mmol) was mixed with a 300 μ l PBS solution consisting of sdAb 2A3 or EG2 (1.5 mg/ml sdAb/PBS); 2A3 or EG2 is estimated to be $\sim 10^{-4}$ mmol based on a molecular weight of ~15 K. The bio-conjugation procedure was the known EDC method,²⁴ using (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide). 22 μ l

Scheme 1. Synthesis of 2A3-conjugated QDs. MUA = mercaptoundecanoic acid, DCC = *N,N'*-dicyclohexylcarbodiimide, EDC = 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide, and NHS = *N*-hydroxysuccinimide. First, the core/shell CdTeSe/CdS QDs are non-water-soluble, and ligand exchange was performed with MUA, together with subsequent cross-linking with lysine. Note, only one of several possible connections with lysine is shown for clarity. The resulting QDs are water-soluble with their surface ligands comprising carboxyl groups and amino groups. Afterwards, the carboxyl groups on the QDs were activated as NHS esters with EDC, and then reacted with the amine groups on sdAb 2A3.

($\sim 10^{-3}$ mmol) of EDC and 20 μl ($\sim 10^{-3}$ mmol) of N-hydroxysuccinimide (NHS) were added at room temperature with vigorous stirring for a maximum of one hour. The EDC stock solution was prepared with 8.1 mg EDC in 100 μl distilled water, while the NHS stock solution was prepared with 5.6 mg NHS in 200 μl distilled water. The resulting bio-conjugates, un-purified and purified, were used for immunocytochemistry. The purification was carried out with a 30 000 MWCO centrifuge filter device. Therefore, with the feed molar ratio of 1QD : 1sdAb, the purified bio-conjugates should have a molecular weight greater than 30 kDa so they were retained on the filter used.

2.3. Cancer Cell Imaging

The specific and compelling targeting of sdAb-conjugated near-IR QDs to cancer cells is demonstrated as proof of principle, with the resulting 2A3-conjugated CdTeSe/CdS QDs on *in vitro* grown BxPC3 human pancreatic cancer cells, and the resulting EG2-conjugated CdTeSe/CdS QDs on *in vitro* grown MDA-MB-468 human breast cancer cells. The tumor cell lines were obtained from American Type Culture Collection (ATCC) and cultured in RPMI (Gibco) medium supplemented with 10% fetal bovine serum (Roche) at 37 °C, 5% CO₂ in 6 well plates (Falcon) with a sterile cover slip in each well. When the cells were about 70% confluence, they were fixed on the cover

slips by using 10% formaldehyde in PBS for 10 minutes. The cells were washed with PBS and then incubated with 2% skimmed milk in PBS for 2 hours. After washing with PBS, cells were incubated with the core/shell QDs alone or sdAb-conjugated QDs (purified or unpurified) for one hour and washed three times with PBS. Finally the cover slips were mounted on a microscopic slide using the Prolong Antifade Kit (Molecular Probes) and were imaged under the epifluorescence microscope (IX-81, Olympus) equipped with a 150x, 1.45 NA oil immersion objective lens (UAPON 150XOTIRF, Olympus) and a back-illuminated EMCCD camera (Cascade 512B, Roper Scientific). The near-IR emitters are excellent providing high signal-to-background sensitivity in targeted cellular imaging. Note that the sdAbs 2A3 and EG2 can be used as both targeting and therapeutic reagents.^{20, 21} To the best of our knowledge, the present work is the first on the targeted imaging of cancer cells with sdAb-conjugated CdTeSe/CdS QDs.

3. RESULTS AND DISCUSSION

Figure 1 (left) shows the absorption and emission of our CdTeSe and corresponding CdTeSe/CdS QDs dispersed in toluene. It is worthy of notice that the QDs with narrow size distribution exhibit rich absorption substructures. Figure 1 (right) shows the absorption and emission

Fig. 1. (left) The optical spectra of the emission (Curve 1, peaking at ca. 710 nm with excitation wavelength of 500 nm) and absorption (Curve 3, peaking at ca. 699 nm) of our purified CdTeSe core QDs in toluene, and of the emission (Curve 2, peaking at ca. 750 nm) and absorption (Curve 4, peaking at ca. 723 nm) of our purified CdTeSe/CdS core/shell QDs in toluene. (right) The emission spectra of the CdTeSe/CdS QDs transferred into double-distilled water before conjugation, 2A3-conjugated CdTeSe/CdS QDs (Curve 2), and EG2-conjugated CdTeSe/CdS QDs (Curve 3). The emission spectra were acquired with the similar amount of CdTeSe/CdS QDs, 2A3-conjugated CdTeSe/CdS QDs, and EG2-conjugated CdTeSe/CdS QDs with 500 nm excitation. Their QY in 18.3 M Ω -cm Milli-Q water was estimated to be ca. 23%, 20%, and 7%, respectively. The PL FWHM was $\leq$ 50 nm. The absorption spectrum of the CdTeSe/CdS QDs transferred into double-distilled water is Curve 4 before the bio-conjugation. It is the same amount of CdTeSe/CdS QDs used for the two conjugation experiments.

