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Recyclable and Robust Optical Nanoprobes with Engineered Enzymes for Sustainable Serodiagnostics

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Recyclable fluorescence assays that can be stored at room temperature would greatly benefit biomedical diagnostics by bringing sustainability and cost-efficiency, especially for point-of-care serodiagnostics in developing regions. Here, a general strategy is proposed to generate recyclable fluorescent probes by using engineered enzymes with enhanced thermo-/chemo-stability, which maintains an outstanding serodiagnostic performance (accuracy >95%) after 10 times of recycling as well as after storage at elevated temperatures (37 °C for 10 days). With these three outstanding properties, recyclable fluorescent probes can be designed to detect various biomarkers of clinical importance by using different enzymes.

1. Introduction

The diagnostics market has experienced significant growth in recent years, driven by factors such as the increasing prevalence of infectious diseases,[1] the growing demand for point-of-care testing,[2] and technological advances that have enabled the development of more accurate and sensitive serodiagnostics.[3] Fluorescent assays that contain enzyme-based probes have attracted great attention since they are highly selective among biomarkers and are widely used in clinical practice and research, especially for the detection of disease-related small biomolecules (e.g., glucose for diabetes or uric acid for hyperuricemia).[4] However, most of these assays are neither affordable, robust, nor deliverable, which are essential elements of the ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users), as defined by the World Health Organization (WHO) for an ideal diagnostic test for the developing world.[5,6]

Currently available probes are single-use, which not only results in serious waste of resources, but also in high costs for serodiagnosis. Moreover, they are criticized for the difficulty of long-term storage in a warm environment (>30 °C) due to progressive denaturation, which leads to a decrease in their biorecognition ability. Therefore, it remains a global challenge to continuously produce sufficient amounts of probes to meet the diagnostic needs while requiring strict storage at low temperatures, especially in low-resource settings.

2. Results and Discussion

In view of this, recyclable and thermostable enzyme-based fluorescent probes will certainly benefit the sustainable development of the diagnostics market and balance the global management of diseases. However, to construct an enzyme-based fluorescent probe, it is necessary to follow certain principles, and therefore, a rational design of structure and composition.

Basically, it can be divided into three main parts: fluorescence donor, fluorescence acceptor, and recognition group (**Figure 1**A). As the diagnostic results depend on the fluorescence signal of the probe, the fluorescence donor should be highly stable, not only in the biofluidic environment but also under repeated excitation. that is, its fluorescence is reliable under various conditions and after recycling. Lanthanide-doped nanophosphors are well-known for their linear-like and non-bleaching fluorescence based on the f-f transition of lanthanide ions.[7] As an intrinsic optical property, the fluorescence of lanthanide mainly depends on the type of dopant and crystal field, and is hardly affected by the environment.[8,9] With a passivation shell coating, the energy migration between emitting dopants and the environment can be further blocked.[10,11] In addition, the lanthanide-doped nanophosphors, especially fluorides, are found

