Analytica Chimica Acta 1221 (2022) 340084

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Development of a biosensor based on a new marine luciferase fused to an affibody to assess Her2 expression in living cells

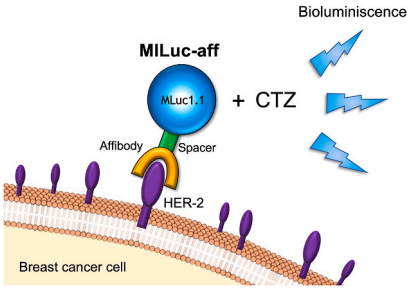
Laura Rodríguez de la Fuente , Irene Golán Cancela , Ánxela M. Estévez-Salguero , Pablo Iglesias , José A. Costoya *

Molecular Oncology Laboratory MOL, Centro Singular de Investigación en Medicina Molecular e Enfermidades Crónicas (CiMUS), Departamento de Fisioloxía, Facultade de Medicina, Universidade de Santiago de Compostela, Instituto de Investigación Sanitaria de Santiago de Compostela (IDIS), 15782, Santiago de Compostela, Spain

HIGHLIGHTS

- Functional optical imaging allows high-sensitivity detection of prognostic biomarkers in cancer.
- MLuc1.1, a thermostable luciferase from *Metridia lucens* with high bioluminescent activity, is presented as a new diagnostic tool in optical imaging.
- Development of a novel biosensor, MlLuc-aff, that recognizes over-expression of HER-2 receptor in breast cancer cell lines.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Cancer
HER2
Affibody
Optical imaging
Bioluminiscence

ABSTRACT

The development of new diagnostic tools in tumor pathology allows the optimization of individualized therapies in cancer patients. The functional optical image provides a unique opportunity to identify the pathophysiological characteristics of each tumor in a non-invasive way. Although fluorescent recombinant affibodies and nanobodies, capable of detecting certain membrane proteins present in tumor cells, has been described, the use of bioluminescent molecules is gaining a great impact in this field due to its high sensitivity. In this work, we characterize a new luciferase from the *Metridia lucens* copepod (MlLuc) and develop a novel bioluminescent recombinant affibody (MlLuc-aff) capable of recognizing the HER2 receptors that are overexpressed in breast cancer tumors. For this purpose, the thermostability and pH sensitivity of MlLuc1.1 were determined, showing no significant changes in the activity among temperatures between 4 and 70 °C, and with a maximum of brightness at pH 8.0. Furthermore, MlLuc-aff was able to accurately detect HER2 receptors expressed in the SK-BR-3 cells. Future applications of this new tracer can contribute to the early diagnosis of breast cancer patients and the assessment of the efficacy of the treatment.

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases can activate different signaling pathways involved in the regulation of cellular proliferation and survival. Among the different members of this family of receptors, HER2 promotes oncogenesis. HER2 gene amplification or gene mutations have been detected in many types of cancers such as, colorectal, lung, bladder, stomach, brain, uterus, skin

* Corresponding author.
E-mail address: josea.costoya@usc.es (J.A. Costoya).

https://doi.org/10.1016/j.aca.2022.340084
Received 17 January 2022; Received in revised form 6 June 2022; Accepted 11 June 2022
Available online 16 June 2022

and breast [1]. In fact, the clinical classification of breast cancer is based on the presence of several receptors, specifically estrogen, progesterone and HER2, a receptor overexpressed or amplified in 25% of patients with breast cancer, and this correlates with poor clinical outcomes [2]. Diverse immunotherapeutic approaches based on HER2-directed monoclonal antibodies has shown an improvement in clinical outcomes of patients with HER2-positive metastatic breast cancer [3–5]. Therefore, HER2 expression is highly used as a biomarker predictive of strong correlations between improved clinical outcomes and treatments with HER2-targeted agents [6].

However, the possibility of analyzing the temporal heterogeneity of metastatic breast cancer with tissue-based biomarkers is difficult because it requires sequential biopsies. Moreover, repeated biopsies may not be informative because the expression may not be found at all metastatic sites. For this reason, molecular imaging can complement the pathological, through a noninvasive and repeatable whole-body assessment of the disease [7].

Noninvasive molecular imaging uses labeled tracers to visualize biomarkers in the whole body. Among them, affibodies meet many requirements to become a remarkable imaging tracer [8]. They have a small size, ensuring an optimal tissue penetration and fast blood clearance, additionally their target specificity has been also demonstrated *in vivo* [9]. These molecules have been used as tools for molecular recognition in therapeutic applications, and many preclinical studies and early clinical evidence is now founding the idea that affibody molecules could be efficacious and safe in human [10].

Small luciferases, and specifically copepod luciferases, have rapidly gained relevance as reporters in *in vitro* and non-invasive *in vivo* assays, especially after water-soluble coelenterazine (s-CTZ) was available [11]. Although, both Renilla and copepod luciferases are coelenterazine-dependent luciferases, these enzymes substantially differ in size and amino acid sequences. Moreover, these luciferases demonstrate a high stability, small size, and strong bioluminescence activity. Although, many *in vivo* assays have been reported during the last years, there are only a few examples of applying them in analytical assays *in vitro*. Considering their exceptional bioluminescent and biochemical properties of these luciferases, the number of reports on their use as labels in binding assays is still reduced. This is mainly due to the difficulty of purifying the recombinant protein in *E. coli* because the correctly folded copepod luciferases must contain five intramolecular disulfide bonds [12].

