Integrin-Targeted Trifunctional Probe for Cancer Cells: A "Seeing and Counting" Approach

ARTICLE *in* ANALYTICAL CHEMISTRY · OCTOBER 2012

Impact Factor: 5.64 · DOI: 10.1021/ac302029w · Source: PubMed

9 AUTHORS, INCLUDING:

Bo Zhang
Xiamen University
30 PUBLICATIONS **245** CITATIONS
SEE PROFILE

Yacui Luo
Xiamen University
5 PUBLICATIONS **83** CITATIONS
SEE PROFILE

Haifeng Chen
Xiamen University
80 PUBLICATIONS **744** CITATIONS
SEE PROFILE

Qiuquan Wang
Xiamen University
80 PUBLICATIONS **1,164** CITATIONS
SEE PROFILE

analytical chemistry

Letter

pubs.acs.org/ac

Integrin-Targeted Trifunctional Probe for Cancer Cells: A "Seeing and Counting" Approach

Zhubao Zhang,[†,∥] Qiang Luo,[§,∥] Xiaowen Yan,[†] Zhaoxin Li,[†] Yacui Luo,[†] Limin Yang,[†] Bo Zhang,[†] Haifeng Chen,*[,§] and Qiuquan Wang*[,†,‡]

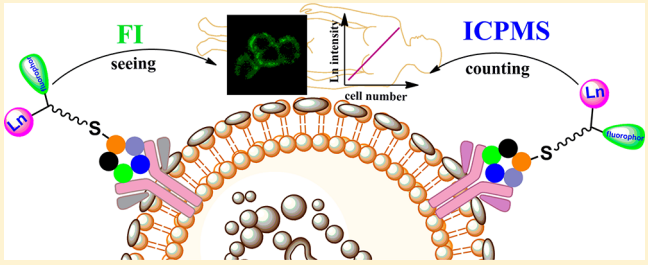
[†]Department of Chemistry & the Key Laboratory of Analytical Science, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China

[‡]State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China

[§]Institute for Biomedical Research, Xiamen University, Xiamen 361005, China

Supporting Information

ABSTRACT: We report the design and synthesis of a trifunctional probe for seeing and counting cancer cells using both fluorescence imaging (FI) and inductively coupled plasma mass spectrometry (ICPMS) for the first time. It consisted of a guiding cyclic RGD peptide unit to catch cancer cells via targeting the $\alpha_v\beta_3$ integrin overexpressed on their surface, a 5-amino-fluorescein moiety for FI using confocal laser scanning microscopy (CLSM) as well as a 2-aminoethyl-monoamide-DOTA group for loading stable europium ion and subsequent ICPMS quantification of the cancer cells without the use of radioactive isotopes. In addition to FI, the LOD (3σ) of the $\alpha_v\beta_3$ integrin was down to 69.2−309.4 amol per cell depending on the type of the $\alpha_v\beta_3$-positive cancer cells when using ICPMS and those of the cancer cell number reached 17−75. This probe developed enables us not only to see but also to count the $\alpha_v\beta_3$-positive cancer cells ultrasensitively, paving a new way for early diagnosis of cancer.