Fig. 2. A typical STEM image of our water-soluble CdTeSe/CdS QDs prepared (shown in Fig. 1 right (Curves 1 and 4)).

of the CdTeSe/CdS QDs, 2A3-conjugated CdTeSe/CdS QDs, and EG2-conjugated CdTeSe/CdS QDs dispersed in mill-Q water with a similar QD concentration. The UV absorption spectrum was acquired from the CdTeSe/CdS

QDs. Here, to transfer the core/shell QDs (shown in Fig. 1 (left)) into aqueous environment, their surface ligands were exchanged with mercaptoundecanoic acid (MUA) and subsequently cross-linked by lysine,^{10,11} as shown in Scheme 1. The resulting CdTeSe/CdS QDs with their surface ligands comprising carboxyl groups and amino groups are water-soluble, highly fluorescent, and stable against flocculation. These CdTeSe/CdS QDs in water exhibited a quantum yield of ca. 23% (excited at 500 nm). The sdAb-conjugated QDs exhibited relatively low PL efficiency as compared to the non-conjugated QDs in water, but little change in the emission peak position and width (which can be expressed as full width at half maximum (FWHM)). For the water-soluble CdTeSe/CdS QDs (shown in Fig. 1 right Curves 1 and 4), see Figure 2 for a typical Scanning Transmission Electron Microscopy (STEM) image. No significant aggregation was observed under the microscopy, while the corresponding core/shell QD water dispersion, kept at room temperature, exhibited little visible precipitation within 3 months of monitor. Figure 3 shows the no cell control fluorescence images that demonstrate a 10,000x intensity increase versus background.

To target BxPC3 a human pancreatic cancer cell line which over expresses CEACAM6²⁵ and MDA-MB-468,

Fig. 3. (top) Fluorescence images of an edge of a drop of the water-soluble CdTeSe/CdS QDs placed on a cover slip, (shown in Fig. 1 right (Curves 1 and 4), emitting at ~ 762 nm), excited at 488 nm and recorded with the fluorescence collected in the 750–822 nm (left) and 514–558 nm (right) ranges. The two images are $54.6 \times 54.6 \mu\text{m}^2$. (bottom) Line intensity profiles of the two fluorescence images (top) measured along the dashed yellow lines indicated. The intensity line profiles are in units of grey level.

a human breast cancer cell line which over expresses EGFR,²⁶ the 2A3-conjugates QDs (shown in Fig. 1 right-2) and EG2-conjugates QDs (shown in Fig. 1 right-3) were used for *in vitro* studies, respectively. The images of the pancreatic and breast cancer cells are shown in Figures 4 and 5, respectively. The images were recorded with the excitation wavelength of 488 nm; also, bandpass

Fig. 4. Representative fluorescence images BxPC3 human pancreatic cancer cells treated with 2A3-conjugated CdTeSe/CdS QDs (a1, a2), unconjugated CdTeSe/CdS QDs (b1, b2), un-treated (c1, c2), and treated with unpurified bio-conjugates (d1, d2). The excitation was at 488 nm and recorded with fluorescence collected in the 514–558 nm (left panel with false-colored green) and 750–822 nm (right panel with false-colored red) ranges. To facilitate intensity comparison, the same brightness and contrast settings were applied to the images recorded with the same band-pass filter. All images are $54.6 \times 54.6 \mu\text{m}^2$.

Fig. 5. Representative fluorescence images MDA-MB-468 human breast cancer cells treated with EG2-conjugated CdTeSe/CdS QDs (a1, a2), unconjugated CdTeSe/CdS QDs (b1, b2), un-treated (c1, c2), and treated with unpurified bio-conjugates (d1, d2). The excitation was at 488 nm and recorded with fluorescence collected in the 514–558 nm (left panel with false-colored green) and 750–822 nm (right panel with false-colored red) ranges. To facilitate intensity comparison, the same brightness and contrast settings were applied to the images recorded with the same bandpass filter. All images are $40.4 \times 40.4 \mu\text{m}^2$.

filters either 514–588 nm or 750–822 nm were used for the fluorescence detection. To facilitate intensity comparison, the same laser power, exposure time, and camera settings were used to record; the same brightness and contrast settings were applied to display the images shown in

Fig. 6. Fluorescence intensity histograms of the 750–822 nm fluorescence images of the BxPC3 human pancreatic cancer cells, (a2) and (c2) shown in Figure 4, represented by thick and thin lines, respectively. The image of the unlabelled cells (Fig. 4(c2)) shows predominantly low intensity background pixels (below 10000); while in the image of the labelled cells (Fig. 4(a2)), much higher intensities dominate. The y axis represents mean pixel counts obtained from the corresponding microscopic images.

