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Review

From fluorescence polarization to Quenchbody: Recent progress in fluorescent reagentless biosensors based on antibody and other binding proteins☆

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ABSTRACT

Recently, antibody-based fluorescent biosensors are receiving considerable attention as a suitable biomolecule for diagnostics, namely, homogeneous immunoassay and also as an imaging probe. To date, several strategies for "reagentless biosensors" based on antibodies and natural and engineered binding proteins have been described. In this review, several approaches are introduced including a recently described fluorescent antibody-based biosensor Quenchbody, which works on the principle of fluorescence quenching of attached dye and its antigen-dependent release. The merits and possible demerits of each approach are discussed. This article is part of a Special Issue entitled: Recent advances in molecular engineering of antibody.

1. Introduction

Until now, antibody-based diagnostics market is steadily growing, and the demand for rapid and accurate immunodiagnostic method is mounting [1]. This is partly because of the rise of personalized medicine, in which majority of antibody-based therapeutics that target a specific disease-related molecular target are classified. Hence, there is a growing need for companion diagnostics (CDx, sometimes referred to as theranostics) to identify patients with a specific biomarker that is predictive of response. The US Food and Drug Administration (FDA) recognized the need for companion diagnostics in recent draft guidance that outlines the agency's preference for simultaneously approving a therapeutic agent with its companion diagnostic agent [2]. Due to its sensitivity and selectivity, antibody-based assay (immunoassay) has an ideal property in terms of CDx, owing to antibody's high affinity and specificity. However, conventional immunoassays like enzyme-linked immunosorbent assay (ELISA) involve many laborious steps, which also need considerable time and labor. In addition, novel probes are constantly needed for solving hard-to-tackle biological as well as biomedical problems.

As a possible solution for these issues, antibody-based biosensors are receiving considerable attention as a suitable biomolecule to probe various types of antigens and their modifications. Historically, the first biosensor was demonstrated by Clark and Lyons in 1962 [3]. They coupled the glucose oxidase to an amperometric electrode for P_{O_2}. The lowering of P_{O_2} in sample solution was sensed by the electrode and shown to be proportional to the concentration of glucose. In 1977, Rechnitz et al. described a 'bio-selective sensor' in which they immobilized living microorganisms at the surface of an ammonia gas-sensing electrode to construct a selective electrode for the amino acid arginine [4].

A biosensor consists of two basic elements: molecule recognition elements and transducer that could transfer the recognition to signals [5–7]. The molecule recognition element is capable of recognizing the presence of a specific analyte in samples, whereas the transducer converts the change in samples into a quantifiable signal. The molecular recognition may be a binding process such as ligand–receptor, protein–protein, or antigen–antibody binding or a biocatalytic reaction such as substrate–enzyme reaction, while the chemical sensors are derived from synthetic compounds [8,9]. The signal transduction may be one of several approaches including electrochemical [10], optical [11] and the measurement of mass [12] or heat [13]. Among them, in recent years fluorescence-based biosensors have been attracting researchers' attention. In most biosensors, the molecule recognition and signal transduction are done separately and involve complex liquid handling, which

Abbreviations: Ab, antibody; Ag, antigen; Fab, antigen binding fragment of antibody; Fd, antibody heavy chain fragment of Fab; Fv, antibody variable region fragment; PBS, phosphate buffered saline; Q-body, Quenchbody; scFv, single chain Fv; UQ-body, Ultra-Quenchbody; V_H, antibody heavy chain variable region fragment; V_L, antibody light chain variable region fragment

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is time-consuming and not suitable for imaging in vivo. A reagentless biosensor integrates the recognition module and transduction module into a compact device within a molecular dimension. Such a biosensor ideally functions without additional reagent, provides quantitative analytical information and follows the concentration of its analyte continuously [14].

In this review, several approaches on fluorescence-based sensing, especially reagentless biosensors are introduced with a special focus on antibody as a recognition molecule. These include a recently described "signal-on" antibody-based biosensor 'Quenchbody', and the possible merits of this approach over others will be discussed. Since fluorescence-based assays are sensitive yet requires no additional reagents, the potential application range is wide including homogeneous immunoassay and bio-imaging studies.

2. Conventional approaches based on fluorescence-labeled antibodies

Before going into detail of recent fluorescent biosensors created by molecular engineering, conventional approaches of antibody-based fluorescent assays in homogeneous solution (homogeneous fluorescent immunoassays) are briefly summarized to facilitate overview of this area.