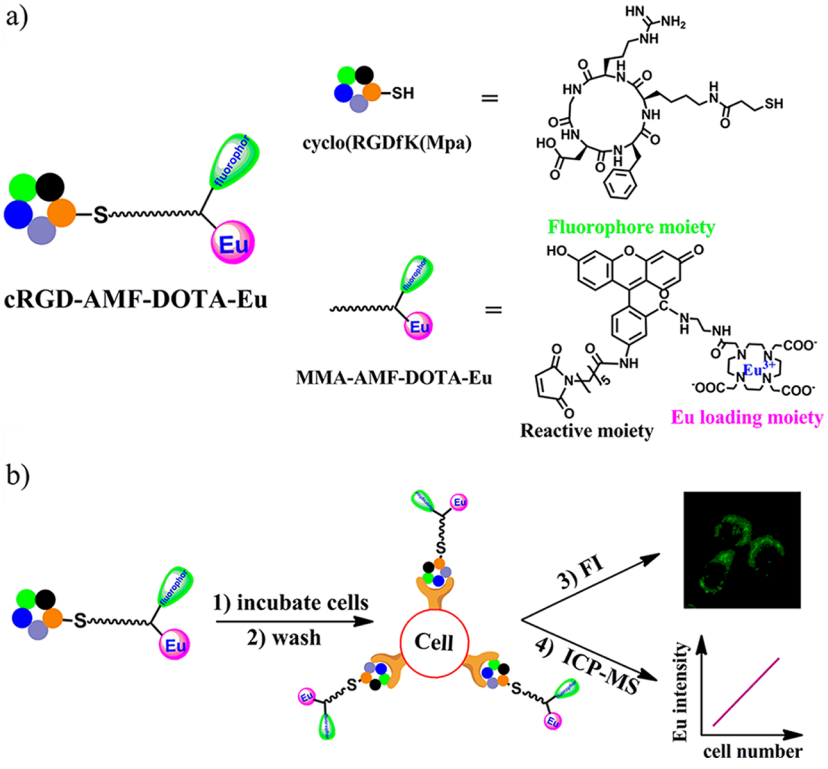
Cancer is the second leading cause of death worldwide.[1] As a consequence, the research never stops for methodological developments which will improve selectivity and/or sensitivity in order to provide reliable decisions concerning the accurate early diagnosis of cancer, and subsequently successful cancer treatment using contemporary therapeutic methods before primary tumors become widely spread. The difficulties of reliable early diagnosis of cancer lie in the fact that both biomarkers and occult cancerous cells are in low abundance and limited number, and they are buried generally in the "ocean" of high abundance proteins and a large number of normal and/or benign cells. These difficulties sometimes lead to false-negative and/or false-positive results. To address these issues, much effort has been directed to the development of various selective probes and thus to enhance the sensitivity of corresponding detection systems, for example, multiple biomedical imaging,[2−5] making it possible to specifically target and with high sensitivity detect cancer cells. Among these, for example, the establishment of a multifunctional platform based on a so-called all-in-one probe,[6] in which multifunctional moieties are conjugated and/or assembled together into one probe via sophisticated design and chemical synthesis, has become a forceful trend. This concept of the all-in-one probe facilitates the selective labeling of targeted biomarkers and thus a subsequent multitechnology based assay of the corresponding cancer cells using various detection techniques,[7−11] offering more comprehensive information over a single assay for early diagnosis of cancer and better patient management. It should be noted, however, that these developed strategies, especially the imaging systems, were aimed primarily at diagnostic imaging of biomarkers and corresponding cancer cells, acting as a "beacon", but were, to a great extent, rare and difficult to offer quantitative information about the limited occult cancer cells, especially in the early or initiatory stages of a cancer. In order to detect the limited number of occult cancer cells at their beginning stage, it is crucial to ultrasensitively quantify the expression levels of the biomarkers on/in cancer cells and the cancer cells themselves. Strategies for meeting these requirements are very scarce and thus are urgently needed.[12]

Here, we report the design and synthesis of a trifunctional probe for the first time to see and to count cancer cells. It consisted of three parts (Scheme 1): (1) an fluorescent dye (5-amino-fluorescein, AMF) for fluorescence imaging (FI); (2) 2-aminoethyl-monoamide-DOTA (MMA-DOTA) for loading stable europium (Eu) and subsequent quantification using inductively coupled plasma mass spectrometry (ICPMS) without the use of radioactive isotopes; as well as (3) a guiding cyclic Arg-Gly-Asp peptide c(RGDfK(Mpa)) (cRGD) to target integrin $\alpha_v\beta_3$ overexpressed on the surface of the $\alpha_v\beta_3$-positive

Received: August 9, 2012
Accepted: October 17, 2012
Published: October 17, 2012

   dx.doi.org/10.1021/ac302029w | *Anal. Chem.* 2012, 84, 8946−8951

Scheme 1. (a) Chemical Structure of the Trifunctional Probe cRGD-AMF-DOTA-Eu and (b) Procedures for the Visualization and Quantification of $\alpha_v\beta_3$ and the Corresponding $\alpha_v\beta_3$-Positive Cancer Cells



cancer cells.[13,14] This trifunctional probe enables us to achieve not only the visualization but also quantification of the targeted $\alpha_v\beta_3$-positive cancer cells via FI and ICPMS.