Figures 4 and 5 obtained with each of the two bandpass filters.

Figures 4 and 5 demonstrate that the CdTeSe/CdS QDs without sdAbs are not targeted and are barely detectable indicating that non-specific adsorption is minimal. While green autofluorescence intensity is comparable in both cancer cell images (as shown in the left panels of Figs. 4 and 5 with false-coloured green), near-IR fluorescence is significantly stronger in the images of Figures 4(a2) and 5(a2) (with false-coloured red), due to the binding of the

Fig. 7. Fluorescence intensity histograms of the 750–822 nm fluorescence images of the MDA-MB-468 human breast cancer cells, (a2) and (c2) shown in Figure 5, represented by thick and thin lines, respectively. The image of the unlabelled cell (Fig. 5(c2)) shows predominantly low intensity background pixels (below ~10000, right y axis); while in the image of the labelled cell (Fig. 5(a2)), much higher intensities (left y axis) are present. The two y axes represent mean pixel counts obtained from the corresponding microscopic images.

conjugated CdTeSe/CdS QDs on the surface of the cancer cells. The intensity difference of the near-IR fluorescence between the labelled (Figs. 4(a2) and 5(a2)) and unlabelled cells (Figs. 4(c2) and 5(c2)) is also evident by examining histograms, as shown in Figures 6 and 7, respectively. Only the labelled cells show intensities (in gray levels) drastically above the background peak. Similarly in the presence of non-conjugated sdAb the fluorescence intensity is diminished (Figs. 4(d2) and 5(d2)) consistent with targeting by the 2A3-CECAM6 interaction. Thus, our 2A3-conjugated near-IR QDs have great potential to detect CECAM6 for the early-stage diagnostics of pancreatic cancer. It is noteworthy that the surface chemistry of the conjugate formation can be easily applied to other bioconjugation, such as leading to the formation of EG2-conjugated near-IR QDs which can be used for the early-stage diagnostics of cancers that express EGFR such as breast cancer.

4. CONCLUSIONS

We have successfully developed 2A3 single-domain antibody and explored targeted imaging of BxPC3 human pancreatic cancer cells with 2A3-conjugated near-IR CdTeSe/CdS QDs. Our successful targeted imaging, with high signal-to-background sensitivity and targeting specificity, demonstrates that sdAb-conjugated near-IR QDs enable great potential in the development of optical molecular imaging aiming at early-stage cancer detection. Further optimization on the synthesis of the targeted contrast agent is in progress, such as to increase the shell thickness of CdTeSe/CdS QDs, to improve colloidal stability in water via other hydrophilic compounds, such as the combination of PEGylation and sdAb conjugation.

Acknowledgments: This work is supported by NRC-Taiwan Initiative Program with two projects entitled “Molecular Imaging for Cancer Diagnostics: Nanobodies meet Nanoparticles” and “Nanoprobe enabled multi-scale multi-modal molecular imaging for cancers by combined higher harmonics photoacoustics and optical-MicroCT tomography.” The authors are grateful to Madam Lorena Macie of the NRC International Relations Office, and to Dr. Jianying Ouyang for the discussion on the determination of quantum yield.

References and Notes

1. M. Bruchez, Jr., M. Moronne, P. Gin, S. Weiss, and A. P. Alivisatos, *Science* 281, 2013 (1998).
2. W. C. W. Chan and S. Nie, *Science* 281, 1616 (1998).
3. X. Michalet, F. F. Pinaud, L. A. J. Bentolila, M. Tsay, S. Doose, J. J. Li, G. Sundaresan, A. M. Wu, S. S. Gambhir, and S. Weiss, *Science* 307, 538 (2005).
4. M. B. Zaman, T. N. Baral, J. Zhang, D. Whitfield, and K. Yu, *J. Phys. Chem. C* 113, 496 (2009).

