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# Article / Book Information

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| Title(English) | Creation of a robust immunosensor enzyme and its application to protocell array-based digital immunodetection systems |
| 著者(和文) | 蘇九龍 |
| Author(English) | Jiulong Su |
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# Outline: Creation of a robust immunosensor enzyme and its application to protocell array-based digital immunodetection systems

## 1. General introduction

Due to the development of economy and society, more and more chemicals that affect our health can be found in the environment and foods, such as caffeine. On the other hand, early-stage diagnosis is very important for the therapy of many diseases. For instance, serum osteocalcin (as known as bone Gla protein) is a biomarker for many bone-related diseases and detection of it can be very valuable in the early-stage diagnosis in these diseases. To detect small molecules such as osteocalcin and caffeine, a fast and effective immunodetection method is needed. The widely used traditional sandwich ELISA and more sensitive digital ELISA need additional separation steps to remove background signals caused by unbound analytes. This makes the assay time-consuming and complicated. To solve this problem, this study developed a homogenous immunosensor using the reconstitution of an enzyme to detect small molecules such as caffeine and osteocalcin in one step. However, due to the homogeneous nature of this system, the contaminated analytes solution many affect the accuracy of the detection. To tackle this drawback, the optimized homogenous immunosensor was integrated into a protocell array-based biosensor system to isolate the reporter enzyme in small compartments.

## 2. Development of a novel immunosensor based on mutant β-glucuronidase [1]

β-glucuronidase (GUS) is a self-assembling tetramer that needs four subunits to be active and whose activity can be easily converted into colorimetric or fluorescence signals by the use of chromogenic or fluorogenic substrates. A set of interface mutations (M516K, Y517E) prohibits the inactive GUS dimers to form tetramer (GUSm) [2]. Meanwhile, the affinity between variable regions of an antibody can be increased by the existence of antigen so that they can bind to each other tightly. In Chapter 2, a homogeneous immunosensor with variable regions of antibodies as detector and GUSm as detector was developed for the one-step detection of small molecules (Figure 1). 4-hydroxy-3-nitrophenylacetyl (NP) and Bone gla protein (BGP) were used as targets detected by this novel immunosensor. NP is one of the well-studied model haptens usually used for immune response evaluations. Bone gla protein (BGP), also known as osteocalcin, synthesized by osteoblasts and measured in blood by radioimmunoassay previously as an index of the rate of bone turnover. After preparation of fusion proteins, both NP and BGP can be detected quantitatively by monitoring GUS activity in both fluorescent and colorimetric signals. The instantaneous response after simply mixing two components of this system and analytes gives this system convenient and timesaving features as an efficient high-throughput analysis method. Also, the rapid and high response makes this system a fairly effective method for

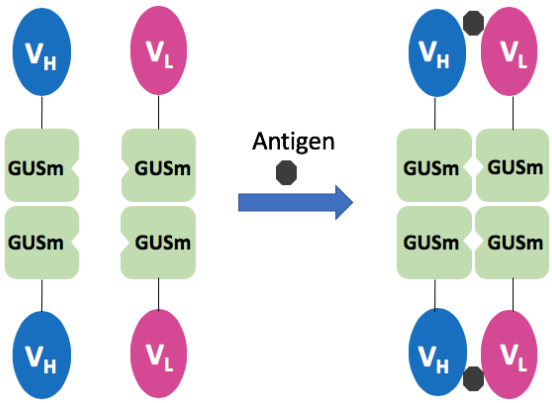


**Figure 1.** Scheme of homogeneous immunosensor

detection of small clinical and environmental molecules. However, the aggregating background signal hampered the performance, and the intrinsic GUS activity in mammalian bodies may complicate the use of this homogeneous immunosensor system in diagnostics such as detection of osteocalcin in blood samples. To solve this problem, a system that separate detector with reporter by isolating optimized GUS mutants in vesicles, such as protocell array, is needed.

3. Optimization of thermostabilized β-glucuronidase mutant [3]

To develop this protocell-array based system, in Chapter 3, the reporter enzyme GUS was optimized by inducing mutations to increase its thermostability and decrease the background signal in the use of immunosensor. In another study, random mutations were introduced and screened to improve the thermostability of the wild-type *E. coli* GUS. After several rounds of randomization and selection, a mutant named $GUS_{IV5}$ with improved thermostability and activity (N27Y, F51Y, A64V, E115D, D185N, I349F, N369S, Y517F, Y525F, G559S, K567R, F582Y, Q585H and G601D) [4] was selected. With these mutations mainly at disordered regions of GUS, the thermostability was significantly increased due to more rigid structure and similar activity as wild type GUS was maintained. The interface mutations that made suitable reporter enzyme for the immunodetection system based on "open sandwich principle" were screened by tethering GUSm to variable regions of an antibody. After screening, a set of interface mutations (516K, 517W) on $GUS_{IV5}$ made $GUS_{IV5}$_KW, which gave low activity before activation and high activity after anti-His antibody-induced dimerization of dimers. Afterwards, $GUS_{IV5}$_KW was tested as the reporter enzyme in previously developed homogeneous immunosensor format, and increased sensitivity was obtained in the detection of NP, probably due to lowered background signal.