First, we started to protect the $-NH_2$ group in the AMF (**1**) with di-*tert*-butyl dicarbonate (Boc_2O) to obtain N-Boc-AMF (**2**) as illustrated in Figure 1. DOTA-tris(*t*-Bu ester) was then used to conjugate **2** via the reaction between the $-NH_2$ and $-COOH$ to obtain Boc-AMF-tBu_3DOTA (**3**). After release of the protected $-NH_2$ and the three $-COOH$ groups in **3** by removing the Boc and tBu in neat trifluoroacetic acid, AMF-DOTA (**4**) was obtained. Subsequently, MMA was conjugated into **4** via the amidation to obtain MMA-AMF-DOTA (**5**). After **5** was subjected to purification using semipreparative RP-HPLC with a yield of 46%, Eu^{3+} was loaded finally into DOTA at pH 5.8 to obtain MMA-AMF-DOTA-Eu (**6**).[15−18] All the intermediates and the MMA-AMF-DOTA-Eu were characterized using liquid chromatography−mass spectrometry (LC−MS) and ^1H NMR and ^{13}C NMR as well as UV−Vis and fluorescence spectrometry (see Figure S1−S8 and the detailed procedures and conditions in the Supporting Information). When the spectral properties of the MMA-AMF-DOTA-Eu in aqueous solution and its Eu-free analogue MMA-AMF-DOTA were compared, the results obtained indicated that they had the same UV−Vis maximum absorption peak at 499 nm (Figure S7 in the Supporting Information) and emitted strong fluorescence (λ_{em} = 520 nm) (Figure S8 in the Supporting Information), suggesting that the metalation of DOTA with Eu^{3+} has no effect on the energy level of the fluorescein moiety. This means that the prepared MMA-AMF-DOTA-Eu can be employed not only for highly sensitive quantification of targeted cancerous cells using ICPMS via the determination of Eu when the cells are labeled with the probe but also for FI of the cells.

Integrins are a family of 24 $\alpha\beta$ heterodimer membrane proteins that mediate dynamic linkages between extracellular matrix proteins and the intracellular actin cytoskeleton.[19] As one key member of the family, integrin $\alpha_v\beta_3$ (230 kDa) is overexpressed significantly on the surface of the cancer cells of, for example, glioblastoma, melanoma and ovarian, colon, lung, and prostate cancers when compared with the minimum expression in most normal resting cells and can serve as a cancer biomarker.[20,21] Different overexpression levels of $\alpha_v\beta_3$ are known to correlate well with tumor angiogenesis, growth, and metastasis as well as disease stages,[22,23] and thus sensitive and accurate quantification of $\alpha_v\beta_3$ allows early diagnosis of the cancers. Fortunately, cRGD has the unique property of interacting specifically with $\alpha_v\beta_3$.[24−29] cRGD was thus used here to conjugate into the prepared MMA-AMF-DOTA-Eu through the reaction between MMA and the sulfhydryl (−SH) in cRGD under the optimum conditions of a 3-fold excess of MMA-AMF-DOTA-Eu to the −SH in 100 mM MOPS (pH 6.8) at 37 °C for 1 h in the dark (Figure 1, and details can be found in the Supporting Information). A novel trifunctional probe, cRGD-AMF-DOTA-Eu (**7**), was then obtained in quantitative yield as confirmed using high-performance liquid chromatography−ultraviolet (HPLC−UV) and HPLC−electrospray (ESI)-MS (Figures S9 and S10 in the Supporting Information), in which cRGD served as a guiding group to target $\alpha_v\beta_3$ overexpressed on the corresponding cancer cells. This trifunctional probe was further purified using semi-preparative RP-HPLC and lyophilized for further use.

In order to quantify $\alpha_v\beta_3$ and the corresponding $\alpha_v\beta_3$-positive cancer cells via the determination of Eu labeled, the response of $Eu(NO_3)_3$, MMA-AMF-DOTA-Eu, and cRGD-AMF-DOTA-Eu on ICPMS was evaluated. The results obtained (Figure S11 in the Supporting Information) indicated that they have the

