COMMUNICATION

Cu-Based Turn-on Fluorescent Sensors for Cu-rich Amyloid β Aggregates

Received 00th January 20xx,
Accepted 00th January 20xx

Yiran Huang,[a] Liang Sun,[a] and Liviu M. Mirica[a,*]

Protein misfolding and metal dishomeostasis are two key pathological factors of Alzheimer's disease. Previous studies have showed that Cu-mediated Aβ aggregation pathways lead to formation of neurotoxic Aβ oligomers. Herein, we reported a series of picolinic acid-based Cu-activatable sensors, which can be used for the fluorescence imaging of Cu-rich Aβ aggregates.

The formation of extracellular amyloid plaques containing the amyloid β (Aβ) peptide is one of pathological hallmark in the Alzheimer's Disease patients' brains.[1] Remarkably high concentration of Cu and Zn have been found within the amyloid plaques in AD patients' brains.[2, 3] Several studies have explored the interactions of metals with monomeric Aβ peptides and their correlation with amyloid plaques and reactive oxygen species formation.[4-10] These studies show that Cu can slow down the aggregation of $A\beta_{42}$ and reduce the fibrilization to a large extent, and is considered that the copper ions can stabilize the $A\beta_{42}$ oligomer species.[11] In this regard, novel molecules that can modulate the interaction of copper ions with the soluble $A\beta_{42}$ species and alleviate the neurotoxicity may serve as novel therapeutic agents for AD.[12-14]

In addition to the development of new therapeutic agents, it is also highly important to detect the Cu-induced Aβ species in AD. A number of fluorescent sensors have been developed to probe biological copper fluxes.[15-19] These reporters can achieve high selectivity and signal-to-noise responses for copper ion imaging from cellular to tissue to whole animal settings.[15, 20-22] However, only very few of the probes were utilized in detecting labile copper pools in AD,[23, 24] even though countless of studies have shown the copper homeostasis were dramatically disrupted. In this regard, to understand and probe the Cu-mediated Aβ aggregation process, it is significantly crucial to develop Cu-Aβ specific probes. Herein, we rationally designed and synthesized a series of Cu-based activable sensors to detect the Cu-Aβ species *in vitro* and *ex vivo*. By linking the picolinic ester moiety with the strong Aβ binding fragments, the copper ions can rapidly catalyze the hydrolysis reaction of the ester bond to generate the high fluorescent Aβ binding molecules *in vitro*. More interestingly, the Cu-responsive sensors can also promptly react with Cu-Aβ oligomers and fibrils, resulting in a significant fluorescence turn-on, and indicating that the probes are also able to detect Cu-Aβ species *in vitro*. To confirm the Aβ binding specificity of the probes, 5xFAD brain sections were stained with the developed sensors. As expected, when the brain sections only stained with the compounds, the fluorescence images show that the sensor has poor ability to detect amyloid plaque, given the low fluorescence intensity. However, to mimic the Cu-rich environment in AD brain, with the addition of excess amounts of Cu(II) to the solution, the fluorescence images clearly indicate that the Cu-responsive sensors were activated by Cu(II) and release the high fluorescent amyloid binding fluorophores which can specifically label the amyloid plaques on the 5xFAD brain sections.

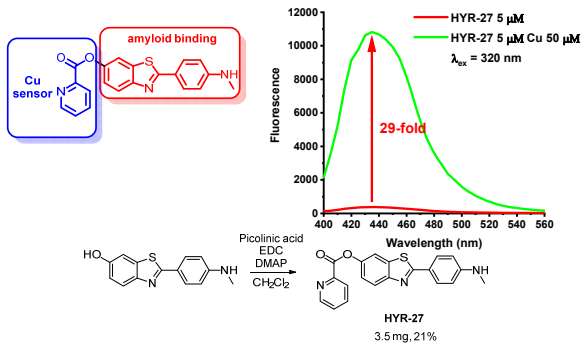


Fig. 1 Design and synthesis of Cu-based activatable sensor **HYR-27**. Cu(II) fluorescence turn-on effects of **HYR-27**. [**HYR-27**] = 5 μM, [Cu] = 50 μM in PBS buffer (pH =7.4).