Although several studies have demonstrated the application of HER2-specific affibody-Alexa Fluor fluorescent conjugates as optimal probes for optical imaging of HER2 *in vivo* [13] and HER2-binding affibody molecule labeled with a radionuclide for PET imaging [14]. A recent study has reported the construction of a hybrid protein consisting of the smallest isoform of copepod luciferase and a murine single-chain variable fragment mini-antibody to the glycoprotein E (gpE) of tick-borne encephalitis virus (TBEV) [15]. Here, we present an innovative bioluminescent biosensor based on a fusion protein comprising a new Metridia luciferase fused to a recombinant affibody (MlLuc-aff), capable of recognizing the HER2 receptors that are overexpressed in breast cancer.

1. Materials and methods

1.1. U87MG cell culture and transfection

The human Uppsala 87 Malignant Glioma cell line (U87MG) was obtained from J. Seoane (Vall d'Hebron Instituto de Oncología, Barcelona, Spain). The human glioma cell line was maintained in low glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher). Cell cultures were maintained at 37 °C and 5% CO_2. The pcDNA3.1(+) plasmid harboring a neomycin (G418) resistance was used for mammalian expression of MlLuc1.1 luciferase. The pcDNA3.1(+)-MlLuc1.1 plasmid was constructed by GenScript Biotech Corporation, subcloning the full-length MlLuc1.1 cDNA sequence into the HindIII/EcoRI site. U87MG cells were grown to 60–70% confluence, the old medium was replaced with DMEM 0% FBS, and the recombinant plasmid called pcDNA3.1 (+)-MlLuc1.1 was transfected with 15 μg/ml of DNA:7.5 μg/ml of branched polyethylenimine (PEI 25, Sigma-Aldrich).

1.2. Determination of the minimal lethal concentration of G418

U87MG cells were seeded in DMEM 10% FBS at a density of 1×10^6 cells/mL in a 6-multiwell plate and they were exposed to different concentrations of Neomycin G418 (sigma-Aldrich; 0, 50, 100, 200, 300 and 400 μg/ml). Cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich) every two days and renewed with complete media and their corresponding G418 concentration. This experiment was performed by triplicate. The minimal lethal concentration of G418 was established as the working concentration for selection of transfected U87MG cells (U87MG-MlLuc1.1).

1.3. Evaluation of luciferase activity and stability in the U87MG-MlLuc1.1 cell line

U87MG-MlLuc1.1 cells were seeded in 10 cm diameter plates at a density of 2×10^6 per plate. MlLuc1.1 secreted to the media was assayed at regular time intervals of 15 h until 75 h post-seed. Determination of the luminescent signal was measured with a Lumat LB9507 luminometer (Berthold Technologies) and quantified as relative light units (RLUs). Briefly, 50 μl of coelenterazine (1 ng/μL) (CTZ, Sigma-Aldrich) was added into 20 μl aliquots of U87MG-MlLuc1.1 media and the exposure time was extended to 10 s. Cell culture medium was used as negative control. Bioluminescent spectra of MlLuc1.1 was measured with a FluoroMax-3 spectrofluorometer (Horiba) after addition of CTZ (1 ng/μL) into U87MG-MlLuc1.1 media aliquots. DMEM media without phenol red was used in this assay. To determine MlLuc1.1 thermostability, U87MG-MlLuc1.1 media samples were diluted 10-fold with 20 mM Tris–HCl (pH 8.0), 50 mM $MgCl_2$ and incubated 30 min over a range of 4–100 °C and then, cooled on ice for 5 min. The bioluminescent activity was measured at room temperature. To determine MlLuc1.1 pH sensitivity, U87MG-MlLuc1.1 media samples were diluted 10-fold with the following buffers: 0.1 M acetate buffer (pH 4.5–5.5), 0.1 M MOPS-NaOH (pH 6.0 and 6.5), 0.1 M HEPES-NaOH (pH 7.0 and 7.5), 0.1 M Tris–HCl (pH 8.0–9.0) and 0.1 M carbonate buffer (pH 9.5 and10.0). All buffers contained 50 mM $MgCl_2$. Samples were incubated for 1 h on ice before measurements. MlLuc1.1 thermostability and pH sensitivity determinations were carried out in triplicate intra and inter-assay with a Lumat LB960 luminometer (Berthold Technologies) by adding 25 μL of CTZ (1 ng/μL) to 10 μl of sample.

1.4. Synthesis and characterization of MlLuc-aff

The fusion of the affibody $Z_{HER2:4}$ with MlLuc1.1 luciferase was carried out by positioning a flexible sequence spacer $(GGGGS)_3$ between both proteins and adding a tail of six histidine (His6-tag) molecules at the C-terminal end of the recombinant protein. The SacI and EcoRI restriction sites were designed for the insertion of the coding sequence MlLuc1.1-$Z_{HER2:4}$-His6-tag in the vector pET-17b. The synthesis of the construct, finally called pET-17b-MlLuc-aff, was commissioned from GenScript Biotech Corporation. *Escherichia coli* BL21(DE3) cells were transformed with pET-17b-MlLuc-aff plasmid by thermal shock. BL21 (DE3)-MlLuc-aff cells were grown at 25 °C until they reached OD_{600} of ~0,5. Then, 100 μM IPTG was added to the culture and temperature was lowered to 23 °C. After 6 h, BL21(DE3)-MlLuc-aff cells were collected and centrifuged at 20,000 g, 20 min at 4 °C. Pellet was frozen at −80 °C for 24 h and then, resuspended in denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 6 M Gu-HCl and 1 mM PMSF). After 1 h of incubation at room temperature, the lysate was centrifuged at 10,000 g, 30 min. The

supernatant, containing MlLuc-aff denatured, was collected and dialyzed for refolding proteins using a Slide-A-Lyzer MINI 10 K MWCO dialysis cassette, (Thermo Fisher). Briefly, supernatants were incubated at 4 °C in agitation during 8–12 h with 50 mL of each the following buffers: the first buffer containing 100 mM NaH_2PO_4, 5 mM Tris-Cl, 2 mM imidazole, 0.1 mM DTT, 4 M Gu-HCl; the second 50 mM NaH_2PO_4, 5 mM Tris-Cl, 150 mM NaCl, 5 mM imidazole, 0.3 mM DTT, 2 M Gu-HCl; and the third 50 mM NaH_2PO_4, 5 mM Tris-Cl, 300 mM NaCl, 10 mM imidazole, 0.5 mM DTT.