Heavy-chain antibody is an antibody found in camelids consisting of only two heavy chains. The variable fragments ($V_HH$) or nanobody separated from a heavy-chain antibody contains an antigen binding site and attains full antigen-binding function. Previously, a $V_HH$ was isolated for small hapten caffeine, which was found to be dimerized after antigen binding [5]. To demonstrate the generality of the immunosensor using thermostabilized reporter enzyme, also to test the ability of $V_HH$ as the antigen detector, a new sensor using the anti-caffeine $V_HH$ as detector was constructed. The constructed sensor succeeded in the detection of caffeine quantitatively at practically useful concentration range.

4. Creation of a protocell-base biosensor

With a strictly controlled enzyme switch developed, a homogeneous protocell array-based biosensor was created with $GUS_{IV5}$_KW as reporter enzyme to amplify the binding signal of external protein as a fluorescent signal was attempted in Chapter 4 (Figure 2). A variety of model membranes have been developed to construct bilayer vesicles in different sizes. Among them, giant unilamellar vesicles (GUVs) have a diameter larger than 1 μm and are ideal vesicles for the protocell array system since its similar size to cells and absence of organelles. High activity at tetramer state and low background at the inactive dimer state make $GUS_{IV5}$_KW an ideal enzyme reporter for a protocell array-based biosensor that demands a strictly controlled enzyme switch to give on/off signals. Transmembrane domain form human epidermal growth factor receptor with a short peptide tag at its N-terminus was tethered to $GUS_{IV5}$_KW to make fusion proteins with transmembrane ability as reporter that convert external binding into internal fluorescence signal. Tag-specific

antibodies were quantitatively detected by the protocell-based biosensor and proved the spontaneously display of small peptides on the surface of protocells. Furthermore, SpyTag/SpyCacher system was used as a connector between $V_HH$(Caf) and protocells to make biosensors that is able to detect caffeine by providing digitalized fluorescence signal. The successful conversion of external binding signal onto internal GUS activity signal give this protocell array-based biosensor a great potential to detect multiple small molecules without disturbance of contaminations.

## 5. Summary and perspectives

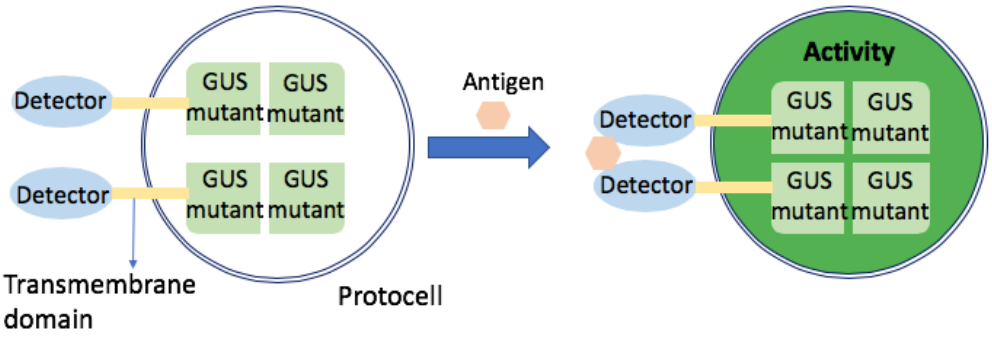


**Figure 2.** Scheme of protocell array-based biosensor

A novel homogeneous immunosensor system based on an optimized β-glucuronidase (GUS) mutant, which can not only be used for the simple and fast detection of small molecules in solution but also be used in a protocell array for the detection of small molecules sensitively by providing digitalized signal, has been developed. The one-step reaction feature makes it a fast and convenient immunodetection method for small molecules. By optimizing the reporter enzyme to develop a strictly controlled enzyme switch, the performance of this immunosensor has been greatly improved due to its on/off manner. Integration of this immunosensor system into bilayer vesicles created a protocell array-based biosensor that can detect tag-specific antibodies and antigens including caffeine, which kept the reporter enzyme complex away from contaminations and gave the possibility to increase sensitivity by providing digital signal.

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