To detect Cu(II) in various Aβ species, the developed fluorescence probes includes two components: a Cu(II)-

[a] *Department of Chemistry, University of Illinois at Urbana-Champaign, 600 S. Mathews Avenue, Urbana, IL 61801, United States*
Correspondence: mirica@illinois.edu (L.M.M.)

† Electronic supplementary information (ESI) available: Experimental procedures, spectroscopic data, and copies of NMR spectra.

responsive 2-picolinic ester group that chelates Cu(II), activating the ester bond for hydrolysis, and a widely used Aβ binding molecule, Pittsburgh compound B (PiB), which has high fluorescence intensity and can strongly interact with amyloid fibrils.

To evaluate the copper-responsive activity of the probe, we firstly performed the Cu turn-on assay in PBS buffer. With the attachment of the picolinic ester moiety, the fluorescence intensity of the probe is dramatically quenched. However, when we treated the Cu(II) with **HYR-27**, the fluorescence intensity significantly increased ~29 folds, indicating that the compounds can coordinates to the Cu(II) to facilitate the hydrolysis reaction to enhance the fluorescence intensity.

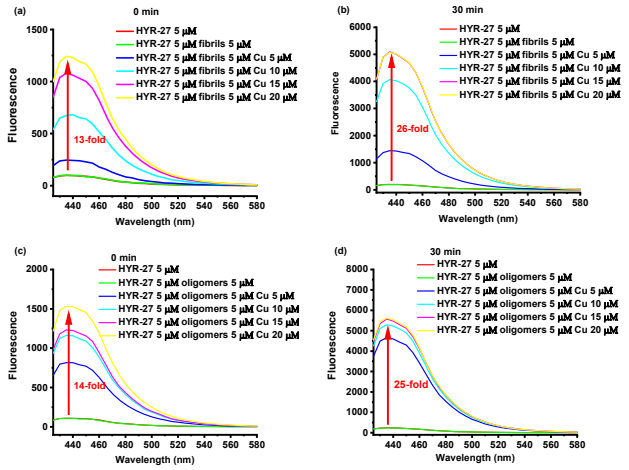


Fig. 2 Cu(II) fluorescence turn-on results of **HYR-27** toward Aβ fibrils and oligomers. [**HYR-27**] = 5 μM, [Aβ] = 5 μM, [Cu] = 0-20 μM in PBS buffer (pH =7.4).

Then, we explored the Cu-activable ability of **HYR-27** with the presence of various Aβ species. When the compound was treated with Aβ fibrils only, no fluorescence enhancement was observed after 30 minutes incubation. However, when Cu-Aβ fibrils were added to the solution, the fluorescence intensity dramatically increased, probably due to the rapid Cu(II)-catalyzed hydrolysis reaction. With more copper ions adding into the solution, all **HYR-27** were hydrolyzed to generate the PiB compounds, exhibiting a similar fluorescence turn-on effect (~26 folds), comparing with the copper-responsive studies. Because Cu(II) can stabilize the formation of Aβ oligomers, it is also highly crucial to investigate if the developed probe can also detect Cu-Aβ oligomers. As a result, firstly, we treated the compounds with Aβ oligomers only. Similar as Aβ fibrils studies, no obvious fluorescence turn-on was observed with 30 minutes incubation. Excitingly, when Cu(II) was added to the solution, the fluorescence intensity immediately increased, indicating that the probe can compete with Aβ oligomers to chelate the Cu(II) to facilitate the hydrolysis reaction to release the high fluorescent PiB probes. Furthermore, it also shows similar fluorescence enhancements, comparing with the control studies. Overall, the Cu-dependent fluorescence turn-on studies with or without various Aβ species clearly demonstrate that the developed probes can be efficiently activated by the Cu(II), Cu(II)-Aβ fibrils and oligomers, exhibiting appreciable fluorescence enhancement *in vitro*.

To further explore the Aβ binding affinity and Cu-activable activity of **HYR-27**, we also performed fluorescence staining studies with the probe on the 11-mon-old 5xFAD brain sections, which can rapidly develop severe amyloid pathology. Firstly, the brain sections were only stained with **HYR-27**. The low fluorescent image shows that the compound cannot efficiently detect amyloid plaques, which is consistent with the *in vitro* studies. To mimic the real Cu-rich environment in AD brain, we further performed the brain sections staining studies with the addition of 50 μM Cu(II). Excitingly, under same conditions, the fluorescence image clearly shows that the fluorescence intensity significantly increased after the incubation with Cu(II), indicating that **HYR-27** was rapidly hydrolyzed to form PiB, which can strongly bind to the amyloid plaques, with the presence of the Cu(II).