dx.doi.org/10.1021/ac302029w | *Anal. Chem.* 2012, 84, 8946−8951

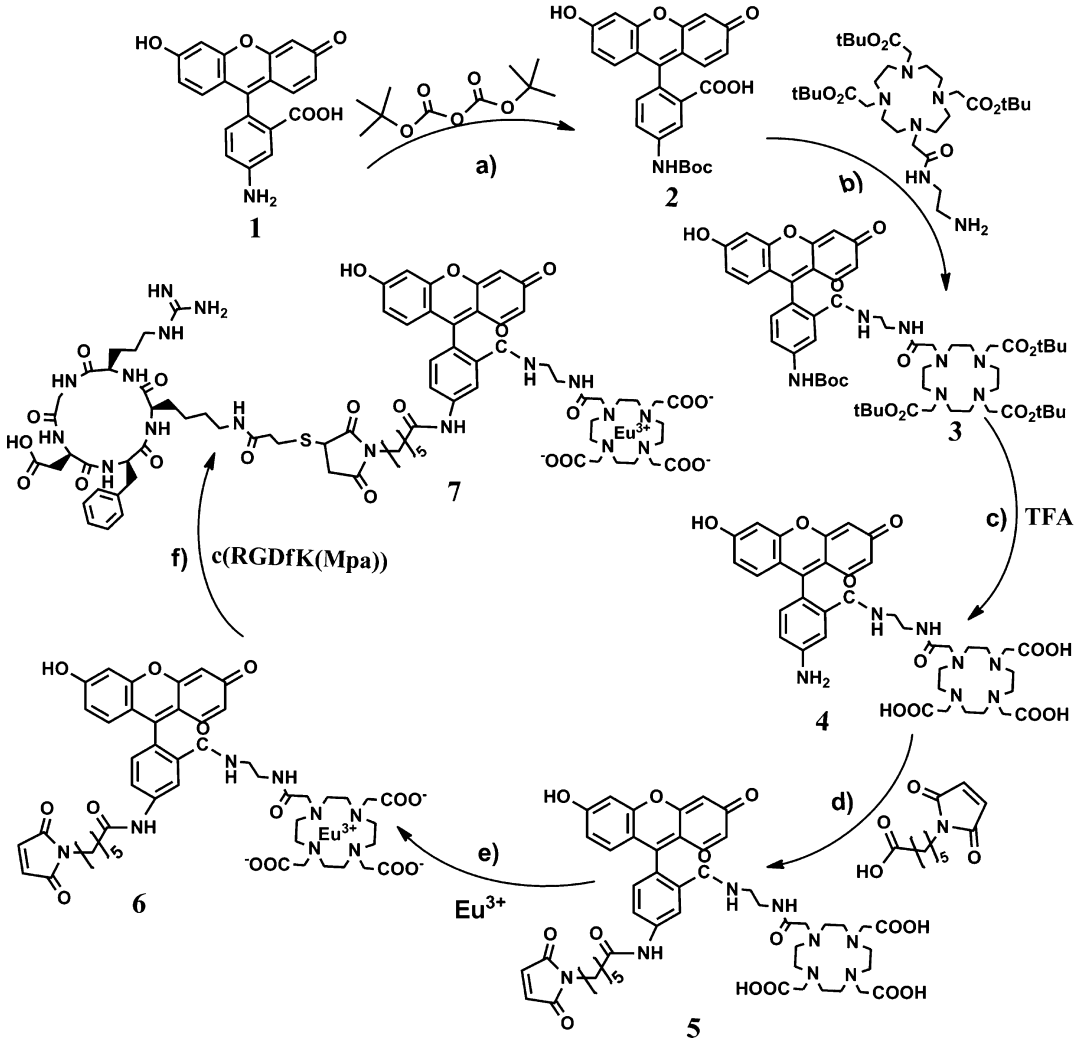


Figure 1. Synthetic route of cRGD-AMF-DOTA-Eu.

same intensity at equal Eu mole concentration, demonstrating no species-dependent ICPMS response as expected because ICP is a very hard ion source. The limit of detection (LOD, 3σ) for Eu reached 6.4 pM with the RSD of 1.7% (n = 6 at 32.7 nM each of them), implying that ICPMS is an ultrasensitive, accurate, and practical tool for the quantification of Eu in different chemical forms, and thus $\alpha_v\beta_3$ and the corresponding $\alpha_v\beta_3$-positive cancer cells when they are labeled with the trifunctional probe developed.

The specificity of the developed trifunctional probe toward $\alpha_v\beta_3$ on the cell membrane and its cell-targeting ability were further tested, in which the HT-29 colon cancer cell line ($\alpha_v\beta_3$-positive)[30,31] was selected while the human breast cancer cell line MCF-7 ($\alpha_v\beta_3$-negative)[32] was used as a control (see the Supporting Information). As shown in Figure 2, a strong green fluorescence was observed on the surface of the HT-29 cells (Figure 2a) after they were incubated with the cRGD-AMF-DOTA-Eu, while no fluorescence was observed with the MMA-AMF-DOTA-Eu without cRGD (Figure 2b) under the same conditions. As expected, the $\alpha_v\beta_3$-negative MCF-7 cells did not show any noticeable fluorescence (Figure 2c). On the other hand, the results from ICPMS (Figure 2a′′−2c′′) demonstrated that Eu was detected only in the case where the HT-29 cells were labeled with cRGD-AMF-DOTA-Eu, in good agreement with the above FI observations, suggesting that cRGD-AMF-DOTA-Eu bound specifically with $\alpha_v\beta_3$ on the HT-29 cancer cells. Moreover, the specificity of the trifunctional probe toward $\alpha_v\beta_3$ overexpressed on the cancer cells was further verified using a blocking study (see the Supporting Information). After the HT-29 cells were blocked with excess cRGD, fluorescence (Figure 2d) decreased remarkably to darkness and ICPMS intensity (Figure 2d′′) decreased to 1.04% when compared with the unblocked (Figure 2a′′). These results supported again the specific interaction of cRGD-AMF-DOTA-Eu with the $\alpha_v\beta_3$-positive tumor cells.

