References

[1] Troilius Rubin A, Lauritzen E, Ljunggren B, Revencu N, Vikkula M, Svensson A, Heredity of port-wine stains: investigation of families without a RASA1 mutation, J Cosmet Laser Ther. 17 (2015) 204-208. https://doi.org/10.3109/14764172.2015.1007060.

[2] Fu Z, Huang J, Xiang Y, Huang J, Tang Z, Chen J, et al, Characterization of Laser-Resistant Port Wine Stain Blood Vessels Using In Vivo Reflectance Confocal Microscopy, Lasers Surg Med. 51 (2019) 841-949. https://doi.org/10.1002/lsm.23134.

[3] Tan W, Zakka LR, Gao L, Wang J, Zhou F, Selig MK, et al, Pathological alterations involve the entire skin physiological milieu in infantile and early-childhood port-wine stain. Br J Dermatol. 177 (2017) 293-296. https://doi.org/10.1111/bjd.15068.

[4] Hagen SL, Grey KR, Korta DZ, Kelly KM, Quality of life in adults with facial port-wine stains, J Am Acad Dermatol. 76 (2017) 695-702. https://doi.org/10.1016/j.jaad.2016.10.039.

[5] Huang YC, Tran N, Shumaker PR, Kelly K, Ross EV, Nelson JS, et al, Blood flow dynamics after laser therapy of port wine stain birthmarks, Lasers Surg Med. 41 (2009) 563-571. https://doi.org/10.1002/lsm.20840.

[6] Zhang Y, Zou X, Chen H, Yang Y, Lin H, Guo X, Clinical study on clinical operation and post-treatment reactions of HMME-PDT in treatment of PWS, Photodiagnosis Photodyn Ther. 20 (2017) 253-256. https://doi.org/10.1016/j.pdpdt.2017.09.013.

[7] Zhao Y, Zhou Z, Zhou G, Tu P, Zheng Q, Tao J, et al, Efficacy and safety of hemoporfin in photodynamic therapy for port-wine stain: a multicenter and open-labeled phase IIa study, Photodermatol Photoimmunol Photomed. 27 (2011) 17-23. https://doi.org/10.1111/j.1600-0781.2010.00555.x.

[8] Zhao Y, Tu P, Zhou G, Zhou Z, Lin X, Yang H, et al, Hemoporfin Photodynamic Therapy for Port-Wine Stain: A Randomized Controlled Trial, PLoS One. 11 (2016) e0156219. https://doi.org/10.1371/journal.pone.0156219.

[9] Shen ZY, Hu B, Wu MF, Correlation between blood flow signal of color flow imaging and nottingham prognostic index in patients with breast carcinoma, Breast Care (Basel). 7 (2012) 126-130. https://doi.org/10.1159/000337766.

[10] Wanitphakdeedecha R, Sudhipongpracha T, Ng JNC, Yan C, Jantarakolica T, Self-stigma and psychosocial burden of patients with port-wine stain (PWS): a systematic review and meta-analysis, J Cosmet Dermatol. 2021. https://doi.org/10.1111/jocd.14199.

[11] Kelly KM, Choi B, McFarlane S, Motosue A, Jung B, Khan MH, et al, Description and analysis of treatments for port-wine stain birthmarks, Arch Facial Plast Surg. 7 (2005) 287-294. https://doi.org/10.1001/archfaci.7.5.287.

[12] Sharif SA, Taydas E, Mazhar A, Rahimian R, Kelly KM, Choi B, et al, Noninvasive clinical assessment of port-wine stain birthmarks using current and future optical imaging technology: a review, Br J Dermatol. 167 (2012) 1215-1223. https://doi.org/10.1111/j.1365-2133.2012.11139.x.

[13] Moy WJ, Ma G, Kelly KM, Choi B, Hemoporfin-mediated photodynamic therapy on normal vasculature: implications for phototherapy of port-wine stain birthmarks, J Clin Transl Res. 2 (2016) 107-111. https://doi.org/10.18053/jctres.02.201603.003.

[14] Ma G, Han Y, Ying H, Zhang X, Yu W, Zhu J, et al, Comparison of two generation photosensitizers of PsD-007 and hematoporphyrin monomethyl ether photodynamic therapy for treatment of port-wine stain: a retrospective study, Photobiomodul Photomed Laser Surg. 37 (2019) 376-380. https://doi.org/10.1089/photob.2018.4593.