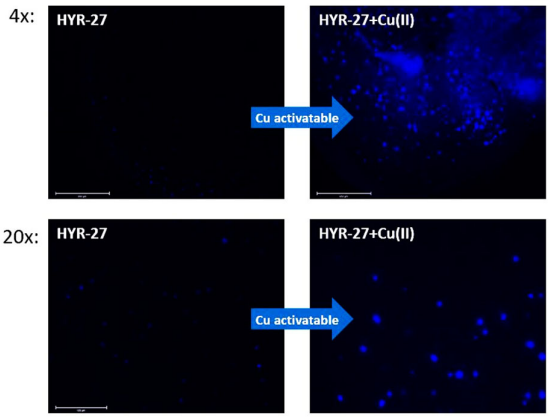


Fig. 3 Fluorescence microscopy images of 5xFAD mice brain sections incubated with compounds **HYR-27** (left panel) and **HYR-27**+Cu (right panel). [**HYR-27**] = 5 μM, [Cu] = 50 μM. Scale bar (20×): 125 μm.

To further confirm the Aβ binding specificity, the brain sections were firstly stained with **HYR-27**, **HYR-27** with Cu(II) and sequentially immunostained with HJ3.4, which can bind to all Aβ species. The fluorescence images show that **HYR-27** did not have good colocalization with HJ3.4 antibody. However, with the presence of Cu(II), the hydrolyzed product of **HYR-27** dramatically increases the colocalization with the pan-Aβ antibody, exhibiting that the **HYR-27** has high Cu-responsive ability which can be utilized to detect the Cu-Aβ amyloid plaques on 5xFAD brain sections.

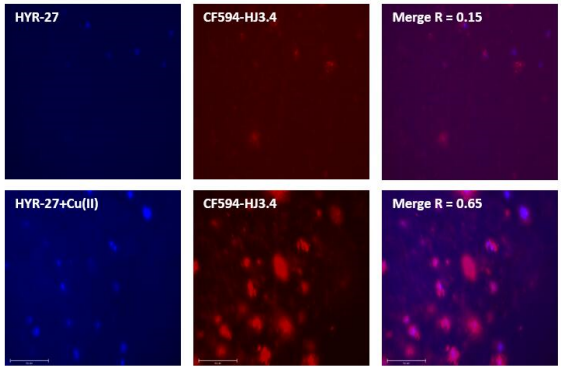
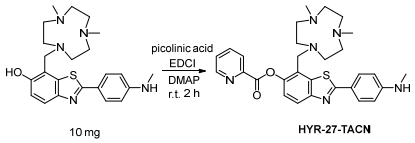


Fig. 4 Fluorescence microscopy images of 5xFAD mice brain sections incubated with compounds **HYR-27** (top left panel), **HYR-27**+Cu (bottom left panel), HJ3.4 (middle panels), and merged images (right panel). [**HYR-27**] = 5 μM, [Cu] = 50 μM, [HJ3.4] = 1 μg/ml. Scale bar: 125 μm.

Since the chelation of Cu(II) can facilitate the hydrolysis of the picolinic ester bond, a strong Cu(II) binding ligand, Me_2HTACN, was introduced to the **HYR-27** fragment. However, when Cu(II) was added, the fluorescence intensity was dramatically decreased, indicating that the copper ions only quenched the fluorescence intensity because of the paramagnetic nature, instead of catalyzing the hydrolysis reaction. The reason that Cu(II) was only served as a fluorescence quencher is probably that the TACN and pyridine ligand can easily chelate with Cu(II) to form 4-coodinates complex, blocking the binding site of the ester bond with copper ions, resulting in loss of esterase activity.