In order to accurately visualize and quantify the expression of $\alpha_v\beta_3$ and the corresponding $\alpha_v\beta_3$-positive cancer cells, we used the HT-29 cancer cell line again as a model to investigate the optimum incubation conditions. All the cell-labeling experiments in this study were performed at 4 °C under which the probe could attach specifically to the cell membrane without internalization at this temperature.[33] The concentration of the cRGD-AMF-DOTA-Eu, which was the main influencing factor on labeling efficiency, was investigated. The results (Figure S12 in the Supporting Information) indicated that after being incubated at 4 °C for 3 h, $\alpha_v\beta_3$ overexpressed on the HT-29 cell membranes (10^5 cells per well) were completely targeted and labeled with cRGD-AMF-DOTA-Eu when its concentration reached 50 nM in the binding buffer (pH 7.4). Time-dependent labeling experiments were also carried out, suggesting that

dx.doi.org/10.1021/ac302029w | Anal. Chem. 2012, 84, 8946−8951

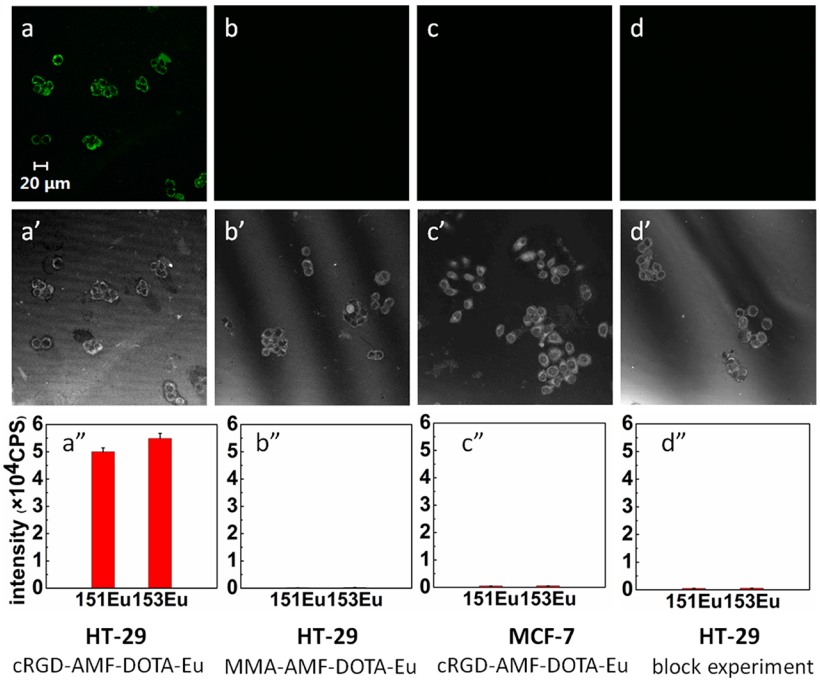


Figure 2. Tests of specificity. Confocal fluorescence (a–d), bright field (a′–d′) images and the corresponding results of ICPMS quantification (a″–d″) of the $\alpha_v\beta_3$-positive HT-29 cells and the $\alpha_v\beta_3$-negative MCF-7 incubated with 100 nM MMA-AMF-DOTA-Eu (b, b′, b″) and cRGD-AMF-DOTA-Eu without (a, a′, a″; c, c′, c″) and with (d, d′, d″) a blocking dose of 2000 nM cRGD for 2.5 h at 4 °C, respectively.

