Quenchbody

Subjects: Engineering, Biomedical

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The detection of viruses, disease biomarkers, physiologically active substances, drugs, and chemicals is of great significance in many areas of our lives. Immunodetection technology is based on the specificity and affinity of antigen–antibody reactions. Compared with other analytical methods such as liquid chromatography coupled with mass spectrometry, which requires a large and expensive instrument, immunodetection has the advantages of simplicity and good selectivity and is thus widely used in disease diagnosis and food/environmental monitoring. Quenchbody (Q-body), a new type of fluorescent immunosensor, is an antibody fragment labeled with fluorescent dyes. When the Q-body binds to its antigen, the fluorescence intensity increases. The detection of antigens by changes in fluorescence intensity is simple, easy to operate, and highly sensitive.

Keywords: antibody ; immunoassay ; fluorescence ; quench ; detection ; biomarker

1. Introduction

The detection of viruses, disease markers, physiologically active substances, drugs, and chemicals is important for many areas of our lives. Immunoassays are based on the specificity and affinity of antigen–antibody reactions. Nowadays, many antibodies against not only proteins but also small molecules (peptides and haptens) with high specificity and affinity can be obtained. As an analytical methodology for such small molecules, liquid/gas chromatography coupled with mass spectrometry (LC/MS and GC/MS, respectively) are frequently used as comprehensive analytical methods. However, compared with LC/MS or GC/MS, which require large and costly equipment and have a relatively long analysis time, immunoassays have the advantages of simple operation, shorter analysis time and good sensitivity as well as selectivity of detection. They are thus widely used in disease diagnosis and food/environmental monitoring.

Irrespective of target size, there are two kinds of immunoassays based on their operation mode: homogeneous and heterogeneous assays. Heterogeneous immunoassays need separation of the solid and liquid phases for separating antigen-bound and free (B/F) antibodies; representative detection technologies include enzyme-linked immunosorbent assay (ELISA) [1], immunofluorescence detection, and so on. Because of the many operation steps and long incubation time for reactions, these assays consume a lot of labor and time. In contrast, homogeneous immunoassays do not require the B/F separation and have the advantage of simple operation. However, we need an alternative way to distinguish the antigen-bound and free antibodies to realize it.

Quantitative analysis of protein location and concentration is key to understand their in situ functions. In principle, scaffolds combined with environment-sensitive fluorophores can detect analytes of interest with high temporal and spatial resolution. However, their adoption can be limited due to the extensive experimental screening required for their development. Wittrup's group recently described their trials to generalize design principle of such "Scaffold conjugated to environment-sensitive fluorophore (SuCESsFul) biosensors" based on various binding protein scaffolds, analytes and fluorescent dyes [2]. After making more than 400 biosensors, they found that the brightest reading can be obtained when a specific binding pocket for the fluorophore is present on the analyte. They also found that the interaction between the fluorophore and binding protein can raise the background fluorescence and limit the dynamic range of the sensor. Islam et al. designed a wavelength-dependent fluorescent immunosensor by integrating a polarity indicator (3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid, Anap) into the specific position of the anti-epidermal growth factor receptor (EGFR) single-chain variable region (scFv) to generate an emission-wavelength-related immunosensor. They found that when binding in the topological neighborhood of the antigen-binding interface, EGFR can titrate the blue shift of the emission wavelength of Anap, and the maximum wavelength shift can reach 20 nm, which has a nanomolar detection limit [3]. However, it is worth noting that these environment-sensitive dye-based approaches are considered effective only for larger protein targets that endow sufficient binding surface near the labeled dye to get distinct signals, and not for smaller molecules such as haptens and peptides.

2. The Working Mechanism and Types of Q-bodies

The Q-body is an antibody-based immunosensor, most frequently constructed by labeling the N-terminal region of scFv or the antigen-binding fragment (Fab) of an antibody with a fluorescent dye. The structure and working principle of a Q-body are shown in Figure 1a. When a specific fluorescent dye such as tetramethylrhodamine (TAMRA), rhodamine 6G (R6G), and ATTO520 is attached near the N-terminus of an antibody with a short (4~25 aa) flexible linker peptide, the fluorescent dye enters the variable regions of the antibody (Fv; V_H + V_L) due to the dye's hydrophobicity. Specific amino acids, namely, several tryptophan (Trp) residues in the antibody variable region weaken the fluorescence of the dye by fluorescence quenching due to photoinduced electron transfer (PET) from Trp to the dye [4]. In general, when the fluorescent dye is illuminated with the excitation light, the electrons transition from a low-energy orbit to a high-energy orbit, and emit fluorescence when these return to their low-energy orbit. However, in the vicinity of an amino acid that can provide electrons, the fluorescent dye takes electrons from the amino acid and does not emit fluorescence, termed as fluorescence quenching. However, when the Q-body binds to an antigen, due to antigen-dependent Fv stabilization and steric hindrance due to bound antigen, the quenched dye can no longer dwell in the interior of the antibody and moves to the exterior, and recovers its fluorescence. The antigen concentration can thus be detected by measuring the positive change in fluorescence intensity. This technique is easy to operate by simply adding the sample to the probe solution, by measuring the fluorescence intensity in a few seconds to minutes.

Figure 1. The working mechanism of Quenchbody. (**a**) The working model of Quenchbody. Trp residues are shown in light green; (**b**) An ELISA to demonstrate the movement of the fluorescent dye on Quenchbody [5].