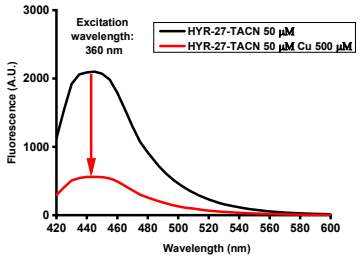
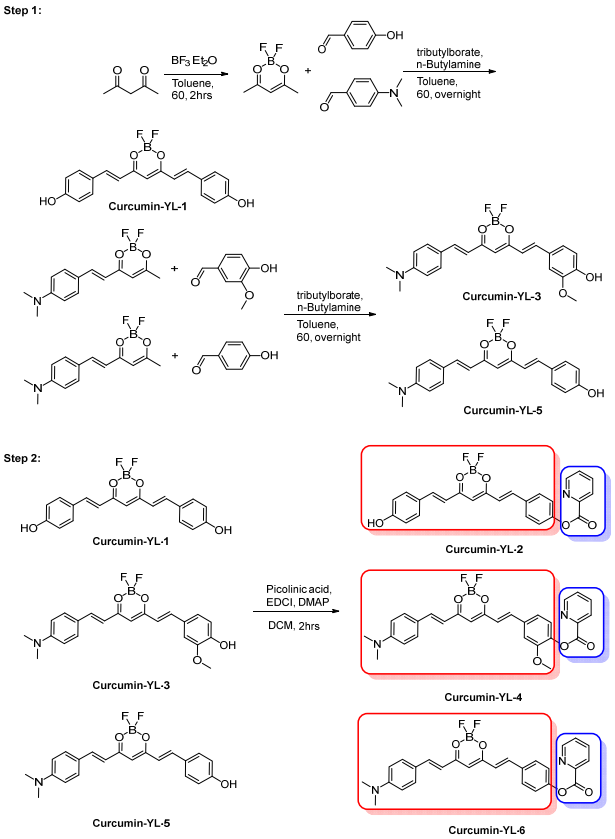


Fig. 5 Cu(II) fluorescence turn-on effects of **HYR-27-TACN**. [**HYR-27-TACN**] = 50 μM, [Cu] = 500 μM in PBS buffer (pH =7.4).

After achieving promising results that **HYR-27** can be activated by Cu(II), a series of curcumin derivatives which have high Aβ binding affinity and near-infrared (NIR) emission properties were integrated with the Cu(II)-responsive picolinic ester. The syntheses of the curcumin precursors, **YL-1**, **-3**, **-5**, were carried out by following previously reported procedures,[25] and we synthesized the final Cu(II)-responsive curcumin derivatives **YL-2**, **-4**, **-6**, via an EDC-mediated coupling reaction between 2-picolinic acid and the curcumin precursors.

Scheme 1 Synthesis of Curcumin Cu-based activatable sensors.



To confirm the Cu-activatable ability of the developed curcumin derivatives, the fluorescence turn-on experiments were performed in PBS buffer. However, the solubility of the compounds is poor in PBS buffer, so methanol was selected as the solvent to perform the Cu(II) turn-on assay. With the presence of Cu(II), all curcumin derivatives with the picolinic ester moiety have a remarkably fluorescence turn-on, proving that the developed compounds can be activated by the Cu(II) (Fig. 6).

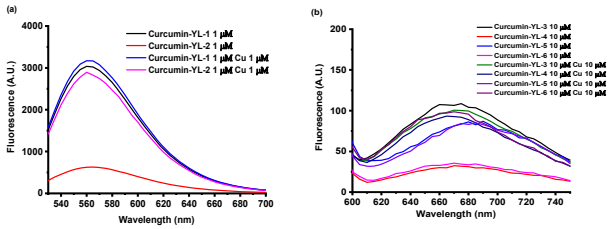


Fig. 6 Cu(II) fluorescence turn-on effects of **YL-1-6**. [**YL**] = 1 or 10 μM, [Cu] =1 or 10 μM in MeOH.

Please do not adjust margins

To investigate the Cu-activatable activity of the curcumin derivatives towards amyloid plaques, the fluorescence staining studies were also exploited with **YL-4** on 5xFAD brain sections. We first stained the brain sections with **YL-4** only, and the fluorescence images show that compound **YL-4** is able to bind amyloid plaques on the brain sections (Fig. 7). However, the fluorescence intensity of the **YL-4**-stained amyloid plaques is low, and it also shows that the compound can only stain the mature fibrils where are the core of the amyloid plaques. By comparison, to mimic the Cu-rich environment in AD brains, the 5xFAD brain sections were stained with **YL-4** and Cu(II). Excitingly, with the addition of Cu(II), the fluorescence intensity of compound-stained amyloid plaques was dramatically increased, indicating that **YL-4** was efficiently activated by Cu(II), generating the more fluorescent curcumin derivative, **YL-3**. More interestingly, with the incubation of **YL-4** and Cu(II), the resulting compound cannot only stain the core but also the peripheral region of the amyloid plaques where possibly Aβ oligomers exist, exhibiting the potential ability to detect Cu-Aβ oligomers of **YL-4** *ex vivo*. For the other curcumin compounds, due to the poor solubility in PBS buffer, the brain sections staining studies were not performed. Overall, these brain section staining studies clearly demonstrate that **YL-4** can be utilized as a Cu-responsive sensor for the detection of Cu-rich amyloid plaques.