Table 1. Quantification of the $\alpha_v\beta_3$ Integrin and the Corresponding Cells Using ICPMS Together with the Trifunctional cRGD-AMF-DOTA-Eu Probe (n = 3)

			numbers of cells	
cell line	$\alpha_v\beta_3$ amol/cell	LODs (3σ) of cell number	ICPMS	flow cytometry
HT-29	116.5 ± 2.7	44	84 208 ± 2157	84 159 ± 1009
MCF-7	1.6 ± 0.1	3320	104 999 ± 1198	104 640 ± 1778
A549	309.4 ± 4.8	17	23 538 ± 364	23 161 ± 232
QSG-7701	5.2 ± 0.5	1000	144 928 ± 6398	141 888 ± 3838
SMMC-7721	69.2 ± 1.6	75	84 602 ± 1878	84 794 ± 1120
HeLa	151.4 ± 3.0	34	26 484 ± 514	26 219 ± 472

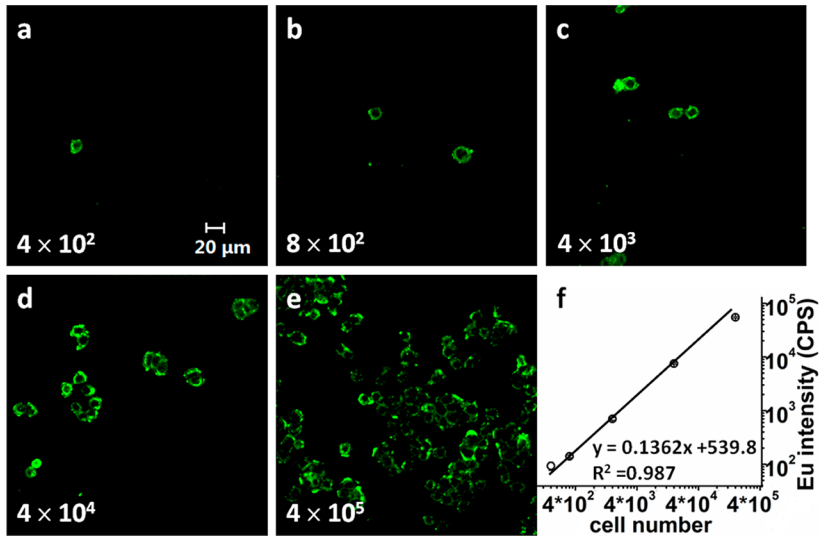


Figure 3. (a–e) Confocal fluorescence images of HT-29 cancer cells of different numbers after labeled with 50 nM cRGD-AMF-DOTA-Eu in binding buffer at 4 °C for 2.5 h. (f) Corresponding linear regression between HT-29 cell number and Eu ICPMS intensity as presented in parts a–e. Each point is the average result from triplicate analysis.

dx.doi.org/10.1021/ac302029w | *Anal. Chem.* 2012, 84, 8946–8951

optimum labeling time was 150 min (Figure S13 in the Supporting Information).

Under the optimal incubation conditions, we visualized and quantified the amount of $\alpha_v\beta_3$ expressed on the cells of different cell lines (Figure S14 in the Supporting Information). Flow cytometry was used in parallel to count the cell number, and the amount of Eu labeled on the cells was determined using ICPMS (see the Supporting Information). The amount of $\alpha_v\beta_3$ expressed per cell was then calculated based on one cRGD-AMF-DOTA-Eu attacks one $\alpha_v\beta_3$.[24−29,31,32] The results listed in Table 1 indicated that 69.2−309.4 amol of $\alpha_v\beta_3$ are expressed per cell of different $\alpha_v\beta_3$-positive cancer cell lines, while only 1.6 and 5.2 amol of $\alpha_v\beta_3$ per $\alpha_v\beta_3$-negative cancer cell MCF-7 and healthy cell QSG-7701. On the basis of the LOD (3σ) of $\alpha_v\beta_3$ and the amount of $\alpha_v\beta_3$ overexpressed on per cell of different $\alpha_v\beta_3$-positive cancer cell lines, 17−75 cancer cells could be determined theoretically depending on their type (Table 1). ICPMS was thus able to quantify the number of $\alpha_v\beta_3$-positive cancer cells when using the trifunctional probe developed in this study. Practically, for example, the linear range of Eu ICPMS intensity versus cell number was from 400 to 400 000 in the case of the HT-29 (R^2 = 0.987) (Figure 3). The cancer cell number counted with flow cytometry and ICPMS were then compared (Table 1), and they are well in agreement with each other, implying that ICPMS together with the trifunctional probe developed is reliable for providing quantitative information regarding $\alpha_v\beta_3$-positive cancer cells. It should be pointed out that when the HT-29 cell number was more than 400 000, the ICPMS intensity of the labeled cells began to deviate from the regression line. We speculated that this phenomenon might be due to cell aggregation when the cell concentration was too high, significantly reducing the cell surface area available for labeling with cRGD-AMF-DOTA-Eu. On the other hand, as can be seen from Figure 3a−e, although the labeled HT-29 could be clearly visualized under fluorescence confocal microscopy, however, much time was spent on finding the targeted cells, and very few cells appeared in our range of vision when the cell number decreased to the level of thousands, especially when the number of cancerous cells is very limited at the very beginning stage of a cancer. Under such a circumstance, ICPMS was much more sensitive and practical to count the cells. It should be also pointed out that the trifunctional probe cRGD-AMF-DOTA-Eu could penetrate into the nucleus of the SMMC 7721 cells even when the incubation temperature was controlled at the same temperature 4 °C (Figure S14e in the Supporting Information). This observation is very interesting and needs to be further investigated considering a possible drug development.