[15] Gao K, Huang Z, Yuan KH, Zhang B, Hu ZQ, Side-by-side comparison of photodynamic therapy and pulsed-dye laser treatment of port-wine stain birthmarks, Br J Dermatol. 168 (2013) 1040-1046. https://doi.org/10.1111/bjd.12130.

[16] Choi B, Tan W, Jia W, White SM, Moy WJ, Yang BY, et al, The role of laser speckle imaging in port-wine stain research: recent advances and opportunities, IEEE J Sel Top Quantum Electron. 2016 (2016). https://doi.org/10.1109/JSTQE.2015.2493961.

[17] Pu Y, Chen W, Yu Z, Research progress of Hemoporfin–part one: preclinical study, Photodiagnosis Photodyn Ther. 9 (2012) 180-185. https://doi.org/10.1016/j.pdpdt.2011.09.004.

[18] Lei TC, Glazner GF, Duffy M, Scherrer L, Pendyala S, Li B, et al, Optical properties of hematoporphyrin monomethyl ether (HMME), a PDT photosensitizer, Photodiagnosis Photodyn Ther. 9 (2012) 232-242. https://doi.org/10.1016/j.pdpdt.2012.01.003.

[19] Yuan KH, Gao JH, Huang Z, Adverse effects associated with photodynamic therapy (PDT) of port-wine stain (PWS) birthmarks, Photodiagnosis Photodyn Ther. 9 (2012) 332-336. https://doi.org/10.1016/j.pdpdt.2012.03.007.

[20] Liang H, Zhou Z, Luo R, Sang M, Liu B, Sun M, et al, Tumor-specific activated photodynamic therapy with an oxidation-regulated strategy for enhancing anti-tumor efficacy, Theranostics. 8 (2018) 5059-5071. https://doi.org/10.7150/thno.28344.

[21] van Drooge AM, Beek JF, van der Veen JP, van der Horst CM, Wolkerstorfer A, Hypertrophy in port-wine stains: prevalence and patient characteristics in a large patient cohort, J Am Acad Dermatol. 67 (2012) 1214-1219. https://doi.org/10.1016/j.jaad.2012.05.027.

[22] Wen L, Zhang Y, Zhang L, Liu X, Wang P, Shen S, et al, Application of different noninvasive diagnostic techniques used in HMME-PDT in the treatment of port wine stains, Photodiagnosis Photodyn Ther. 25 (2019) 369-375. https://doi.org/10.1016/j.pdpdt.2019.01.008.

[23] Zhu YC, Zhang Y, Deng SH, Jiang Q, A prospective study to compare superb microvascular imaging with grayscale ultrasound and color doppler flow imaging of vascular distribution and morphology in thyroid nodules, Med Sci Monit. 24 (2018) 9223-9231. https://doi.org/10.12659/MSM.911695.

doi: 10.1016/j.pdpdt.2024.104181

Gold nanoparticle-based two-photon fluorescent nanoprobe for monitoring intracellular nitric oxide levels

Carla Arnau del Valle [a], Paul Thomas [b], Francisco Galindo [c], M. Paz Muñoz-Herranz [d], María J. Marín [d]

[a] *Fischell Department of Bioengineering, University of Maryland, College Park, Maryland, United States*

[b] *School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, UK*

[c] *Departamento de Química Inorgánica y Orgánica, Universitat Jaume I, Castellón, Spain, School of Chemistry*

[d] *University of East Anglia, Norwich Research Park, Norwich, UK*

Significance: Nitric oxide (NO) is involved in the regulation of physiological and pathological mechanisms of the cardiovascular, nervous and immune systems; and plays an important role in cancer being involved in tumor growth and suppressing processes depending on its concentration.

Approach: The development of a near-infrared excitable nanoprobe, consisting of gold nanoparticles functionalized with a two-photon excitable NO probe, for the detection of intracellular NO is reported.

Results: The nanoprobe showed good selectivity towards NO over cellular interferences and excellent stability in aqueous-medium over time. The nanoprobe was able to selectively detect endogenous and exogenous NO in different cell lines and it accumulated in the acidic organelles showing negligible toxicity. Importantly, the nanoprobe showed potential to quantify intracellular NO concentrations in breast cancer cells.