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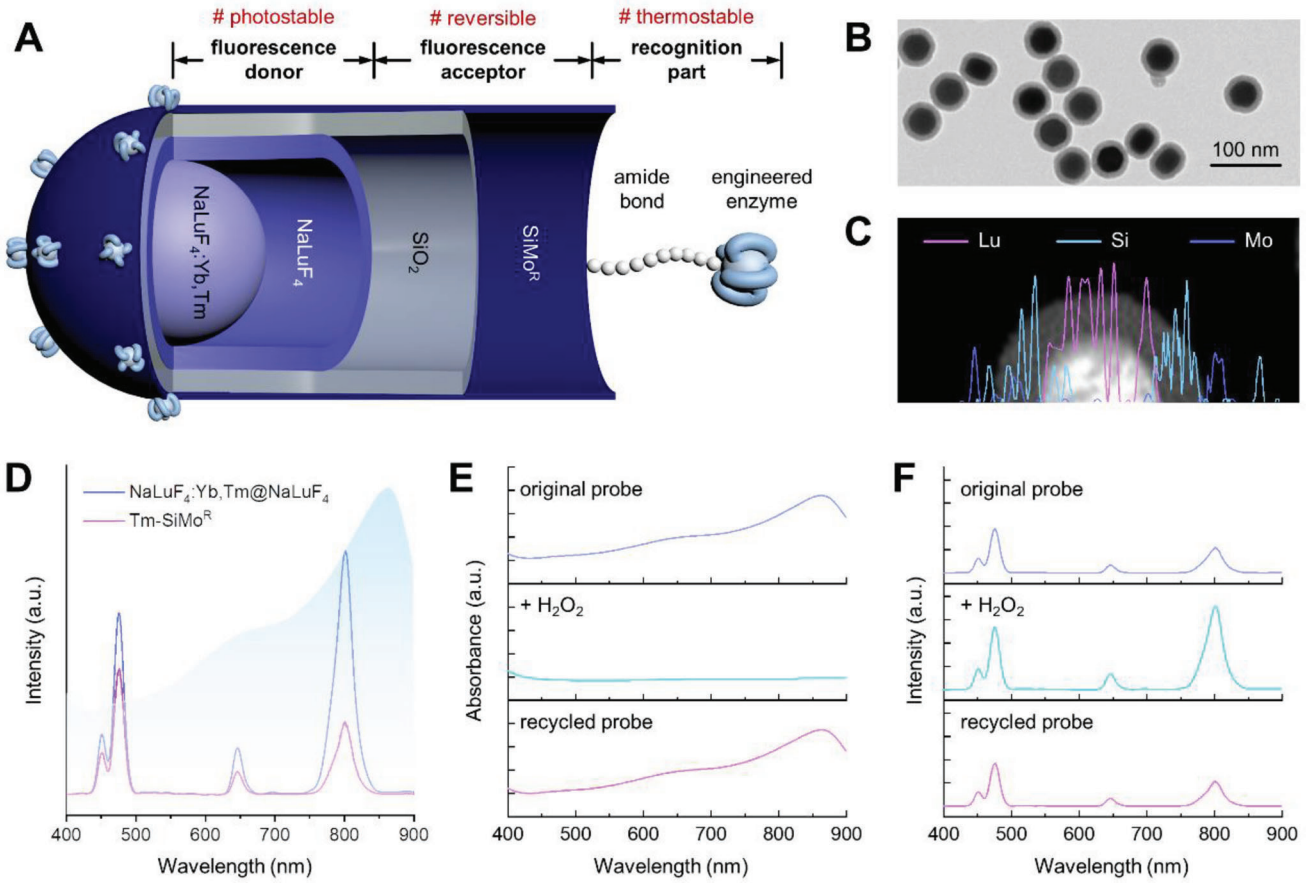

Figure 1. Stable and recyclable fluorescent probe. A) Rational design of the recyclable fluorescent probe based on engineered enzymes. B) Transmission electron microscopy image, C) electron dispersive X-ray line scan, and D) absorbance and fluorescence spectra of Tm-SiMo^R. E) Absorbance and F) fluorescence spectra of original Tm-SiMo^R (top), after H_2O_2 addition (center), and after recycling (bottom).

to be stable and biocompatible in various biological systems.[12,13] Due to their high signal-to-noise fluorescence and excellent stability, lanthanide-doped nanophosphors are widely used in biological applications, such as in-vivo bioimaging,[14] photodynamic therapy,[15] and optogenetic research.[16] Lanthanide-doped nanophosphors coated with a passivation shell could be suitable fluorescence donors in the probe.