In conclusion, the trifunctional probe developed here enables us to see and to count the $\alpha_v\beta_3$-positive cancer cells via FI and ICPMS. Especially, ICPMS together with the trifunctional probe is able to quantify ultra trace $\alpha_v\beta_3$ and a limited-number of the corresponding $\alpha_v\beta_3$-positive cancer cells, not only paving an alternative way for quantifying $\alpha_v\beta_3$-positive cancer cells but also extending the ever more important ability of ICPMS, a powerful nonradioactive element/isotope analysis tool for the quantification of biomolecules and cells. It is worthy of note that this approach is not limited to the $\alpha_v\beta_3$-positive cancer cells studied here. The design and synthesis of other novel multifunctional probes, in which different cell membrane biomarker-targeted groups together with fluorescent agents and corresponding lanthanides are chemically assembled, are greatly expected to perform a more comprehensive multiplex assay of cancer cells, achieving more accurate early diagnosis of cancers. This kind of research is ongoing in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (+86) 592 2181796. Fax: (+86) 592 2187400. E-mail: qqwang@xmu.edu.cn (Q.W.); Haifeng@xmu.edu.cn (H.C.).

Author Contributions

‖These authors contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was financially supported by the National Natural Science Foundation of China (Grants 21035006 and 21275120) and the National Basic Research 973 Program (Grant 2009CB421605). We thank Professor John Hodgkiss of the University of Hong Kong for his help with the English.

■ REFERENCES

(1) World Health Organization. *The Global Burden of Disease: 2004 Update*; World Health Organization: Geneva, Switzerland, 2008.

(2) Michalet, X.; Pinaud, F. F.; Bentolila, L. A.; Tsay, J. M.; Doose, S.; Li, J.; Sundaresan, G.; Wu, A.; Gambhir, S. S.; Weiss, S. *Science* **2005**, *307*, 538−544.

(3) Fass, L. *Mol. Oncol.* **2008**, *2*, 115−152.

(4) Louie, A. *Chem. Rev.* **2010**, *110*, 3146−3195.

(5) Lee, S.; Xie, J.; Chen, X. *Chem. Rev.* **2010**, *110*, 3087−3111.

(6) Lee, J. H.; Lee, K.; Moon, S. H.; Lee, Y.; Park, T. G.; Cheon, J. *Angew. Chem. Int. Ed.* **2009**, *48*, 4174−4179.

(7) Xu, H.; Baidoo, K.; Gunn, A. J.; Boswell, C. A.; Milenic, D. E.; Choyke, P. L.; Brechbiel, M. W. *J. Med. Chem.* **2007**, *50*, 4759−4765.

(8) Edwards, W. B.; Akers, W. J.; Ye, Y.; Cheney, P. P.; Bloch, S.; Laforest, R.; Achilefu, S. *Mol. Imaging* **2009**, *8*, 101−110.

(9) Joshi, B. P.; Wang, T. D. *Cancers* **2010**, *2*, 1251−1287.

(10) Schooeveld, M. M.; Cormode, D. P.; Koole, R.; Wijingaarden, J. T.; Calcagno, C.; Skajaa, T.; Hilhorst, J.; Hart, D. C.; Fayad, Z. A.; Mulder, W. J. M.; Meijerink, A. *Contrast Media Mol. Imaging* **2010**, *5*, 231−236.