Conclusions: The novel gold-based two-photon nanoprobe showed an excellent performance and versatility and could potentially be applied for the spatiotemporal monitoring of in vivo NO levels.

Keywords: photodiagnosis, nitric oxide, nanoparticles, cancer, two-photon, near-infrared

1. Introduction and Background

NO regulates physiological processes when present at low concentrations; however, at high levels, it is related to pathologies including cancer [1,2]. NO can be monitored using fluorescent probes, however, most of these are excited using ultraviolet or visible light, which results in poor penetration and high photodamage to cells. Alternatively, near-infrared light provides high photostability and tissue penetration, low level of autofluorescence and minimal photodamage upon long-term irradiation [3]. Although NO probes have been reported, the development of novel tools for the detection and quantification of NO is required for a better understanding of its role in biological processes.

2. Aims

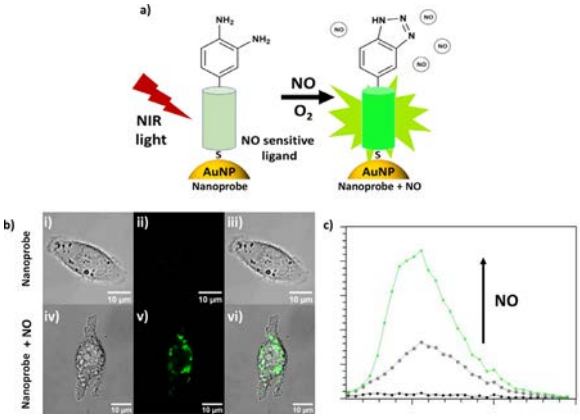
The importance of NO in cancer, motivates the development of tools for monitoring intracellular levels of NO. The research presented here aims to develop a novel near-infrared excitable fluorescent nanoprobes to improve the detection and quantification of NO in biological environments and to, potentially, combine it with photodynamic therapy.

3. Methods

A NO-sensitive ligand was synthesized similarly to the molecular NO probe published in our previous work [4] containing an *o*-phenylenediamine moiety, a naphthalimide core and a thiolated chain. The fluorescence emission intensity of the probe is quenched in the absence of NO *via* photoinduced electron transfer (PET). In the presence of NO, the PET is cancelled and the fluorescence emission of the probe is restored. The NO-sensitive ligand was used to functionalized gold nanoparticles yielding the desired nanoprobe and it was characterized by means of UV-Vis and steady state fluorescence spectroscopies, and transmission electron microscopy. Selectivity, sensibility and stability of the nanoprobe was investigated in solution. *In vitro* application of the nanoprobe was evaluated in mouse macrophages (RAW264.7ϒ NO^-), human leukemia macrophages (THP-1), endothelial and breast cancer cells (MDA-MB-231). Confocal laser scanning (images and emission spectra) and two-photon microscopies were used to confirm the internalization and exogenous and endogenous NO detection by the nanoprobe. LysoTracker™ Red DND-99 was used to study the colocalization of the nanoprobe within the cells. Cell viability studies were performed using CellTiter-Blue® cell viability assay. *S*-nitroso-*N*-acetylpenicillamine (SNAP) was used as NO donor, Ca^{2+} ionophore A-23187 was used to stimulated NO production in endothelial cells and lipopolysaccharide (LPS) and interferon gamma (IFN-ϒ) were used to stimulate NO production in macrophages. *N*-nitroarginine methyl ester (L-NAME) inhibited NO production. Flow cytometry was used to study a large population of cells looking at internalization and NO detection. An intracellular calibration curve was obtained using the nanoprobe within MDA-MB-231 cells.