As for the fluorescence acceptor, it has to be reversible to meet the recyclability requirement. Typical reversible fluorescence acceptors are organic dyes[17,18] or metal complexes[19,20] that can selectively absorb the fluorescence of the donor in a specific wavelength range. However, most of them are either hydrophobic or unstable under storage, which makes them unfavorable as diagnostic tools applied in aqueous-based biofluids. On the other hand, hydrophilic and reversible inorganic acceptors are more preferable for this specific application. In particular, some of them can grow on the fluorescence donor in situ, contributing to a stable base for the fluorescent probe. The SiO_2 shell is a widely-used stable base for in situ growth of various materials and preparation of heterostructures, especially on lanthanide-doped nanophosphors.[21–24] Therefore, a lanthanide-doped and SiO_2-coated nanocomposite with in situ grown fluorescence acceptors could be a good start to design our fluorescent probe.

Based on this, a fluorescent probe of $NaLuF_4$:Yb,Tm@$NaLuF_4$@SiO_2-reduced silicomolybdate (Tm-SiMo^R) was designed and constructed. The lanthanide-doped $NaLuF_4$:Yb,Tm core is stable, while the $NaLuF_4$ passivation shell limits the energy loss due to migration from Tm^{3+} to the environment. In addition, the coated SiO_2 is not only a hydrophilic shell, but also provides a stable surface for in situ growth of SiMo^R.[25,26] Therefore, this rationally designed nanostructure will not be easily disassembled or fluorescence quenched in typical biofluidic environments. Morphological, elemental, and crystallographic analyses indicate the successful construction of the probe as designed (Figure 1B,C and Figure S1,S2, Supporting Information). Due to the overlap between the absorption of SiMo^R and the fluorescence of the Tm^{3+}, the fluorescence is quenched by both the inner filter effect and the fluorescence resonance energy transfer pathways (Figure 1D). As the absorbance of SiMo^R originates from the intervalence charge transfer between Mo^V and Mo^{VI}, the addition of H_2O_2 oxidizes Mo^V, thereby weakening the absorbance (Figure 1E and Figure S3, Supporting Information) and, thus, the fluorescence recovers (Figure 1F). This oxidation step is finished within 3 min, which enables rapid detection and diagnosis (Figure S4, Supporting Information). The Tm-SiMo^R-BTUO is relatively stable in the standard biological pH range (pH 5 – 8), which may be attributed to the

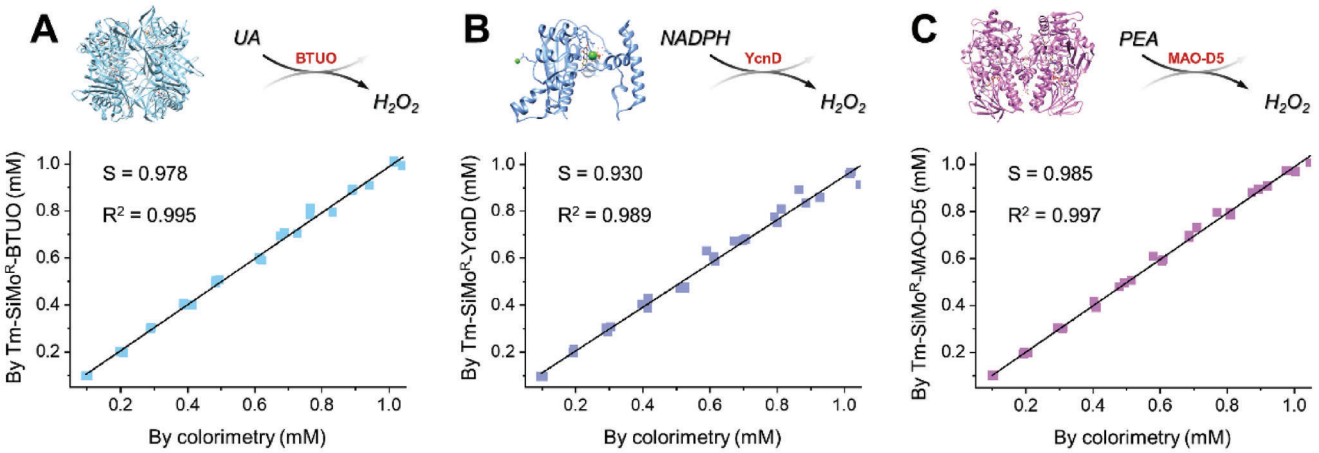

Figure 2. Generalizability of the concept. Quantification of UA, NADPH, and PEA, in 30 buffer samples each, comparing the performance with the reference TMB colorimetric assay. A) UA by engineered UA oxidase-conjugated Tm-SiMo^R-BTUO, B) NADPH by NADPH oxidase-conjugated Tm-SiMo^R-YcnD, and C) PEA by engineered monoamine oxidase-conjugated Tm-SiMo^R-MAO-D5.