After the dialysis process, MlLuc-aff was purified under native conditions using the HisPur Ni-NTA, 0,2 ml kit (Thermo Fisher). To eliminate the remaining imidazole from the final eluate containing MlLuc-aff, an extra dialysis was performed against 50 mL of PBS. Finally, protein expression was monitored by Coomassie blue staining and Western blot. The final concentration of MlLuc-aff was quantified by the Bradford assay method.

1.5. Binding assay performed with SK-BR-3 and MDA-MB-231 cells

Breast adenocarcinoma SK-BR-3 and triple-negative breast MDA-MB-231 cancer cells (ATCC) were seeded at density 2.5×10^5 per well in a 35 mm diameter plate. Both cancer cell lines were cultured with high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS, at 37 °C and 5% CO_2. After 48 h, MlLuc-aff (0.5 μg/μL) was added to each plate by dilutions of 1:10, 1:50 and 1:100. SK-BR-3 and MDA-MB-231 cells were incubated with MlLuc-aff for 2 h in the same conditions described above. Then, cells were washed twice with phosphate-buffered saline (PBS, Sigma-Aldrich) and bioluminescence data was registered with IVIS® Spectrum system after 10 s of exposure time upon addition of 250 μL CTZ (1 ng/μL) to each plate.

2. Results

2.1. High and stable expression of MlLuc1.1 in U87MG-MlLuc1.1 cell line

Bioluminescent spectra of MlLuc1.1, secreted to the culture media by U87MG-MlLuc1.1., was analyzed with FluoroMax-3 spectrofluorometer (Horiba) by adding CTZ into aliquots with media from U87MG-MlLuc1.1. The luciferase activity reached its maximum intensity at wavelength 475 nm (Fig. 1a), which correspond with blue light emission.

MlLuc1.1 secreted to the culture media was measured using the luminometer Lumat LB9507 (Berthold Technologies). After seeding U87MG-MlLuc1.1 cells, the luciferase activity was assayed at regular time intervals of 15 h until 75 h post-seed. The bioluminescence detected increased with the time elapsed since cells were seeded (Fig. 1b). This suggests that there is a correlation between luciferase expression and the number of cells in cultured, showing a stable accumulation of MlLuc1.1