2.1. Methods to monitor Brownian motion

One of the most popular fluorescence-based homogeneous immunoassays is the fluorescence polarization immunoassay (FPIA) (Fig. 1a). This is a simple method of monitoring changes in molecular tumbling rate of fluorescence-labeled molecules due to Brownian motion. In the assay utilizing this method, light is differentially absorbed by molecules as a function of their orientation relative to the direction and polarization of the exciting light. The light subsequently emitted as fluorescence by each of the resulting electronically excited molecules will usually be polarized. Rotation during the lifetime of the excited states randomizes the orientation of the excited molecules leading to a net reduction in fluorescence polarization. The more rapid the tumbling the less polarization is observed. This phenomenon was first applied in a homogeneous immunoassay by Dandliker et al. [15,16].

FPIA has been applied almost exclusively to small molecule analysis. The sample and antibody are combined and the antigen in the sample competes with fluorolabeled antigen for binding to the antibody. Increasing concentrations of the antigen produce decreased polarization. However, high molecular weight analytes including most protein antigens are difficult to analyze by FPIA for two reasons. First, the binding of a large antigen to an antibody that may be of similar mass produces a smaller relative change in the tumbling rate and thus a smaller change in polarization. Second, most fluorescent labels have excited state lifetimes in the range of 1–100 ns, which is too short to permit rational rotational reorientation of large bio-polymers. A rhenium (Re)-based fluorophore has been identified that shows polarized fluorescence with a lifetime of 3 ms. This extends the theoretically accessible molecular weight range for special applications [17]. However, interference from quenching impurities becomes problematic with these fluorophores since quenching affects the measured polarization.

There is a similar method to monitor the Brownian motion of fluorescent molecules called fluorescence correlation spectroscopy (FCS) [18] (Fig. 1b). The method is one of single molecule detection method, which monitors fluorescence fluctuation derived from a sample containing fluorescent molecules moving in and out of a laser beam. If the frequency is high, the Brownian motion is fast. If the frequency is lowered, slower motion due to interaction with other molecule is suggested. The method can be applied to higher molecular weight antigens than FPIA, and it is applicable to simultaneous monitoring of two proteins of interest, each labeled with different colors (fluorescence cross-correlation spectroscopy, FCCS) [19,20]. While these methods provide valuable information from a limited amount of sample, a costly equipment, either dedicated or as an option of laser scanning microscope, might potentially limit their wider use.

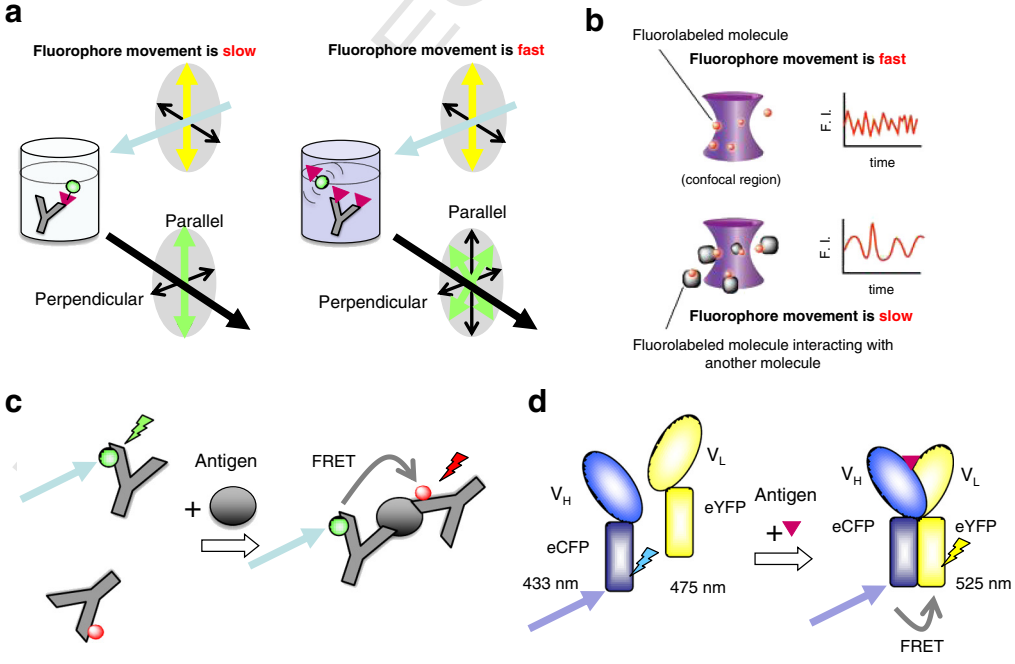

Fig. 1. Scheme of fluorescence-based immunoassays. (a) Fluorescence polarization immunoassay (FPIA). In the absence of antigen, all labeled antigens bind antibody, and its Brownian motion is slow (left). In the presence of antigen, the unbound labeled antigen shows faster Brownian motion (right). (b) Fluorescence correlation spectroscopy (FCS). The Brownian motion of fluorolabeled molecule is monitored by a confocal laser. The presence of interacting molecule is detected by the slower fluorescence signal fluctuation (modified from Amalgaam website). (c) Fluorescence resonance energy transfer immunoassay (FETI). When the donor and acceptor fluorophores each labeled to an antibody come sufficiently close due to antigen binding, FRET between them occurs. (d) Open sandwich fluoroimmunoassay (OS-FIA). The donor and acceptor (in this case eCFP and eYFP, respectively) are each labeled with the V_H and V_L fragments (or vice versa) of an antibody, respectively. Antigen-dependent association of the two fragments is monitored by FRET. Low molecular weight antigens can be noncompetitively detected.