protection by the functionalization with the modified enzyme (Figure S5, Supporting Information). It is worth noting that, unlike traditional H_2O_2 fluorescent probes, the oxidation of SiMo^R by H_2O_2 is reversible, allowing the recycling of Tm-SiMo^R after use (Figure S6, Supporting Information). By adding excess ascorbate, the used probes can be reduced, thereby restoring the absorbance and quenching the fluorescence. Therefore, in addition to the reversibility, the Tm-SiMo^R could be used as an H_2O_2 fluorescent probe, often used in clinical practice in combination with a substrate-specific oxidase for biomarker detection.

The last and critical part of the probe is the recognition moiety that is the immobilized enzyme, which determines the ability of the fluorescent probe to recognize biomarkers. In principle, the recognition moiety of the probe could be any oxidase that converts the selected biomarker of clinical importance as the substrate and produces H_2O_2 as the byproduct. Wild-type oxidases are widely used in commercial assays but commonly suffer from moderate stability and catalytic performance under the conditions required for our probe. Therefore, probes based on such oxidases are difficult to store and are unfavorable for providing timely and reliable diagnostic results. Therefore, appropriate selection and engineering of enzymes is important to improve specific properties, such as thermostability through selective mutation and enhanced intramolecular interactions.

Here, by utilizing an engineered uric acid (UA) oxidase from *Bacillus* sp. (5AYJ, *abbr.* BTUO, Figure S7, Supporting Information),[27] a nicotinamide adenine dinucleotide phosphate (NADP) oxidase from *Bacillus subtilis* (1ZCH, *abbr.* YcnD),[28,29] and an engineered monoamine oxidase from *Aspergillus niger* (2VVM, *abbr.* MAO-D5, Figure S8, Supporting Information),[30,31] three fluorescent probes were prepared for the determination of the model disease biomarker UA, the model co-enzyme NADPH, and the model molecular medicine phenylethylamine (PEA). These three model analytes are valuable for early diagnosis of disease (e.g., hyperuricemia and depression), progression monitoring, and evaluation of therapeutic efficacy, respectively. All three fluorescent probes showed good correlation with the reference 3,3′,5,5′-tetramethylbenzidine (TMB) colorimetric assay currently used in clinical practice (**Figure 2** and Figures S9 and S10, Supporting Information), which not only confirms the generalizability of our strategy, but also validates the practicality of our constructed probes for diagnostic applications.

To validate the stability and recyclability of the probe, Tm-SiMo^R-BTUO is selected for the following detailed studies. The BTUO is an engineered enzyme from *Bacillus sp.* TB-90 with substitution of the arginine 298 residue with a cysteine, which enables the spontaneous formation of an inter-subunit bridge, thus improving its thermostability. Therefore, Tm-SiMo^R-BTUO has a prolonged half-life in a warm environment (37 °C) ($t_{1/2}$ = 17.4 days), compared to commercially available probes based on wild-type UA oxidase (4D12, Tm-SiMo^R-wtUO, $t_{1/2}$ = 1.8 days) (**Figure 3**A). After 10 days of storage, Tm-SiMo^R-BTUO retains a recognition efficiency of 95% in response to uric acid, which is similar to the performance of the freshly prepared probe (Table S1, Supporting Information and Figure 3B).