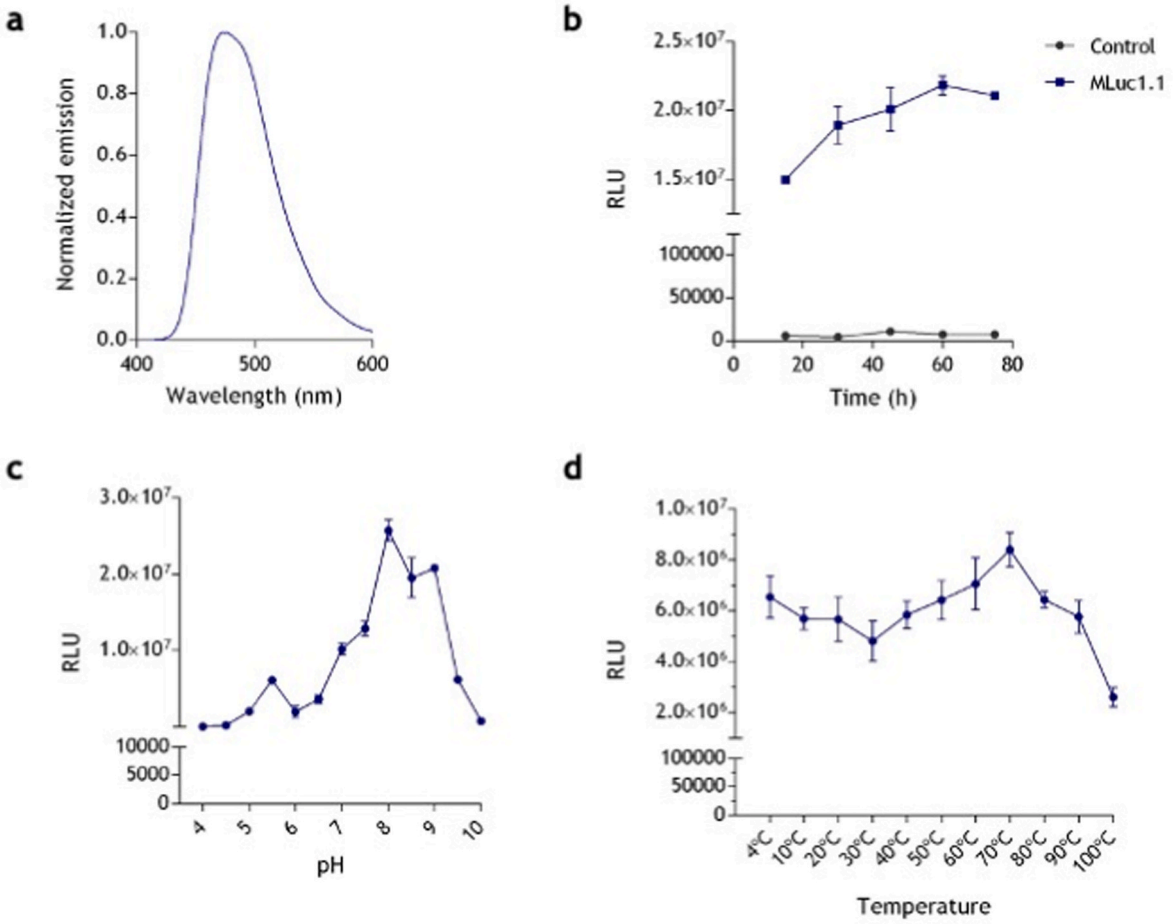


Fig. 1. Bioluminescence of MlLuc1.1. a) Bioluminescence spectra of MlLuc1.1. Luciferase activity in media from U87MG-MlLuc1.1 was measured after addition of 1 ng/μL CTZ. MlLuc1.1 shows its maximum intensity at 475 nm. **b)** Correlation of bioluminescence activity with time. **c)** pH sensitivity of MlLuc1.1 was measured in media from U87MG-MlLuc1.1 at different pH values upon addition of CTZ. MlLuc1.1 reaches its maximum level of bioluminescence at pH 8.0. **d)** Thermostability of MlLuc1.1. Media from U87MG-MlLuc1.1 was heated at different temperatures for 30 min and measured after adding 25 μl of CTZ 1 ng/μl. As shown, MlLuc1.1 activity is maintained and at 80 °C and steeply declines at higher temperatures.

in the media. As occurs in both luciferin-dependent and coelenterazine-dependent luciferases [16], the intensity of light emitted by MlLuc1.1 varies depending on pH value, reaching its maximum activity at pH 8.0 (Fig. 1c).

We also examined the thermostability of MlLuc1.1, and after 30 min of incubation at different temperatures in a range of 4–100 °C [17], bioluminescence of MlLuc1.1 remained constant up 90 °C (Fig. 1d). In fact, even at 100 °C the level of bioluminescence continues being detectable. Based on this data we can conclude that MlLuc1.1 shows a high level of thermostability.

2.2. Design and expression of MlLuc-aff

In order to use MlLuc1.1 as a probe in cancer diagnosis, the affibody $Z_{HER2:4}$ [18], against HER2 receptors, was fused to the luciferase. Expression and purification of the recombinant protein, denominated MlLuc-aff, was carried out in *E. coli* under denaturing conditions. The presence of our recombinant protein in the lysate was monitored by Coomassie staining and Western blot (Fig. 2C, D). Although MlLuc-aff was obtained mostly as a soluble protein, it can be also detected in the insoluble fraction (Fig. 2a and b). Lysates were dyalized prior to column purification in order to remove denaturing salts preventing the refolding of our protein. Finally, MlLuc-aff was purified using HisPur Ni-NTA columns and quantified by Bradford assay method.

The presence of the bioluminescence produced by MILuc-aff was quantified in each step of the purification process (Fig. 3). One aliquot was taken from each step and measured after addition of CTZ. As shown in Fig. 3, part of the bioluminescence activity can be appreciated in the