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2.2. FRET-based sensors

Fluorescence resonance energy transfer, or Förster resonance energy transfer refers to the transfer of energy from an electronically excited donor molecule to a nearby acceptor molecule that has an energetically accessible excited state. The excited state of one or both of the donor and acceptor can decay with fluorescence emission. When both are fluorescent, energy transfer is observed by a reduction in the emission intensity of the donor with an accompanying increase in the intensity of the longer wavelength emission from the acceptor. The most relevant energy transfer mechanism in immunoassays depends on through-space coupling of the electronic transition dipoles [21]. The rate of energy transfer is inversely dependent on the sixth power of the distance between the donor and acceptor, and is directly related to the spectral overlap of the donor emission and acceptor absorption spectra. The distance at which the donor fluorescence is reduced by 50%, R_0, is usually 30–80 Å depending on the dyes used.

Fluorescence energy transfer immunoassays (FETI) using this phenomenon provide an attractive alternative to FPIA [22,23] (Fig. 1c). The method needs a simple fluorometer and is applicable to both small and large molecules. However, due to the limitation in R_0, sandwich detection of large proteins with two labeled antibodies is difficult due to noises derived from sample autofluorescence and direct excitation of the donor fluorophore.

An important improvement in the method was the development of longer life fluorophore such as rare earth chelates including ruthenium and europium that permit the use of time-resolved fluorescence. These labels, which have fluorescent lifetimes in the order of milliseconds, are used as energy donors. Using the acceptors that are conventional dyes absorbing at the emission wavelengths of the chelate, irradiation with a brief pulse of light is followed by rapid decay of the short-lived fluorescence of most sample impurities and any acceptor molecules that are directly excited by the light. The decayed fluorescence that is subsequently measured is associated only with emission from those acceptor molecules that are excited by FRET together with emission from the donor. The best reported sensitivity in a sandwich immunoassay for human chorionic gonadotropin in serum is about 10 pM [24], which is at least one order of magnitude more sensitive than can be obtained using steady-state fluorescence.

A characteristic derivative of FRET-based homogeneous immunoassay is open-sandwich fluoroimmunoassay (OS-FIA) (Fig. 1d). The assay is based on a novel immunoassay approach 'open sandwich principle' that is based on the antigen-dependent association of the two variable region fragments V_H and V_L [25,26]. A most important and unique character of OS immunoassay is that it can noncompetitively detect small molecules of less than 1000 in molecular weight, which have only been subjects of competitive immunoassays. Owing to its noncompetitive format, the assay has been proven to have superior sensitivity and wider measurable antigen concentration range. The assay principle was shown to be applicable to FRET-based homogeneous assays including OS-FIA, which can quickly monitor antigen concentration in a sample solution without any separation steps [27,28]. Since the distance between V_H and V_L is far shorter than that between two full-sized antibodies, OS-FIA has a merit of stronger signal. Although it has a greater merit in small molecule detection, the assay can be conducted with some protein antigens and their modifications, if an antibody with suitable property is employed. For example, Sasajima et al. succeeded in rapid detection of protein modification with this method by using recombinant fusion proteins of anti-phosphotyrosine (PY) variable region fragments and GFP variants [29].

3. Reagentless biosensors

3.1. Reagentless biosensors based on binding proteins

More recently, by site-specifically labeling the proteins with environment sensitive fluorophore at or near the ligand binding site, various proteins were successfully converted to fluorescence-based sensors (Fig. 2a). Since the development of environment sensitive dyes such as 7-nitrobenz-2-oxa-1,3-diazole (NBD) and 8-(phenylamino)-1-naphthalenesulfonate (ANS), a couple of proteins were site-specifically labeled to transform them into a fluorescent reagentless biosensor. These dyes show low fluorescence when exposed to the solvent, but emit stronger fluorescence if it is buried in the protein. Hence, if an appropriate labeling position is selected, the binding of protein ligand or ligand-induced conformational change of the protein, if any, can be detected as an increase of fluorescence. Since early in this millennium, sensors for glutamine [31,32], IgG [33], ligands of periplasmic binding proteins [34], maltose [35], phosphonate [36], lipid transfer protein ligands [37], glucose [38,39], and single strand DNA [40] were reported. Typically, the protein used as a receptor is mutated to remove cysteine (Cys) residues, and a site for labeling is mutated to Cys to conjugate with sulfur-reactive dye such as those with maleimide or iodoacetamide group. The sites for labeling are often chosen based on the known tertiary structure, but the best labeling position that gives best response should be experimentally determined.