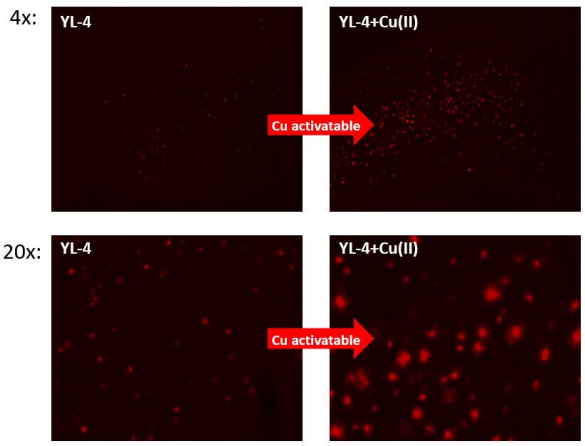


Fig. 7 Fluorescence microscopy images of 5xFAD mice brain sections incubated with compounds **YL-4** (left panel) and **YL-4**+Cu (right panel). [**YL-4**] = 50 μM, [Cu] = 50 μM. Scale bar: 125 μm.

In conclusion, we rationally designed and synthesized a series of Cu-based activatable sensors to detect the Cu-Aβ species *in vitro* and *ex vivo*. With the introduction of the picolinate ester moiety to the strong Aβ-binding fragment, the developed sensors can efficiently chelate the Cu(II) to facilitate the hydrolysis of the ester bond to release the high fluorescent Aβ-binding molecules *in vitro*. Furthermore, the Cu-responsive sensors can also rapidly react with Cu-Aβ oligomers and fibrils, resulting in a significant fluorescence turn-on, and indicating that the probes are also able to detect Cu-Aβ species *in vitro*. To confirm the Aβ binding specificity of the probes, brain section imaging studies were also performed with the compounds. As expected, if the brain sections were only stained with the compounds, the fluorescence images show that the sensor has poor ability to detect amyloid plaques, exhibiting low fluorescence intensity. However, to mimic the Cu-rich environment in AD brain, excess Cu(II) was added to the incubation solution, and the resulting fluorescence images clearly demonstrate that the Cu-responsive sensors are activated by the copper ions and generate the highly fluorescent amyloid-binding fluorophores which can specifically label the amyloid plaques on the 5xFAD brain sections. Overall, the developed Cu-based activatable sensors can be utilized to detect the Cu-mediated Aβ species both *in vitro* and *ex vivo*.

Conflicts of interest

There are no conflicts to declare.

ORCID

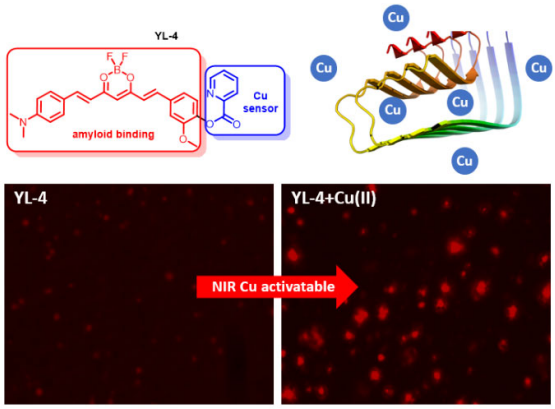
Liviu M. Mirica: 0000-0003-0584-9508
Yiran Huang: 0000-0003-1755-9874
Liang Sun: 0000-0002-0080-0855