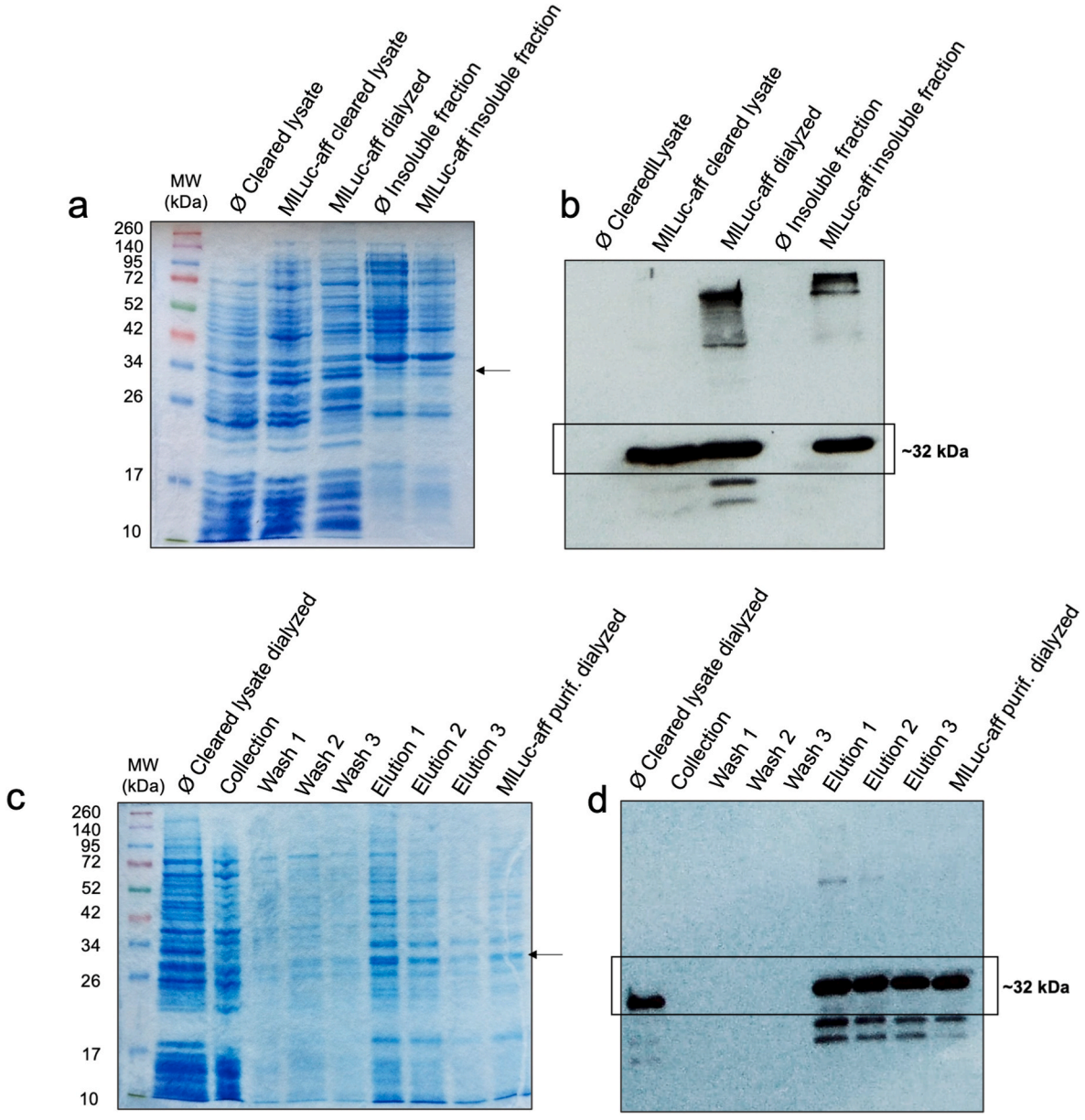


Fig. 2. Coomassie staining and Western blot to check the expression and purification of MlLuc-aff. *E. coli* BL21(DE3) cells were transformed with the plasmid containing MlLuc-aff and grown until they reached OD_{600} of ~0,5. Then, 100 μM IPTG was added to the culture to induce protein expression. After 6 h, BL21(DE3)-MlLuc-aff bacteria were centrifuged and resuspended in denaturing buffer. The expression of MlLuc-aff was monitored by Coomassie staining (a) and Western Blot (b). Although a small portion of MlLuc-aff remained in the insoluble fraction, MlLuc-aff was also obtained from the soluble fraction (cleared lysate). Purification of MlLuc-aff was performed under native conditions and monitored by Coomassie staining (c) and Western Blot (d). Dialysis after purification resulted in an increased concentration of MILuc-aff (appreciated in Coomassie staining, Fig. 2c).

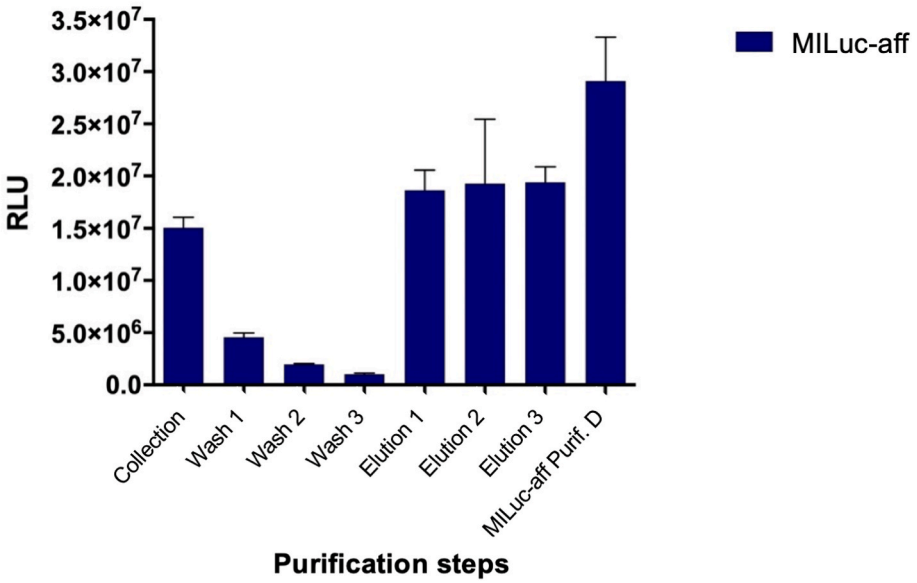


Fig. 3. Bioluminescence quantification of the purification process. Aliquots from each step were diluted 1:100 before analysis, and their bioluminescent activity recorded upon addition of CTZ. Highest bioluminescence values were registered in the elution and dialyzed fractions.

first flowthrough (purification step: collection) from the purification column. This bioluminescence detection can be due because a small fraction of MlLuc-aff that did not properly bind to the resin column. However, most of the bioluminescence is coming from the aliquot with MlLuc-aff already purified and dialyzed, which indicates a satisfactory purification of MlLuc-aff. The concentration of protein in the sample MlLuc-aff purified and dialyzed was quantified by Bradford assay, obtaining a total of 0.6 μg/μl.

2.3. Specific binding of MILuc-aff to HER2 receptors

To check the ability of MlLuc-aff to recognize the receptor on the membrane of breast cancer cells, a binding assay was performed with SK-BR-3 cell line, which overexpress the target receptor, and MDA-MB-231 cell line with low expression of the same receptor [19]. Two hours before the binding assay was performed, MlLuc1-aff was incubated with the cells. Cells were washed with PBS twice before adding CTZ and measuring bioluminescence activity with an IVIS® Spectrum system. As indicated in Fig. 4, MILuc1.1 was retained mostly in the plates containing SK-BR-3 HER2 receptors. MlLuc-aff was able to recognize specifically the HER2 receptors expressed in both cell lines (Fig. 4). Higher levels of brightness can be appreciated in the wells containing SK-BR-3 cells, which corresponds with the overexpression of HER2 for this cell line. However, MlLuc-aff is also able to recognize the low expression of HER2 in the MDA-MB-231 cell line. This suggests a high affinity of MlLuc-aff for its target receptor HER2.