As another approach, fluorescent proteins (FPs), most typically the variants of green fluorescent protein (GFP), were used as a reporter fluorophore. Engineering of FP, such as circular permutation and insertion of binding domains, or insertion of metal binding site, could successfully modulate the fluorescence of FP. An example is an HcRed mutant, whose red fluorescence is quenched by Cu^{2+} [41]. More extensive survey on FP-based biosensors can be found elsewhere [42,43].

3.2. Antibody-based reagentless biosensors

The same approach was successfully applied to antibodies for the detection of their target antigens. Renard et al. made reagentless biosensors based on an anti-hen egg lysozyme antibody D1.3 [44,45] (Fig. 2b). Based on the crystal structure, several residues near complementary determining region (CDR) of single chain variable region (scFv) were individually mutated to yield mutant scFv fragments. The labeled scFv with IANBD (N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole) showed various responses up to 2.8-fold antigen-dependent increase. It is worth noting that while individual V domain (V_H and V_L) has one intradomain disulfide bond, these original Cys residues were not labeled even after reduction with 2 mM dithiothreitol before labeling. This was probably due to the rigid β-sandwich structure of Ig V domain, with a disulfide bond already formed. Similarly, anti-dengue virus mAb4E11 was successfully converted to a reagentless biosensor with its antigen-dependent fluorescence increase up to 2.8-fold [46].

As an extension of this approach, Jespers et al. applied phage-display selection to obtain functional Cys mutant scFvs after labeling with the same fluorophore IANBD [47]. After the selection, a clone that responds to phOx conjugated to bovine serum albumin (BSA) was successfully obtained. Interestingly, the obtained clone did not show significant binding to phOx-BSA before labeling with IANBD.

The merit of antibody-based sensor is that it has an almost infinite possibility of recognition specificity. However, at this moment extensive engineering is necessary to obtain a good responder, especially when structural information of the antigen–antibody complex is unavailable. Also, successful reports are limited to protein antigen sensors.

It is worth noting that the use of fluorescent antibody fragments made by conventional labeling methodology sometimes gives sufficient information depending on the needs, especially in the case of cellular imaging. This is because antigen-dependent translocation of the probes will give spatiotemporally recognizable difference. For example, fluorolabeled Fab fragment recognizing phosphorylation of anti-histone H3 Ser10 (H3S10) was successfully used to visualize the intracellular histone modification upon injection of the Fab protein in live cells [48]. H3S10 phosphorylation occurs during chromosome condensation in mitosis mediated by the aurora B kinase. This was successfully monitored by the translocation of the probe from cytosol to nucleus. The

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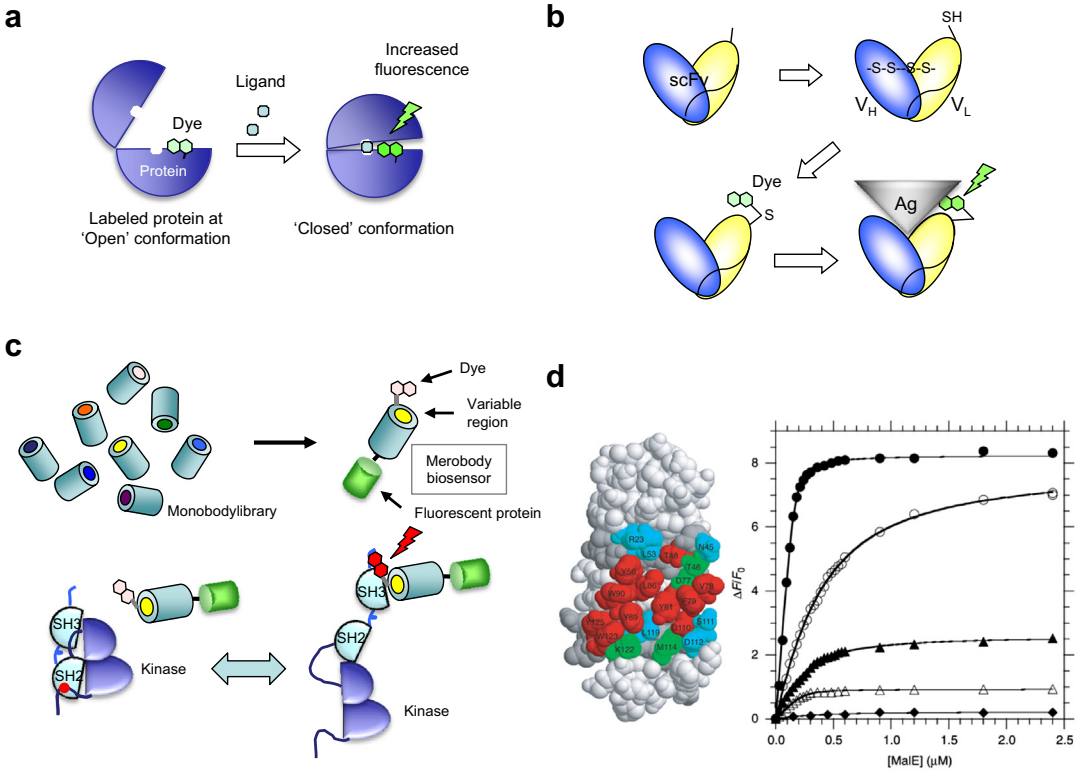