4. Results

A goldNP-based NO nanoprobe was developed by self-assembling a thiolated NO-sensitive ligand onto the surface of goldNPs (Figure 1.a). The nanoprobe (2.4 ± 0.7 nm) was characterized in solution by studying its selectivity and sensitivity towards NO (limit of detection of 1.30 μM), and its stability in a range of pH values of intracellular relevance (4.7 - 8) and over time. The nanoprobe was employed for the intracellular detection of NO in mouse macrophages (RAW264.7ϒ NO^-), human leukaemia macrophages (THP-1), endothelial and breast cancer cells (MDA-MB-231). Confocal images (Figure 1.b) and fluorescence emission spectra (Figure 1.c) of the cells demonstrated the successful intracellular NO detection by the nanoprobe. Upon stimulation of the cells to produce NO or treatment with NO donor, enhancement of the fluorescence emission intensity of the nanoprobe was evidenced confirming the NO detection. The nanoprobe was able to detect endogenous NO in macrophages and endothelial cells and exogenous NO in endothelial and breast cancer cells; and inhibition of the NO-synthase enzyme allowed confirmation of selective NO detection. Great biocompatibility was observed for all tested cells. Colocalization studies proved the accumulation of the nanoprobe in the acidic organelles of the cells. The ability of the nanoprobe to be excitable *via* two-photon was demonstrated and the NO detection was also visualized through multiphoton microscopy. The potential application of nanoprobe to quantify intracellular NO concentrations was explored in MDA-MB-231 breast cancer cells showing a linear correlation between the nanoprobe's fluorescence intensity and the concentration of NO released in the cells.



5. Conclusion

The combination of a fluorescent NO probe and goldNPs to develop a two-photon fluorescent NO nanoprobe is reported. The research described here includes 1) synthesis and characterisation of the first two-photon nanoprobe based on goldNPs; 2) ability of the nanoprobe to be visualised under NIR light using a multiphoton microscope; 3) excellent performance of the nanoprobe to detect, in a selective manner, exogenous and endogenous NO levels produced in a variety of cellular environments; 4) great biocompatibility of the nanoprobe in all the cellular systems investigated; and 5) successful use of different analytical techniques to monitor the performance of the nanoprobe including confocal, multiphoton microscopy and flow cytometry. All these results suggested the potential of the nanoprobe to be used as a nanoplatform for the development of more sophisticated nanosystems including ratiometric nanoprobes for NO quantification or theranostic nanotools when combining the NO probe with a photosensitizer for photodynamic therapy.

Disclosures if required

There are no conflicts to declare.

Acknowledgments

The authors acknowledge Dr Derek Warren and Dr Anastasia Sobolewski (School of Pharmacy, University of East Anglia) for kindly providing the endothelial cells and THP-1 cells, respectively. The authors acknowledge Dr. A. Goldson for training and guidance on the flow cytometer; Dr. P. Wilson for training and guidance on the multiphoton microscope. The authors would like to thank the Faculty of Sciences and School of Chemistry at the University of East Anglia and Mr. and Mrs. Whittaker oncology fellowship for financial support, and the EPSRC (Grant

EP/S017909/1) that supported the purchase of the Edinburgh Instrument FS5 fluorescence spectrometer used in this work.

References

[1] S. Korde Choudhari, M. Chaudhary, S. Bagde, A.R. Gadbail, V. Joshi, Nitric oxide and cancer: a review. World J. Surg. Oncol. 11 (2013) 118. https://doi.org/10.1186/1477-7819-11-118
[2] T. Nagano, T. Bioimaging Probes for Reactive Oxygen Species and Reactive Nitrogen Species, J. Clin. Biochem. Nutr. 45 (2009) 111-124. https://doi.org/10.3164/jcbn.R09-66
[3] G. Hong, A. L. Antaris, H. Dai, Near-infrared fluorophores for biomedical imaging. Nat. Biomed. Eng. 1 (2017) 0010. https://doi.org/10.1038/s41551-016-0010
[4] C. Arnau del Valle, L. Williams, P. Thomas, R. Johnson, S. Raveenthiraraj, D. Warren, A. Sobolewski, M. P. Muñoz, F. Galindo, M. J. Marín, A highly photostable and versatile two-photon fluorescent probe for the detection of a wide range of intracellular nitric oxide concentrations in macrophages and endothelial cells. J. Photochem. Photobiol. B: Biol. 234 (2022) 112512. https://doi.org/10.1016/j.jphotobiol.2022.112512

doi: 10.1016/j.pdpdt.2024.104182

Design and Synthesis of Difluoroboronite Curcuminoid derivatives for application in photodynamic therapy

Sneha Mishra, Neeraj Agarwal

UM-DAE, Centre for Excellence in Basic Sciences, University of Mumbai, Santacruz (E), Mumbai, 400098, India

This research paper describes the synthesis of two derivatives, 1 and 2, using aldehydes containing acid-sensitive groups. Both derivatives are of the donor-acceptor-donor type and exhibit absorption and emission in the red region. Derivative 1 has an absorption peak at 647 nm and an emission peak at approximately 713 nm. These molecules possess intramolecular charge transfer properties, allowing them to populate a triplet state that produces singlet oxygen, which plays a crucial role in cell death. Furthermore, the acid-sensitive groups on these derivatives enable selective accumulation in slightly acidic cancer cells. The cellular uptake of these derivatives can be enhanced through the formation of nanoaggregates, with average particle sizes of 112 nm and 81 nm for 1 and 2, respectively. While derivative 2 has a higher capacity for singlet oxygen production ($\varphi\Delta$~0.40) and better fluorescence quantum yield than derivative 1, both molecules have high molar absorption coefficients and can serve as potential photosensitizers for cancer treatment.