To further illustrate the practicality of our fluorescent probe for diagnosis, uric acid in artificial urine samples of simulated hyperuricemia cases were tested. The freshly prepared Tm-SiMo^R-BTUO shows 98.3% accuracy for correctly distinguishing hyperuricemia cases from normal cases. Notably, after 10 days storage in a warm environment, the Tm-SiMo^R-BTUO retains a high diagnostic accuracy of 95.0%, which is comparable to the freshly prepared ones. Therefore, contributed by the outstanding thermostability of BTUO, the diagnostic precision of Tm-SiMo^R-BTUO is very reliable, even after storage in warm environments, which would be useful for (sub)tropical regions that are in lack of appropriate cooling facilities.

Furthermore, the recyclability of Tm-SiMo^R-BTUO was investigated. By using ascorbate in a weak acidic solution, the oxidized SiMo in the probe is reduced. Thereby, the absorbance is regenerated, which can be repeated multiple times (Figure S11, Supporting Information). Regeneration of the absorbance induces the corresponding change in fluorescence. After 10 cycles, the Tm-SiMo^R-BTUO remains stable and applicable as a uric acid fluorescent probe (**Figure 4**A). The superimposed fluorescence peaks centered at 801 nm ($^3H_4 \rightarrow {}^3H_6$) for the ten cycles indicate

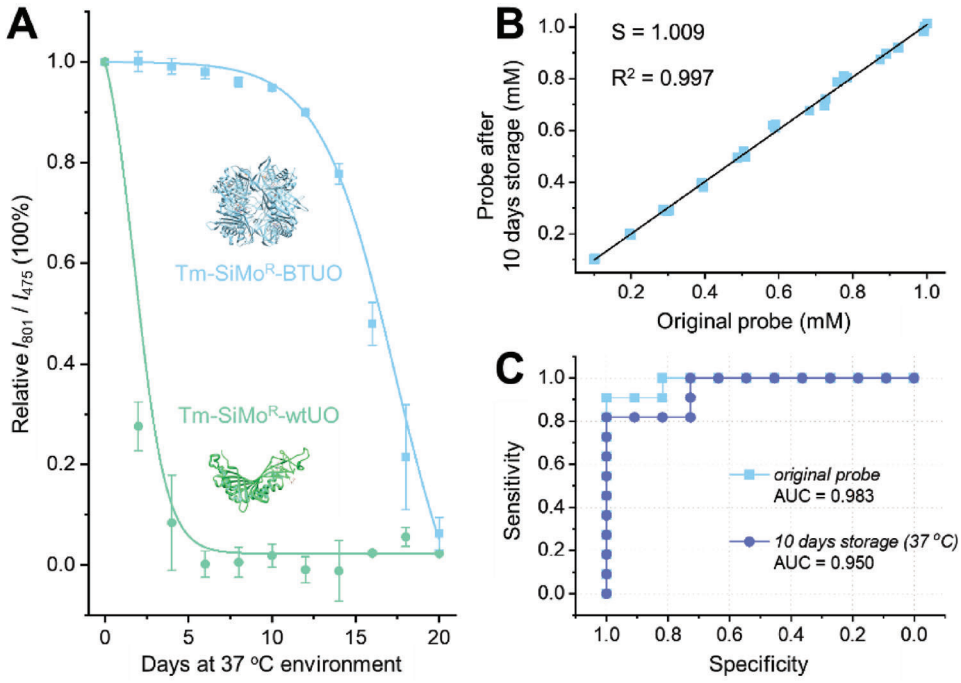

Figure 3. Stability of the Tm-SiMo^R-BTUO fluorescent probe. A) Relative I_{801}/I_{475} fluorescence ratios of Tm-SiMo^R-BTUO and Tm-SiMo^R-wtUO with addition of 1 mM of uric acid after storage in warm environment (37 °C). B) Quantification of UA in 30 artificial urine samples. Results obtained by using Tm-SiMo^R-BTUO after 10 days storage in a warm environment (37 °C) are plotted against results obtained using freshly prepared Tm-SiMo^R-BTUO. C) Receiver operating characteristic (ROC) curve showing the probability for the assay to correctly distinguish between normal and hyperuricemia cases based on the UA content in artificial urine samples determined by fluorescence spectra. Data are presented as mean ± SD ($n = 3$).