Fig. 2. Reagentless biosensors. (a) Scheme of biosensors based on various binding proteins. A protein of interest is labeled with an environment-sensitive fluorescent dye near its active site, and the ligand-dependent conformation change of the protein around the dye is monitored. (b) Antibody-based biosensors. Similar to (a), a residue of scFv comprised of V_H and V_L near the antigen paratopes is mutated to cysteine, in order to introduce an environment-sensitive dye. The binding of protein antigen makes the environment of the dye more hydrophobic, thus making it more fluorogenic (modified from [46]). (c) Merobody, which is a reagentless biosensor made of monobody and merocyanine dye. A monobody, which is a mimic of antibody V region made of a fibronectin domain 3, specific to Src SH3 domain is selected by phage display, and is labeled with merocyanine dye to make a sensor that can detect active Src proteins inside cells. The sensor is tagged with a fluorescent protein to perform ratiometric imaging (modified from [50]). (d) Structure and response of DARPin-based biosensors for MalE (reproduced from [63]). Peripheral residues (shown in green and cyan) around the antigen (MalE) binding site (red) of the DARPin are mutated to Cys and labeled to yield sensors with their responses as shown right. Open triangles, position Arg23; closed circles, Asn45; open circles, Thr46; closed triangles, Leu53; closed diamonds, Lys68 (negative control on the backside of the molecule).

results suggested that, during interphase, moderate aurora B activity or H3S10 phosphorylation is required for accurate chromosome segregation. Recently, similar approach was used to monitor histone H3 K9 acetylation by using GFP-fused scFv expressed in the cell (termed Mintbody after modification-specific intracellular antibody) [49].

3.3. Merobody

Recently, a reagentless biosensor based on the fibronectin type III (FN3)-based binding molecule (monobody) for quantifying Src dynamics in the cell was reported (Fig. 2c) [50]. The environmental sensitive dyes used were merocyan dyes, which have similar structures to cyanine dyes, and had been successfully used for monitoring Cdc42 activation upon conjugation with the Cdc42 binding domain of WASP [51]. The monobody specific to the 'open' conformation of Src family kinases was obtained by phage display screening with SH3 domain of Src as a bait, and it was found not to inhibit or activate kinase activity. Four solvent-sensitive merocyanine dyes were screened at four positions near the variable loops of the monobody. Of all the variations tested, three showed substantial response, with a maximum of ~110% increase for mero87 dye at residue 53. The 'merobody' thus made was successfully used for monitoring the intracellular distribution of Src kinase activity after microinjection into NIH3T3 cells.

3.4. DARPin-based biosensors

Target binding activity was also found in other protein families such as ankyrin and armadillo. These proteins with repeat sequences are ubiquitous binding molecules fundamental to many biological processes [52,53]. According to the structural studies of ankyrin repeat proteins, in most cases, the binding sites of the proteins are built from contiguous loops connecting B strands or helices. Furthermore, the binding surfaces for proteins are engineered on the faces of helical bundles [54,55]. Based on these findings, a new family of target binding proteins could be developed by engineering a stable polypeptide scaffold, which can imitate antibodies in many of their applications [56,57]. The designed ankyrin repeat proteins (DARPins) have been demonstrated to bear the potential of these artificial families of antigen binding proteins. The repeated ankyrin modules are present in thousands of proteins from all phyla and involved in specific recognition between proteins [58,59]. The consensus sequences of the modules were revealed to have some remarkable biophysical properties such as high stability, high solubility and robust expression in *Escherichia coli* [60–62].

Based on the above knowledge, Brient-Litzler et al. reported the rules for the design of reagentless biosensor from DARPins when the three-dimensional structure of the complex with their target is known [63] (Fig. 2d). They also validated these rules with Off7, a DARPin which is directed against maltose binding protein (MBP) from *E. coli* [64]. The design and construction of the reagentless biosensors from DARPin Off7 involved three steps: the choice of a target residue, the change of this residue into cysteine by site-directed mutagenesis and the chemical coupling of a fluorophore to the mutant Cys. The sites for labeling should satisfy two principles. One is that the environment of the coupling residue should change between the free and bound states. The other is that the residue should not be involved in the interaction between protein and its binding target. According to the structural

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analyses and calculation of ASA, they selected eight residues as potential coupling sites. The eight targeted residues were changed individually into cysteine by site-directed mutagenesis of the coding gene. The mutant DARPins were produced in *E. coli*, purified and labeled with IANBD ester to make potential biosensors. As a result, all the eight conjugates were sensitive to the binding of their target MBP, with signal increase between 0.73 and 14. Similar result was also reported [65].