References

1. S. Tiwari, V. Atluri, A. Kaushik, A. Yndart and M. Nair, *Int J Nanomed*, 2019, **14**, 5541-5554.
2. K. P. Kepp, *Coord. Chem. Rev.*, 2017, **351**, 127-159.
3. K. P. Kepp, *Chem. Rev.*, 2012, **112**, 5193-5239.
4. C. J. Maynard, A. I. Bush, C. L. Masters, R. Cappai and Q. X. Li, *International Journal of Experimental Pathology*, 2005, **86**, 147-159.
5. M. A. Smith, G. Perry, P. L. Richey, L. M. Sayre, V. E. Anderson, M. F. Beal and N. Kowall, *Nature*, 1996, **382**, 120-121.
6. D. P. Smith, G. D. Ciccotosto, D. J. Tew, M. T. Fodero-Tavoletti, T. Johanssen, C. L. Masters, K. J. Barnham and R. Cappai, *Biochemistry*, 2007, **46**, 2881-2891.
7. B. Alies, C. Bijani, S. Sayen, E. Guillon, P. Faller and C. Hureau, *Inorg. Chem.*, 2012, **51**, 12988-13000.
8. J. T. Pedersen, J. Ostergaard, N. Rozlosnik, B. Gammelgaard and N. H. Heegaard, *J Biol Chem*, 2011, **286**, 26952-26963.
9. S. Bagheri, R. Squitti, T. Haertle, M. Siotto and A. A. Saboury, *Front. Aging Neurosci.*, 2017, **9**, 446.
10. G. z. Eskici and P. H. Axelsen, *Biochemistry*, 2012, **51**, 6289-6311.
11. A. K. Sharma, S. T. Pavlova, J. Kim, J. Kim and L. M. Mirica, *Metallomics*, 2013, **5**, 1529-1536.
12. C. Esmieu, D. Guettas, A. Conte-Daban, L. Sabater, P. Faller and C. Hureau, *Inorg. Chem.*, 2019, **58**, 13509-13527.
13. C. Hureau, *J. Biol. Inorg. Chem.*, 2017, **22**, S230-S230.
14. H. J. Yu, W. Zhao, Y. Zhou, G. J. Cheng, M. Sun, L. Wang, L. Yu, S. H. Liang and C. Z. Ran, *Anal. Chim. Acta*, 2020, **1097**, 144-152.
15. T. Hirayama, G. C. Van de Bittner, L. W. Gray, S. Lutsenko and C. J. Chang, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 2228-2233.
16. S. Lee, C. Y. S. Chung, P. Liu, L. Craciun, Y. Nishikawa, K. J. Bruemmer, I. Hamachi, K. Saijo, E. W. Miller and C. J. Chang, *J. Am. Chem. Soc.*, 2020, **142**, 14993-15003.
17. K. J. Bruemmer, S. W. M. Crossley and C. J. Chang, *Angew. Chem., Int. Ed.*, 2020, **59**, 13734-13762.
18. D. A. Iovan, S. Jia and C. J. Chang, *Inorg. Chem.*, 2019, **58**, 13546-13560.
19. H. Li, P. Zhang, L. P. Smaga, R. A. Hoffman and J. Chan, *J. Am. Chem. Soc.*, 2015, **137**, 15628-15631.
20. M. Saleem, M. Rafiq, M. Hanif, M. A. Shaheen and S. Y. Seo, *J. Fluoresc.*, 2018, **28**, 97-165.
21. L. Zeng, E. W. Miller, A. Pralle, E. Y. Isacoff and C. J. Chang, *J. Am. Chem. Soc.*, 2006, **128**, 10-11.
22. D. W. Domaille, L. Zeng and C. J. Chang, *J. Am. Chem. Soc.*, 2010, **132**, 1194-1195.
23. B. Muthuraj, S. Layek, S. N. Balaji, V. Trivedi and P. K. Iyer, *ACS Chem. Neurosci.*, 2015, **6**, 1880-1891.
24. Y. Y. Yu, P. Wang, X. D. Zhu, Q. W. Peng, Y. Zhou, T. X. Yin, Y. X. Liang and X. X. Yin, *Analyst*, 2018, **143**, 323-331.
25. K. Liu, J. M. Chen, J. Chojnacki and S. J. Zhang, *Tetrahedron Lett.*, 2013, **54**, 2070-2073.

Please do not adjust margins

TOC Graphic


YL-4
amyloid binding
Cu
sensor
Cu
Cu
Cu
Cu
Cu
Cu
YL-4
YL-4+Cu(II)
NIR Cu activatable