Tryptophan endowed with an indole side chain is an effective electron donor in the reaction with dye molecules, as it is one of the most easily oxidized functional groups among natural amino acids. Antibody fragments quench the coupled fluorescent dye through the Trp residue semiconserved in its variable region, as shown in Figure 1a. There are four conserved (>94%) Trp residues in an Fv region (36H, 47H, 103H, and 35L, according to Kabat numbering scheme [6]), and many antibodies have more in their complementarity determining region (CDR). In the absence of antigens, fluorescent groups enter the interface of the variable region of the heavy chain (V_H) and variable region of the light chain (V_L), and interact with Trp residues either directly through hydrophobic and π-π stacking interactions, or indirectly through the protein. It is known that such electron tunneling can happen effectively within 1 nm distance, as exemplified in the photosynthetic reaction center [7]. Hence, the position of the dye that causes optimal quenching is not very easy to predict even if the positions of the Trp residue are known. However, it is worth mentioning that the two conserved core Trp residues (36H and 35L) play a significant role in quenching [4], showing the potential generality of Q-body principle.

To explore the antigen-dependent characteristics of the Q-body and the intramolecular mechanism of its action, Ohashi et al. analyzed the dynamics of dye movement in the reaction between the Q-body and antigen by ELISA as well as fluorescence polarization techniques (FP) [5]. When they analyzed the available TAMRA dye attached to the Q-body against osteocalcin (BGP), as shown in Figure 1b, the more fluorescent dyes moved from the inside of the antibody to the outside, the more easily they were captured by the anti-dye antibody. The ELISA results showed that the signal in the presence of the antigen osteocalcin peptide (BGP-C7) was significantly higher than that in the absence of the antigen. Therefore, they carried out further experiments to detect the differences in signals in the presence of different antigen concentrations. As expected, the results were consistent with the fluorescent intensity detection. The more the antigen, the more fluorescent dyes were detected. This also explains why the fluorescence intensity increases with increasing

antigen concentration. The effect of the linker length between the antibody and dye on the dye movement was also analyzed. The results indicated that a longer linker resulted in a stronger signal, as in the case of Q-body response. To confirm this conclusion, an experiment with an anti-bisphenol A (BPA) antibody was also conducted, and similar results were obtained. The FP results used to analyze the molecular tumbling of fluorescent dye indicated that the antigen concentration- and linker length-dependencies were also consistent with the ELISA results. The higher the antigen concentration, the more fluorescent dyes were released to the outside of the antibody; thus, the Brownian motion rate of the dye molecules increased and the degree of polarization decreased.

The ELISA and FP results indicated that enhancement of fluorescence intensity was caused by the outward movement of fluorescent dyes in the molecule, and the same results were also obtained for other two Fab-type Q-bodies for small molecular weight antigens, namely, methamphetamine analog and deoxynivalenol. Hence, the primary working mechanism of the Q-bodies for small molecules (haptens or peptides) will be summarized as the intramolecular competition between the antibody-tethered dye and the antigen in a sample. For the Q-bodies for protein antigens, another quenching mechanism also plays an important role, which is explained elsewhere [8].

3. Prospects of Q-body Technology

As a rapid fluorescent homogeneous immunoassay probe, Q-body can be used to detect a series of substances, such as small molecules, peptides, and proteins. Q-bodies labeled with a variety of dyes may be used to simultaneously detect a variety of substances in the same sample in future.

The merits of Q-body technology over other immunoassays can be summarized as follows.

(1) Simplicity. No necessity of other reagents and steps, especially washing.

(2) Quickness of the assay. The assay time only depends on the binding kinetics of antibody.

(3) Allows noncompetitive detection for small molecules. Signal increases, unlike competitive assays. Competition of the dye and antigen only happens inside the Q-body, hence it ensures lower experimental error. In addition, preparation of antigen-dye (or carrier) conjugate is not necessary.

We also can make various types of Q-bodies using scFv [4], Fab [8], IgG [9] and nanobody [10]. Each format has merit, for example, scFv and nanobody are easy to express, and Fab and IgG can be more sensitive, especially when introduced with multiple dyes. On the other hand, scFv is not very stable and Fab manifests low yield when expressed. IgG protein can be chemically modified easily, but its engineering is just its beginning. To overcome the previous limitations of Q-bodies, e.g., the need considerable genetic engineering to clone the antibody gene and testing for several clones with different combinations of dye and linkers, fluorescence-labeled antibody binding proteins (Quench probes, Q-probes) have been developed, which can easily convert the available antibody protein to a Q-body [9].

At present, semirational design of Q-body can be performed as follows:

For detecting small antigens:

- By using a PM Q-probe, find a suitable antibody that shows deep quenching upon binding and high dequenching upon subsequent antigen addition.
- Clone V_H/V_L genes to construct single- (or double-) labeled Q-body (scFv- or Fab-type).
- Optimize the dye position (H or L chain N-terminus) and linker length.

For detecting proteins:

- Prepare candidate antibody genes with a sufficient number of Trp residues in its V_H/V_L region.
- Make double-labeled Fab-type Q-bodies with relatively short linker peptides.
- Perform antigen detection assay to find the best clone.

Another potentially promising approach is molecular evolution, construction of a suitable antibody library (with Trp-rich CDR as exemplified in anti-HER2 Fab37 [11]), and selection based on fluorescent responses. We envisage that the combination of appropriate display technologies such as phage display and yeast display will give us an efficient and timely selection of good recombinant Q-bodies. Yet another potential approach in the near future is the use of molecular dynamics simulation to design high-performance Q-bodies in silico [12].

Thus, the Q-body has important theoretical significance and broad application prospects in biosensing and biological imaging as a new type of immunosensor.

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