More recently, DARPin-based biosensors for monitoring intracellular ERK activity was developed, and cellular ERK activity was successfully visualized with merocyanine-labeled DARPins [66]. The stable DARPins constitute a promising alternative to antibody fragments for the construction of reagentless biosensor, especially in the intracellular reducing milieu where normal antibody fragments with an intradomain disulfide bond are difficult to fold. In spite of their fundamental limitation as a protein binder in nature, these biosensors can be, and will be used for the quantification of target molecules in complex mixtures and in real time.

4. Quenchbody

Recently, a novel strategy of reagentless biosensor based on the principle of fluorescence quenching by the intrinsic amino acids in antibody variable region was proposed [67]. According to the proposed mechanism, the phenomenon was considered to have a generality.

4.1. Discovery of Quenchbody

The phenomenon was discovered rather fortuitously using a position-specific protein labeling method based on fluorolabeled aminoacyl tRNA and a cell-free translation system (Fig. 3a). The system is capable of incorporating many types of unnatural amino acids during in vitro translation, at the specific position encoded by amber codon or four-base codon [68]. If these codons are placed near the N-terminus of the protein, for example in the tag sequence, many organic fluorescent dyes conjugated to phenylalanine are efficiently incorporated regardless of their size [69]. Hence, the system has been utilized as an efficient site-specific fluorescence labeling method, and successfully applied to specific dual labeling of calmodulin [70] and MBP [71] to make FRET-based biosensors.

In this case, the system was used for constructing an open-sandwich FRET-immunoassay (OS-FIA) employing TAMRA-labeled V_H fragment and rhodamine 110 (R110)-labeled V_L fragment of anti-human osteocalcin (also called bone gla protein, BGP) antibody (Fig. 3b). When the purified two fragments were mixed and measured for the