The results suggest that our new molecule MlLuc-aff is able to detect specifically the targeted receptor on the surface of cells. There is more emission of light on wells containing cells that overexpress the studied receptor (Fig. 4). Additionally, the high level of brightness produced by the luciferase MlLuc1.1, together with the high affinity of the anti-HER2 affibody, allows detecting with high sensitivity the receptor HER2 even at high dilutions rates (up to 1:500).

3. Discussion

Despite the confinement of functional optical imaging to preclinical settings, it already shows a great potential to have a great impact on the management of cancer patients, especially those with solid tumors. One of the reasons is that this method would facilitate the detection of the tumor mass without the need to resort to invasive techniques. This work describes the design and characterization of a new biosensor (MlLuc-aff), formed by a novel luciferase fused to an affibody targeting the HER2 membrane receptor. Furthermore, the high affinity of affibody for its HER2 target could be a way to improve tumor specificity and, consequently, early diagnosis.

The small size of MlLuc-aff will also be a great advantage when it comes to its application as a bioluminescent tracer *in vivo*. Given its size, it is predictable that it will present low retention times, as other molecules that have a molecular weight less than 50 kDa. Small size molecules are rapidly eliminated from the circulatory stream through glomerular filtration of the kidneys [20]. Accordingly, lower retention times reduce the likelihood that an adverse reaction will occur [21]. Likewise, the small size of the affibodies favors extravasation into the tissues, allowing the tumor to be detected more easily [22]. Besides, the structure of affibodies is endowed with low immunogenicity [23]. It would be necessary to study the biodistribution and toxicity of MlLuc-aff, but, together with its possible thermostability and its small size, it can be proposed as an interesting tool in the field of clinical oncology. Previous studies have described molecules like MlLuc-aff, capable of detecting the HER2 receptor in the tumor cell membrane but combining affibody with radioisotopes [24] or fluorochromes [25]. None of them use bioluminescent molecules in combination with affibody. It is important to remember that, although the fluorescent signals are brighter, the bioluminescence presents lower levels of background signal [26], so the sensitivity in the detection of tumor cells will increase considerably [27].

The results of this study indicate that the bioluminescent signal of MlLuc-aff is suitable for the identification with high specificity and sensitivity of tumors positive for HER2. For this reason, among the biomedical expectations of MlLuc-aff as a bioluminescent tracer are the visualization of cancer cells and the evaluation of the progression of the disease based on the different treatments used. It would also be interesting to study the ability of MlLuc-aff to block the HER2 receptor signaling pathway. Currently, there are therapies directed against this molecule, such as Trastuzumab, in which it has been seen that there is an inhibition of the growth of breast tumors [28]. In the event that MlLuc-aff were able to act in a similar way to these drugs, the molecule may offer diagnostic and therapeutic capabilities.