5. S. Kim, Y. T. Lim, E. G. Soltesz, A. M. De Grand, J. Lee, A. Nakayama, J. A. Parker, T. Mihaljevic, R. G. Laurence, D. M. Dor, L. H. Cohn, M. G. Bawendi, and J. V. Frangioni, *Nat. Biotechnol.* 22, 93 (2003).
6. (a) J. Sun, M. Q. Zhu, K. Fu, N. Lewinski, and R. A. Drezek, *International J. Nanomedicine* 2, 235 (2007); (b) K. T. Yong, *Nanotechnology* 20, 015102 (2009); (c) C. Shi, Y. Zhu, Z. Xie, W. Qian, C. L. Hsleh, S. Nie, Y. Su, H. E. Zhau, and L. W. K. Chung, *J. Urology* 74, 446 (2009); (d) P. M. Allen, W. Liu, V. P. Chauhan, J. Lee, A. Y. Ting, D. Fukumura, R. K. Jain, and M. G. Bawendi, *J. Am. Chem. Soc.* 132, 470 (2010).
7. H. Kobayashi, M. Ogawa, R. Alford, P. L. Choyke, and Y. Urano, *Chem. Rev.* 110, 2620 (2010).
8. R. E. Bailey and S. Nie, *J. Am. Chem. Soc.* 125, 7100 (2003).
9. (a) K. Yu, B. Zaman, S. Romanova, D. Wang, J. A. Ripmeester, *Small* 3, 332 (2005); (b) C. I. Ratcliffe, K. Yu, J. A. Ripmeester, M. B. Zaman, C. Badarau, and S. Singh, *Phys. Chem. Chem. Phys.* 8, 3510 (2006).
10. W. Jiang, A. Singhal, J. Zheng, C. Wang, and W. C. W. Chan, *Chem. Mater.* 18, 4845 (2006).
11. K. T. Yong, I. Roy, H. Ding, E. J. Eergey, and P. N. Prasad, *Small* 5, 1997 (2004).
12. A. Jemal, R. Siegel, E. Ward, Y. Hao, J. Xu, and M. J. Thun, *CA Cancer J. Clin.* 59, 225 (2009).
13. R. D. Blumenthal, E. Leon, H. J. Hansen, and D. M. Goldenberg, *BMC Cancer* 7, 2 (2007).
14. M. S. Duxbury, H. Ito, S. W. Ashley, and E. E. Whang, *J. Biol. Chem.* 279, 23176 (2004).
15. M. Tempero, W. Plunkett, V. R. Van Haperen, J. Hainsworth, H. Hochster, R. Lenzi, and J. Abbruzzese, *J. Clin. Oncol.* 21, 3402 (2003).
16. C. Hamers-Casterman, T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E. B. Songa, N. Bendahman, and R. Hamers, *Nature* 363, 446 (1993).
17. M. A. Ghahroudi, A. Desmyter, L. Wyns, R. Hamers, and S. Muyldermans, *FEBS Lett.* 414, 521 (1997).
18. S. Li, W. Zheng, R. Kuolee, T. Hiram, M. Henry, S. Makvandi-Nejad, T. Fjallman, W. Chen, and J. Zhang, *Mol. Immunol.* 46, 1718 (2009).
19. M. Dumoulin, K. Conrath, A. Van Meirhaeghe, F. Meersman, K. Heremans, L. G. Frenken, S. Muyldermans, L. Wyns, and A. Matagne, *Protein Sci.* 11, 500 (2002).
20. V. Cortez-Retamozo, M. Lauwereys, G. Hassanzadeh Gh, M. Gobert, K. Conrath, S. Muyldermans, P. De Baetselier, and H. Revets, *Int. J. Cancer* 98, 456 (2002).
21. R. C. Roovers, T. Laeremans, L. Huang, S. De Taeye, A. J. Verkleij, H. Revets, H. J. de Haard, and P. M. van Bergen en Henegouwen, *Cancer Immunol. Immunother.* 56, 303 (2007).
22. (a) J. Zhang, M. O'Connor-McCourt, T. N. Baral, and Y. Murad, Anti-Cecam6 Antibodies and Uses Thereof, US Provisional Application No. 61/388,746 filed on October 1, (2010); (b) T. N. Baral, Y. Murad, T. D. Nguyen, and J. Zhang, Isolation of functional single domain antibody by whole cell immunization: Implications for cancer treatment submitted to Molecular immunology.
23. (a) We would like to point out that 2A3 is not the first sdAb against CEACAM 6 AFAI sdAb was reported before by Dr. J. Zhang and others. However, the affinity of 2A3 is much higher than that of AFAI. (b) J. Zhang, Q. Li, T. D. Nguyen, T. L. Tremblay, E. Stone, R. To, J. Kelly, and R. C. MacKenzie, *J. Mol. Biol.* 341, 161 (2004).
24. G. T. Hermanson, *Bioconjugate Techniques*, 2nd edn., Elsevier Science, San Diego, CA (2008), pp. 216–223.
25. M. S. Duxbury, H. Ito, E. Benoit, S. W. Ashley, and E. E. Whang, *British Journal of Cancer* 91, 1384 (2004).
26. R. E. Payne, E. Yagüe, M. J. Slade, C. Apostolopoulos, L. R. Jiao, B. Ward, R. C. Coombes, and J. Stebbing, *Pharmacogenomics* 10, 51 (2009).

Received: 9 November 2010. Accepted: 4 February 2011.