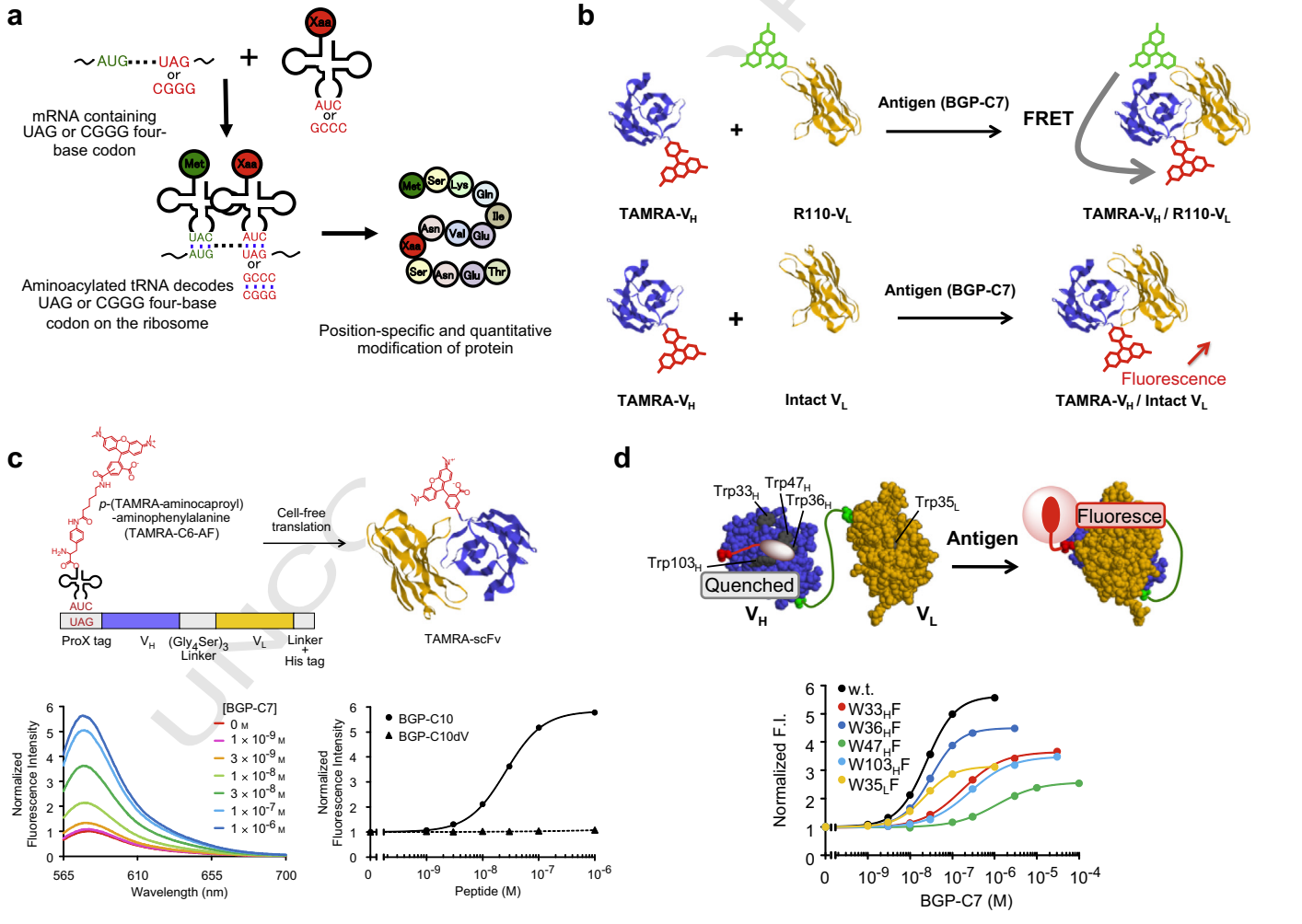

Fig. 3. Quenchbody. (a) The scheme to position and specifically introduce unnatural amino acids into proteins by in vitro translation. (b) A cartoon showing the experiment that leads to the discovery of Quenchbody. (c) The scheme to prepare TAMRA-labeled scFv (Q-body) and results of its antigen-dependent fluorescence [67]. When a peptide devoid of a C-terminal valine (BGP-C10dV) is used instead of C10, no response was observed, showing its specific recognition (right). (d) The proposed working mechanism of Quenchbody and its verification by the mutagenesis of Trp residues in the scFv to less quenching Phe residues [67]. The red oval indicates tethered TAMRA dye, either at quenched or fluorescent state. Green line indicates interdomain linker. The mutated Q-bodies at each position all showed attenuated antigen-dependent fluorescence increase, showing the involvement of all the Trp residues in the quenching of TAMRA dye in its 'open' conformation (upper left).

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change in fluorescent spectra by adding the C-terminal 7-mer peptide of BGP, expectedly, a good antigen-dependent increase in FRET efficiency was observed (data not shown). However, when a control experiment with TAMRA-labeled V_H and intact V_L was performed, a strange phenomenon of antigen-dependent fluorescence enhancement up to 1.7-fold was observed. In addition, when the V_H and V_L fragments were connected via a flexible linker to make scFv, expressed and labeled with TAMRA at its N-terminal tag region, a higher antigen-dependent fluorescence increment up to 5.6-fold with a low EC_{50} of 25 nM was observed (Fig. 3c). The fluorescence enhancement was observed for both intact BGP and its C-terminal peptide, but not for the peptide without C-terminal Val residue that is essential for recognition by this antibody [72]. It was also found that inclusion of 50% plasma in the fluorescence assay manifested little effect on its response, if the background fluorescence was subtracted, suggesting its robustness against interfering substances.

4.2. Analysis of working mechanism

An obvious question that arose was why such phenomenon was observed. Energy transfer was not plausible explanation since no other fluorescent dyes than single TAMRA dye per molecule exist in the system. There was a hint in the Hohsaka's group experiment that quenching of BODIPY-FL occurs when the dye was introduced near a Tryptophan (Trp) residue [71]. Also, there was a literature describing the eminent quenching of many organic dyes including TAMRA by free Trp at high concentration (30 mM), and it was attributed to photo-induced electron transfer (PeT) mechanism [73]. Hence, according to the assumption that Trp residues in scFv are involved in this phenomenon, a mutagenesis study was performed wherein each of the five Trp residues of scFv was mutated to non-quenching phenylalanine, and tested for the effect on fluorescence behavior (Fig. 3d). It was revealed that all the mutant scFvs showed an attenuated antigen-dependent fluorescence increase. The result indicates that in the absence of antigen, all the Trp residues in the V region more or less serve as a naturally occurring quencher, probably by PeT mechanism, and the addition of antigen suppresses such quenching. A fluorescence lifetime measurement of TAMRA-scFv supported this mechanism. When antigen was absent, the proportion of shorter lifetime species corresponding to quenched dye took major part. However, when antigen or denaturant (7 M guanidine hydrochloride, 100 mM dithiothreitol) was added, the proportion of this species significantly decreased. The result suggested that antigen- or denaturant-dependent conformational change of the scFv resulted in spatial separation of TAMRA dye from the Trp residues, resulting in its de-quenching. Such antigen-dependent conformational change probably corresponds to antigen-dependent Fv stabilization also observed in OS-FIA, which tightens the 'open' V_H/V_L interface to 'closed' one, thus expels the penetrating TAMRA dye.