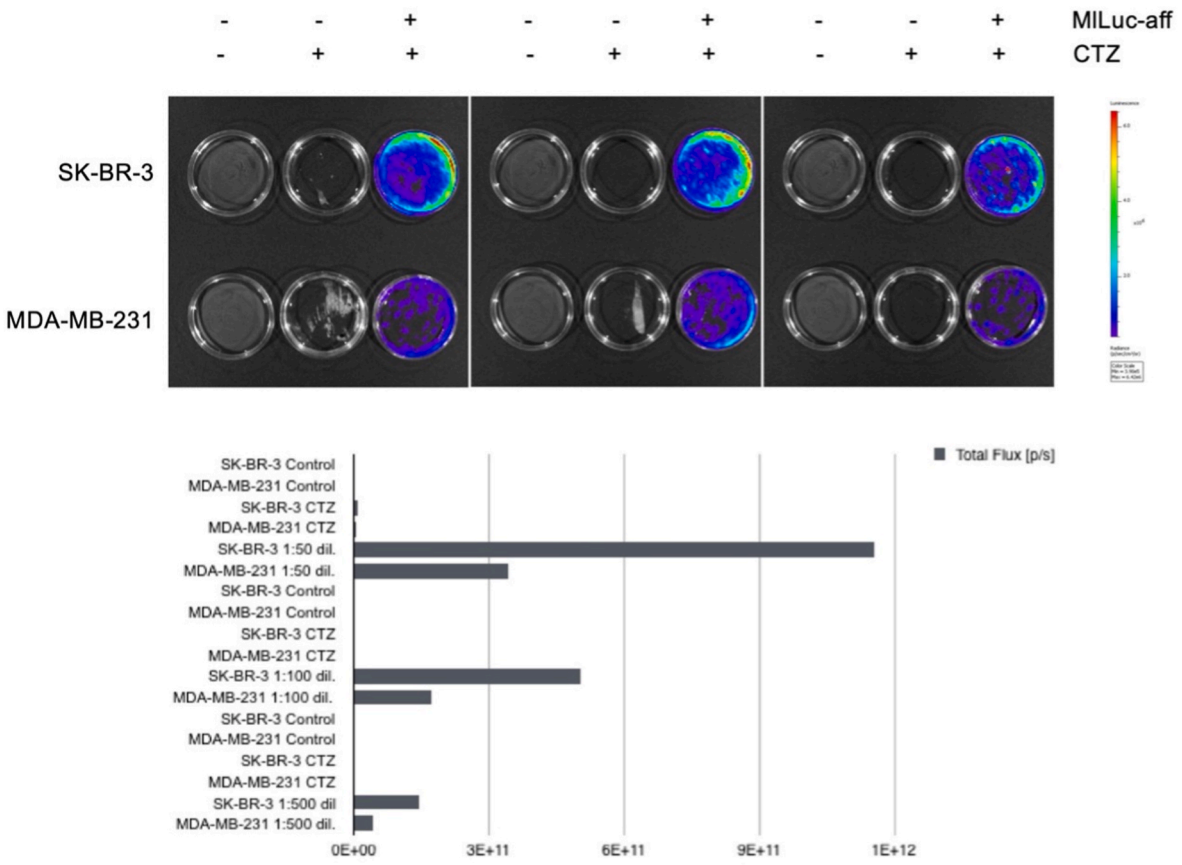


Fig. 4. Binding assay of MlLuc-aff. MlLuc-aff was incubated with the cells (seeded in in 35 mm diameter plates) after dilutions 1:50, 1:100 and 1:500. After 2 h, the cells on the wells were rinsed with PBS three times and bioluminescent activity with the IVIS® Spectrum system by adding the substrate (CTZ) immediately before the measurement. Pseudocolor representation of the bioluminescence intensity from MlLuc-aff. Wells containing medium only with or without CTZ served as negative controls. Quantitation of bioluminescence signal emitted (Total Flux) is expressed in photons/second (p/s).

4. Conclusions

MlLuc1.1 is proposed as a new, small, secretable and ATP-independent luciferase with high thermostability that shows its maximum functional level at pH 8.0 (6.5–9), which is favorable for its application *in vivo*. The fusion of MlLuc1.1 with an affibody against HER2 resulted in a new functional bioluminescent recombinant protein capable of recognizing HER2 membrane receptors present in breast cancer tumor cells with high specificity. MlLuc-aff, as a new sensor for optical imaging, has a broad range of possible applications in diagnostic medicine, from screening and early diagnosis of cancer, disease and therapy monitoring to applications during surgery.

Funding sources

This work received financial support from the Ministerio de Ciencia e Innovación (SAF2009-08629; J.A.C.) and ISCIII, Ministerio de Economía y Competitividad (PI15/01129), the Consellería de Cultura, Educación e Ordenación Universitaria (GPC2014/030, INCITE08PXIB208091PR and PXIB208091PR; J.A.C.), the Axencia Galega de Innovación (Galician Agency of Innovation; 2020-PG068), the Centro Singular de Investigación de Galicia accreditation 2016–2019, ED431G/05) and the European Regional Development Fund (ERDF).

CRediT authorship contribution statement

Laura Rodríguez de la Fuente: Conceptualization, Methodology, Data curation, Validation, Writing – review & editing. **Irene Golán Cancela:** Investigation, Resources. **Ánxela M. Estévez-Salguero:** Investigation, Resources. **Pablo Iglesias:** Investigation, Writing – review & editing, Resources, Data curation. **José A. Costoya:** Conceptualization, Methodology, Data curation, Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors acknowledge Miguel González-Blanco and Raquel Carreira-Rodríguez (DNA Repair and Genome Integrity-CiMUS-USC), Xulio Maside-Rodríguez and Carolina Bartolomé-Husson (Genomics and Bioinformatics Group-CiMUS-USC) and Eugenio Vázquez-Sentís (CIQUS-USC) for helpful discussions.

Abbreviations

CTZ	coelenterazine
DMEM	Dulbecco's modified Eagle's medium
EGFR	epidermal growth factor receptor
gpE	glycoprotein E
HER2	human epidermal growth factor receptor 2
PET	positron emission tomography scan
TBEV	tick-borne encephalitis virus

References

[1] C.M. Connell, G.J. Doherty, Activating HER2 mutations as emerging targets in multiple solid cancers, ESMO Open 2 (2017), e000279.

[2] D.J. Slamon, G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, W.L. McGuire, Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene, Science 235 (1987) 177–182.

[3] S.M. Swain, J. Baselga, S.B. Kim, J. Ro, V. Semiglazov, M. Campone, E. Ciruelos, J. M. Ferrero, A. Schneeweiss, S. Heeson, E. Clark, G. Ross, M.C. Benyunes, J. Cortés, CLEOPATRA Study Group. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer, N. Engl. J. Med. 372 (2015) 724–734.

[4] R.L.B. Costa, H. Soliman, B.J. Czerniecki, The clinical development of vaccines for HER2 $^{+}$ breast cancer: current landscape and future perspectives, Cancer Treat Rev. 61 (2017) 107–115.

[5] R.L.B. Costa, B.J. Czerniecki, Clinical development of immunotherapies for HER2 $^{+}$breast cancer: a review of HER2-directed monoclonal antibodies and beyond, NPJ Breast Cancer 6 (2020) 10.

[6] L. Harris, H. Fritsche, R. Mennel, L. Norton, P. Ravdin, S. Taube, M.R. Somerfield, D.F. Hayes, R.C. Bast Jr., American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer, J. Clin. Oncol. 25 (2007) 5287–5312.

[7] G.A. Ulaner, C.C. Riedl, M.N. Dickler, K. Jhaveri, N. Pandit-Taskar, W. Weber, Molecular imaging of biomarkers in breast cancer, J. Nucl. Med. 57 (Suppl 1) (2016), 53S–9S.

[8] J. Löfblom, J. Feldwisch, V. Tolmachev, J. Carlsson, S. Ståhl, F.Y. Frejd, Affibody molecules: engineered proteins for therapeutic, diagnostic and biotechnological applications, FEBS Lett. 584 (2010) 2670–2680.