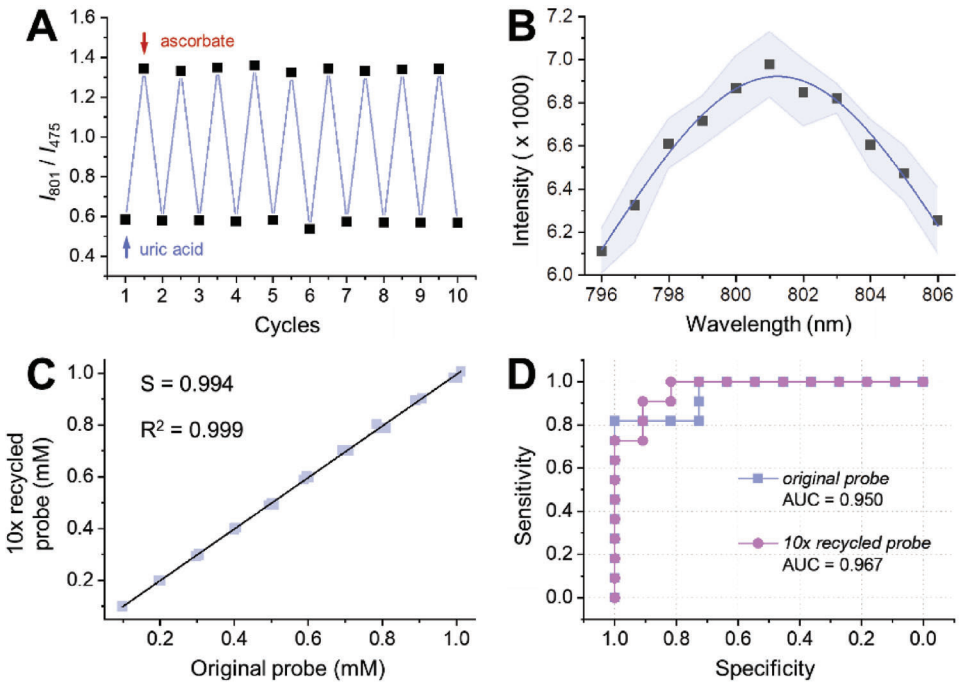

Figure 4. Recyclability of the Tm-SiMo^R-BTUO fluorescent probe. I_{801}/I_{475} fluorescence ratios A) and fluorescence peaks centered at 801 nm B) for 10 recycling processes. C) Quantification of UA in 30 artificial urine samples. Results obtained by using 10x recycled Tm-SiMo^R-BTUO, plotted against results obtained by original Tm-SiMo^R-BTUO. D) Receiver operating characteristic (ROC) curve showing the probability of the assay to correctly distinguish between normal and hyperuricemia cases based on the UA content in artificial urine samples determined by fluorescence analysis.