Keywords: Intramolecular charger transfer, Donor-acceptor-donor molecule, singlet oxygen

1. Introduction:

In photodynamic therapy, a photosensitizer with high molar absorptivity within the therapeutic window of 600-850 nm, zero dark cell toxicity, is required. Most importantly, PS should be capable of producing a significant amount of singlet oxygen.[1,2] To enhance singlet oxygen production, heavy atoms can be introduced to induce spin orbit coupling, which populates the triplet state of the photosensitizer. Alternatively, charge transfer properties of donor-acceptor systems can be utilized to achieve the same effect without the need for heavy atoms.[3] Difluoroboronite curcuminoid derivatives possess a central dioxaborine ring that acts as a strong electron-acceptor unit and two terminal electron-donor groups, creating a donor-acceptor-donor system that exhibits charge transfer properties. Electrons can be transferred from the charge transfer state to populate the triplet states of similar energy, thus making these derivatives suitable as photosensitizers for photodynamic therapy. Additionally, difluoroboronite curcuminoid derivatives have been previously studied and utilized as a near-infrared cell-imaging probe.[4]

2. Aims

The objective of this study is to synthesize novel Donor-acceptor-donor type photosensitizers that absorb in the red-NIR region and are suitable for use in photodynamic therapy. The ultimate aim is to develop photosensitizers that have a high selectivity towards cancerous cells.

3. Methods

(a) Difluoroboronite curcuminoid derivatives 1 and 2 were synthesized in two steps using the scheme presented below.

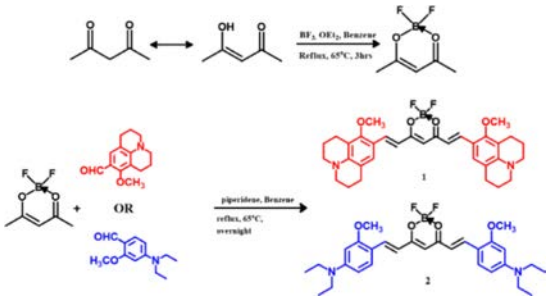


Fig 1: Scheme of Synthesis of **1** and **2**

(b) The synthesis of these derivatives was confirmed using 1H NMR and MALDI-TOF.
(c) Detailed photophysical studies were carried out in solvents with varying polarities.
(d) Nanoaggregates of these derivatives were synthesized using PEG 1500 to increase their surface area, water solubility, and cellular uptake. The particle size of these nanoaggregates was characterized using DLS.
(e) Singlet oxygen generation studies were performed for these derivatives using the DPBF assay. The photooxidation of DPBF was monitored at different time intervals in solvents with varying polarities. Similarly, singlet oxygen generation studies were carried out for their nanoaggregates.

4. Results and Discussion

Compounds **1** and **2** were synthesized using Knoevenagel condensation with different aldehydes to fine-tune their photophysical properties. The aldehydes contained amine groups, which act as acid-sensitive moieties that can be selectively activated in cancerous cells due to their slightly acidic nature. Both derivatives exhibit strong absorption and emission bands in the red region. Photophysical studies were conducted on 1 and 2 in solvents with different polarities. The absorption spectra of both compounds showed a **bathochromic shift** with an **increase in solvent polarity**, indicating their polar nature in the ground state. Additionally, the emission spectra of both compounds exhibited a bathochromic shift with an increase in solvent polarity, but their intensity decreased, indicating the formation of a charge transfer state in these derivatives as charge transfer state depends on solvent polarity. The nanoaggregates of 1 and 2 also absorbed and emitted in the red region. These nanoaggregates have good water solubility and have been incorporated into a biocompatible polymer, PEG 1500, which is commonly used for drug loading in cell lines.