(11) Banerjee, S. R.; Pullambhatla, M.; Byun, Y.; Nimmagadda, S.; Foss, C. A.; Green, G.; Fox, J. J.; Lupold, S. E.; Mease, R. C.; Pomper, M. G. *Angew. Chem. Int. Ed.* **2011**, *50*, 9167−9170.

(12) Bendall, S. C.; Simonds, E. F.; Qiu, P.; Amir, E. D.; Krutzik, P. O.; Finck, R.; Bruggner, R. V.; Melamed, R.; Trejo, A.; Ornatsky, O. I.; Balderas, R. S.; Plevritis, S. K.; Sachs, K.; Peer, D.; Tanner, S. D.; Nolan, G. P. *Science* **2011**, *332*, 687−696.

(13) Mizejewski, G. J. *Proc. Soc. Exp. Biol. Med.* **1999**, *222*, 124−138.

(14) Jin, H.; Varner, J. *Br. J. Cancer* **2004**, *90*, 561−565.

(15) Yan, X. W.; Xu, M.; Yang, L. M.; Wang, Q. Q. *Anal. Chem.* **2010**, *82*, 1261−1269.

(16) Yan, X. W.; Yang, L. M.; Wang, Q. Q. *Angew. Chem. Int. Ed.* **2011**, *50*, 5130−5133.

(17) Zhang, Z. B.; Yan, X. W.; Yang, L. M.; Wang, Q. Q. *J. Anal. At. Spectrom.* **2011**, *26*, 1175−1177.

(18) Yan, X. W.; Luo, Y. C.; Zhang, Z. B.; Li, Z. X.; Luo, Q.; Yang, L. M.; Zhang, B.; Chen, H. F.; Bai, P. M.; Wang, Q. Q. *Angew. Chem. Int. Ed.* **2012**, *51*, 3358−3363.

(19) Hynes, R. O. *Cell* **2002**, *110*, 673−687.

(20) Cheresh, D. A. *Cancer Metastasis Rev.* **1991**, *10*, 3−10.

(21) Gasparini, G.; Brooks, P. C.; Biganzoli, E.; Vermeulen, P. B.; Bono, di E.; Dirix, L. Y.; Ranieri, G.; Miceli, R.; Cheresh, D. A. *Clin. Cancer Res.* **1998**, *4*, 2625−2634.
(22) Vonlaufen, A.; Wiedle, G.; Borisch, B.; Birrer, S.; Luder, P.; Imhof, B. A. *Mod. Pathol.* **2001**, *14*, 1126−1132.
(23) Desgrosellier, J. S.; Cheresh, D. A. *Nat. Rev. Cancer* **2010**, *10*, 9−22.
(24) Pierschbacher, M. D.; Ruoslahti, E. *Nature* **1984**, *309*, 30−33.
(25) Haubner, R.; Gratias, R.; Diefenbach, B.; Goodman, S. L.; Jonczyk, A.; Kessler, H. *J. Am. Chem. Soc.* **1996**, *118*, 7461−7472.
(26) Xiong, J.; Stehle, T.; Zhang, R.; Joachimiak, A.; Frech, M.; Godman, S. L.; Arnaout, M. A. *Science* **2002**, *296*, 151−155.
(27) Marinelli, L.; Gottschalk, K. E.; Meyer, A.; Novellino, E.; Lessler, H. *J. Med. Chem.* **2004**, *47*, 4166−4177.
(28) Cai, W.; Chen, X. *Nat. Protoc.* **2008**, *3*, 89−96.
(29) Xiong, L. Q.; Chen, Z. G.; Tian, Q. W.; Cao, T. Y.; Xu, C. J.; Li, F. Y. *Anal. Chem.* **2009**, *81*, 8687−8694.
(30) Kemperman, H.; Wijnands, Y. M.; Roos, E. *Exp. Cell Res.* **1997**, *234*, 156−164.
(31) Enns, A.; Korb, T.; Schlüter, K.; Gassmann, P.; Spiegel, H. U.; Senninger, N.; Mitians, F.; Haier, J. *Eur. J. Cancer.* **2005**, *41*, 1065−1072.
(32) Cao, Q.; Cai, W.; Li, T.; Yang, Y.; Chen, K.; Xing, L.; Chen, X. Y. *Biochem. Biophys. Res. Commum.* **2006**, *351*, 726−732.
(33) Wu, X.; Liu, H.; Liu, J.; Haley, K. N.; Treadway, J. A.; Larson, J. P.; Ge, N.; Peale, F.; Bruchez, M. P. *Nat. Biotechnol.* **2003**, *21*, 41−46.