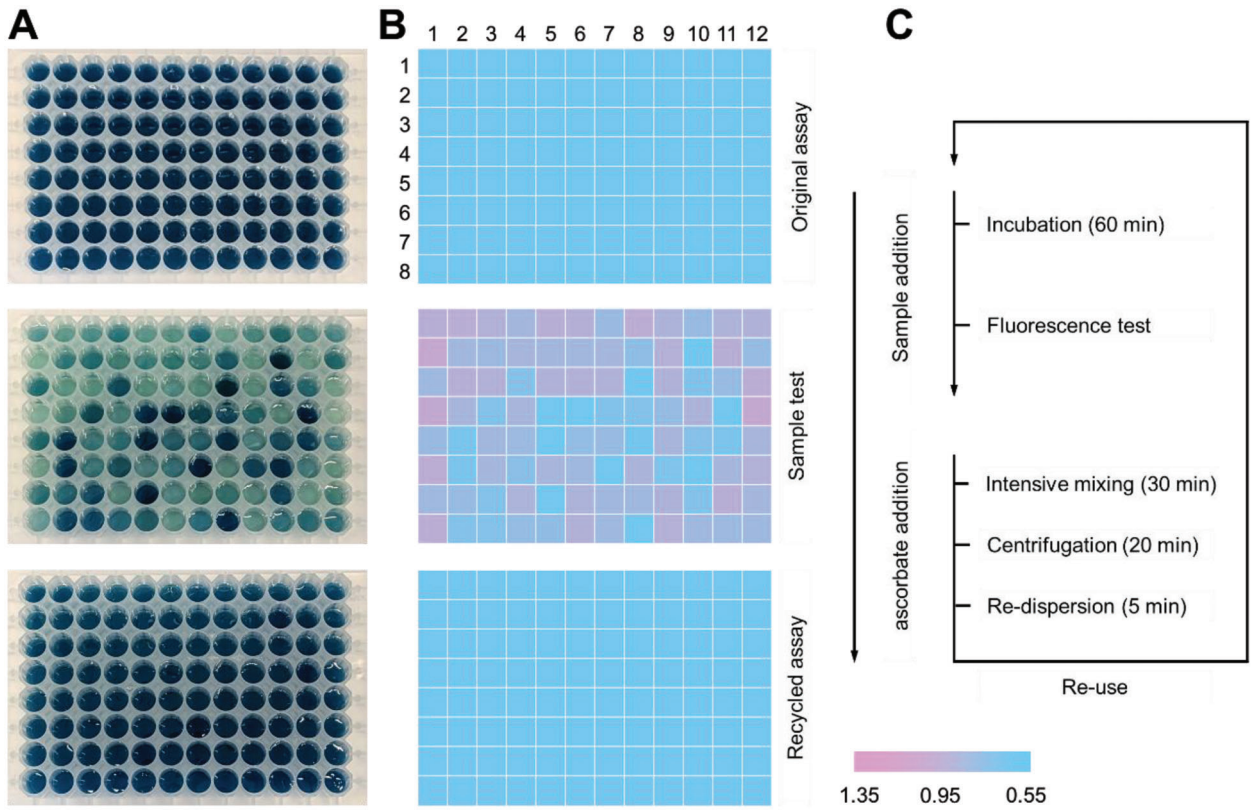

Figure 5. Fluorescence assay in a 96-well plate with recycling, using Tm-SiMo^R-BTUO probes. Photographs A) and I_{801}/I_{475} fluorescence ratios B) of original Tm-SiMo^R-BTUO probes (top), probes after incubation with samples (center), and recycled probes (bottom) in a 96-well plate. Photographs were taken with a smart phone (Google Pixel 5, f/1.7, 1/50, 4.38 mm, ISO134) under warm-white light in the laboratory. Except for cropping, no changes to the images were made. C) Scheme of the diagnostic and recycling process using the fluorescence assay based on the Tm-SiMo^R-BTUO probe.

that the fluorescence of recycled probes could be quenched to a similar level, which ensures the reliability of the recycled probe (Table S2, Supporting Information and Figure 4B). Notably, the probe recycled for the 10th time shows comparable quantification performance for uric acid as the original probes (Figure 4C). This still allows for an accurate identification of hyperuricemia cases (>95%) (Figure 4D). In addition to the urine samples, this recycling process also works for human serum and (artificial) sweat, which indicates a wide sample scope for uric acid detection (Figure S12, Supporting Information). These results show that the Tm-SiMo^R-BTUO probe can be recycled by a mild reduction process and it is reliable within certain cycles of use. This can significantly reduce the workload for the production of such probes.