[9] C. Grönwall, S. Ståhl, Engineered affinity proteins–generation and applications, J. Biotechnol. 140 (2009) 254–269.

[10] F. Frejd, K. Kim, Affibody molecules as engineered protein drugs, Exp. Mol. Med. 49 (2017) e306.

[11] D. Morse, B.A. Tannous, A water-soluble coelenterazine for sensitive in vivo imaging of coelenterate luciferases, Mol. Ther. 20 (2012) 692–693.

[12] S.V. Markova, M.D. Larionova, E.S. Vysotski, Shining light on the secreted luciferases of marine copepods: current knowledge and applications, Photochem. Photobiol. 95 (2019) 705–721.

[13] S.B. Lee, M. Hassan, R. Fisher, O. Chertov, V. Chernomordik, G. Kramer-Marek, A. Gandjbakhche, J. Capala, Affibody molecules for in vivo characterization of HER2-positive tumors by near-infrared imaging, Clin. Cancer Res. 14 (2008) 3840–3849.

[14] G. Kramer-Marek, D.O. Kiesewetter, L. Martiniova, E. Jagoda, S.B. Lee, J. Capala, [^{18}F]FBEM-$Z_{HER2:342}$-Affibody molecule—a new molecular tracer for in vivo monitoring of HER2 expression by positron emission tomography, Eur. J. Nucl. Med. Mol. Imag. 35 (2008) 1008–1018.

[15] M.D. Larionova, S.V. Markova, N.V. Tikunova, E.S. Vysotski, The smallest isoform of Metridia longaLuciferase as a fusion partner for hybrid proteins, Int. J. Mol. Sci. 21 (2020) 4971.

[16] C.G. England, E.B. Ehlerding, W. Cai, NanoLuc: a small luciferase is brightening up the field of bioluminescence, Bioconjugate Chem. 27 (2016) 1175–1187.

[17] Y. Takenaka, H. Masuda, A. Yamaguchi, S. Nishikawa, Y. Shigeri, Y. Yoshida, H. Mizuno, Two forms of secreted and thermostable luciferases from the marine copepod crustacean, Metridia pacifica. Gene. 425 (2008) 28–35.

[18] A.C. Steffen, A. Orlova, M. Wikman, F.Y. Nilsson, S. Ståhl, G.P. Adams, V. Tolmachev, J. Carlsson, Affibody-mediated tumour targeting of HER-2 expressing xenografts in mice, Eur. J. Nucl. Med. Mol. Imag. 33 (2006) 631–638.

[19] K. Subik, J.F. Lee, L. Baxter, T. Strzepek, D. Costello, P. Crowley, L. Xing, M. C. Hung, T. Bonfiglio, D.G. Hicks, P. Tang, The expression patterns of ER, PR, HER2, CK5/6, EGFR, ki-67 and AR by immunohistochemical analysis in breast cancer cell lines, Breast Cancer Basic Clin. Res. 4 (2010) 35–41.

[20] S. Oliveira, G.A. van Dongen, M. Stigter-van Walsum, R.C. Roovers, J.C. Stam, W. Mali, P.J. van Diest, P.M. van Bergen en Henegouwen, Rapid visualization of human tumor xenografts through optical imaging with a near-infrared fluorescent anti-epidermal growth factor receptor nanobody, Mol. Imag. 11 (2012) 33–46.

[21] L.O. Gainkam, L. Huang, V. Caveliers, M. Keyaerts, S. Hernot, I. Vaneycken, C. Vanhove, H. Revets, P. De Baetselier, T. Lahoutte, Comparison of the biodistribution and tumor targeting of two 99mTc-labeled anti-EGFR nanobodies in mice, using pinhole SPECT/micro-CT, J. Nucl. Med. 49 (2008) 788–795.

[22] Y. Hu, C. Liu, S. Muyldermans, Nanobody-based delivery systems for diagnosis and targeted tumor therapy, Front. Immunol. 8 (2017) 1442.

[23] S. Steeland, R.E. Vandenbroucke, C. Libert, Nanobodies as therapeutics: big opportunities for small antibodies, Drug Discov. Today 21 (2016) 1076–1113.

[24] M. Kijanka, F.J. Warnders, M. El Khattabi, M. Lub-de Hooge, G.M. van Dam, V. Ntziachristos, L. de Vries, S. Oliveira, P.M. van Bergen En Henegouwen, Rapid optical imaging of human breast tumour xenografts using anti-HER2 VHHs site-directly conjugated to IRDye 800CW for image-guided surgery, Eur. J. Nucl. Med. Mol. Imag. 40 (2013) 1718–1729.

[25] M. Satpathy, R. Zielinski, I. Lyakhov, L. Yang, Optical imaging of ovarian cancer using HER-2 affibody conjugated nanoparticles, Methods Mol. Biol. 1219 (2015) 171–185.

[26] T. Troy, D. Jekic-McMullen, L. Sambucetti, B. Rice, Quantitative comparison of the sensitivity of detection of fluorescent and bioluminescent reporters in animal models, Mol. Imag. 3 (2004) 9–23.

[27] D.K. Tiwari, M. Tiwari, J. Takashi, Near-infrared fluorescent protein and bioluminescence-based probes for high-resolution in vivo optical imaging, Mater Adv 1 (2020) 967–987.

[28] K. McKeage, C. M. Perry Trastuzumab, A review of its use in the treatment of metastatic breast cancer overexpressing HER2, Drugs 62 (2002) 209–243.