Notably, the Tm-SiMo^R-BTUO showed both high physical and chemical stability during the recycling process. Due to the mild reduction conditions, the probe maintained its morphological characteristics (Figure S13A, Supporting Information), while its dispersibility in aqueous buffer did not change (Figure S13B, Supporting Information). In addition, neither Mo leakage nor obvious changes in chemical composition were observed in the recycling (Figure S14, Supporting Information). Moreover, the intrinsic electron structure of the lanthanide ions makes the Tm-SiMo^R-BTUO photostable under repeated irradiation cycles (Figure S15, Supporting Information).

Based on the recyclable and thermostable Tm-SiMo^R-BTUO probe, a fluorescence assay for uric acid detection and hyperuricemia diagnosis is constructed. After incubation with 96 different test samples (ranging from 0–1 mM concentr. UA) in a 96-well plate, the probes show different visible colors (**Figure 5**A). Accordingly, the I_{801}/I_{475} fluorescence ratios change and indicate the concentration of uric acid in each well (Figure 5B and Table S4, Supporting Information). Therefore, the assay can provide either quantitative colorimetric and fluorescence results or qualitative naked eye colorimetric evaluation to meet the needs of different laboratories. By mixing all used probes from different wells together, the probes can be recycled easily within 60 min, including reduction, separation, and re-dispersion steps, to enable the next assay (Figure 5C and Table S3–S5, Supporting Information). The reduction process typically takes 30 min, but could be reduced further if required, since the probes react with the ascorbate within 2 min (Movie S1, Supporting Information). Therefore, the fluorescence assay can be easily re-used by following this rapid and facile treatment.

3. Conclusion

In conclusion, we combined materials chemistry with enzyme selection and engineering to construct probes for the determination of biomarkers in biofluids. The probes are recyclable after use and can be stored in a warm environment, due to the

reversible oxidation of the polyoxometalate acceptor and the enhanced thermostability of the engineered oxidases. This significantly saves resources, reduces costs, and lowers the requirement for storage, which will improve diagnostics and ultimately the management of various diseases. Apart from the fluorescence, the obvious visible color change may also enable equipment-free colorimetric diagnostics, which would be highly favorable for rapid point-of-care testing. As a result, we provide a general strategy to advance enzyme-based optical diagnostic assays, showing better performance than a current commercial assay (Table S6, Supporting Information) and meeting the WHO ASSURED criteria for point-of-care diagnostics in the developing world.

However, there are shortcomings that need to be addressed in the future. First, regarding the accessibility of equipment, besides the fluorescence and colorimetric methods shown here, we will further explore other methods that can provide quantitative results in low-cost settings. For example, the temperature determined by a thermometer can also provide reliable results for diagnosis (Figure S16, Supporting Information), and it may be solar powered with appropriate optimization, which is more accessible for developing regions. Another research priority should be the in-depth exploration of thermostable enzymes for construction of probes. Here, we have shown that the use of engineered enzymes can apparently prolong the stability of probes in a warm environment, which is a good starting point and demonstrates the possibility of obtaining much more robust probes in the future. In addition, although the price of recyclable lanthanide-doped nanoparticles is comparable to traditional dyes (Table S7, Supporting Information), the cost of probes could be further reduced by using cheaper alternative fluorescent cores (e.g., chalcogenide quantum dots and carbon nanotubes) or by exploring bulk synthesis methods. Finally, the study of engineered enzymes should also be considered to broaden the scope of detectable biomarkers, with a particular focus on H_2O_2-producing oxidases (Table S8, Supporting Information). In principle, all oxidases could be used to modify the probe, thereby enabling the detection of various biomolecules and the diagnosis of related diseases.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Y.L. and Z.W. contributed equally to this work. F.F.L., F.G.M., and H.Z. supervised the project and acquired the financial support. Y.L. conceived the project and analyzed the data. Y.L. and Z.W. designed the methodology and established the model for the study. Y.L., Z.W., M.D., X.Z., and T.K. performed the experiments. H.Z. and F.G.M. provided main resource support. Y.L., F.F.L., F.G.M., and H.Z. decided on the visualization and data presentation. Y.L. and Z.W. wrote and decided the original manuscript. All authors contributed to the review and editing of manuscript.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords

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