It was rather unexpected since the four out of five Trp residues ($Trp36_H$, $Trp47_H$, $Trp103_H$, and $Trp35_L$) are in the framework region and situates far from the V_H N-terminus. The C5 linker between the TAMRA dye and phenylalanine side chain, as well as the following four amino acids preceding the N-terminus of scFv seemed to allow motional freedom and approximation of the dye to the Trp residues.

4.3. Application to other antibodies

Since at least four Trp residues important for the quenching are highly (>96%) conserved in mouse and human V domains, the possibility of applying the same strategy to other antibodies recognizing other antigens was investigated. As candidate antibody clones, anti-hen egg lysozyme (HyHEL-10), anti-serum albumin, anti-phosphotyrosine, and anti-bisphenol A were used. The scFv genes were prepared, and expressed in vitro in the presence of TAMRA-tRNA. This time, a short flexible linker $(GGGS)_2$ was appended between the dye-containing tag and the N-terminus of scFv, to endow more flexibility to the dye. The result of fluorescence measurement showed significant antigen-dependent fluorescent intensity increments in all the above clones, with a variation in sensitivity and response. Therefore, thereafter Q9 these TAMRA-labeled scFvs were called Quenchbodies or Q-bodies, after antibodies with their quenched fluorescence. In addition, an anti-morphine antibody was successfully converted to Q-body, and confirmed for its fluorescence increase with similar EC_{50} value to reported K_d of this antibody.

It was also found that in addition to TAMRA, other rhodamine dyes such as R110 and ATTO520, and an oxanine dye ATTO655 were also suitable dyes for Q-body, with somewhat reduced responses but at different wavelength peaks. These dyes share rather hydrophobic planer scaffold, and essential moiety will be a three ring structure. Their quenching propensity in Q-body is not directly proportional to that observed with free Trp. For example, compared with TAMRA, ATTO655 is more quenched by free Trp but less responsive in Q-body (max. 2.0-fold). Probably, its shape and hydrophobicity (i.e. affinity to hydrophobic V_H/V_L interface) also influence the dye's quenching property.

4.4. Application to vimentin phosphorylation detection

Protein phosphorylation is a key event in intracellular signal transduction, and fluorescent biosensor for the specific phosphorylation event in a target protein is considered highly useful as a tool of cellular biology and drug screening. Vimentin, the most abundant intermediate filament protein, is phosphorylated at its specific serine (Ser) residues in a cell cycle dependent manner [55,56]. Its structural and functional characteristics are modified by the phosphorylation, which affects biological properties of the cell. The detection of the vimentin Ser71 phosphorylation (PS71) and the vimentin Ser82 phosphorylation (PS82) by Q-body was reported [74]. Interestingly, in these cases rhodamine 6G (R6G)-labeled Q-bodies showed a superior response than TAMRA-labeled ones. After optimization of reaction condition, the fluorescence intensity of V_H–V_L type PS71 Quenchbody labeled with R6G at two positions (N-terminal and middle of interdomain linker) increased up to 4.0-fold in an antigen dependent manner. On the other hand, the fluorescence intensity of doubly R6G-labeled V_L–V_H type PS82 Quenchbody was increased to 6.7-fold after adding antigen peptide. In general, higher performances were obtained for dual-labeled Q-bodies, suggesting their deeper initial quenching. This was probably due to additional quenching by dye–dye interaction, namely H-dimer formation between the dyes [14,75]. It is worth noting that a similar dual-labeled biosensor (TAMRA-labeled ParM protein for detecting ADP [76]) is reported. The Q-body based phosphorylation detection will be particularly valuable as an intracellular imaging probe, where lower background fluorescence than attained with conventional fluorolabeled antibody, is expected.

4.5. Application of Q-body strategy to Fab fragment

While scFv-based Q-body showed applicability to many targets, its lower thermostability than that of parental antibody and low fluorescent response to some target antigens constituted its potential problems. With the aim of further extending the Q-body approach, production of Fab-type Q-body (Ultra Q-body, UQ-body) was attempted [77]. In this case, the genes for Fd ($=V_H + C_H1$) and light chain ($=V_L + C_L$) were co-translated in the same in vitro reaction, and UQ-bodies with one or two dyes incorporated at the N-terminal tag region were successfully prepared (Fig. 4a). A merit of this system is that depending on the constructs and their combinations, either H or L chain N-terminus, or both of the two N-termini can be labeled with a dye of choice. Also, with the use of different codons (amber and four-base etc.), two different dyes can be simultaneously incorporated to a single Fab fragment.

Using this system, anti-BGP Fab fragment TAMRA-labeled at the H chain was prepared and compared with TAMRA-scFv. Surprisingly, the Fab showed a superior fluorescence increase up to 9.6-fold. Also,

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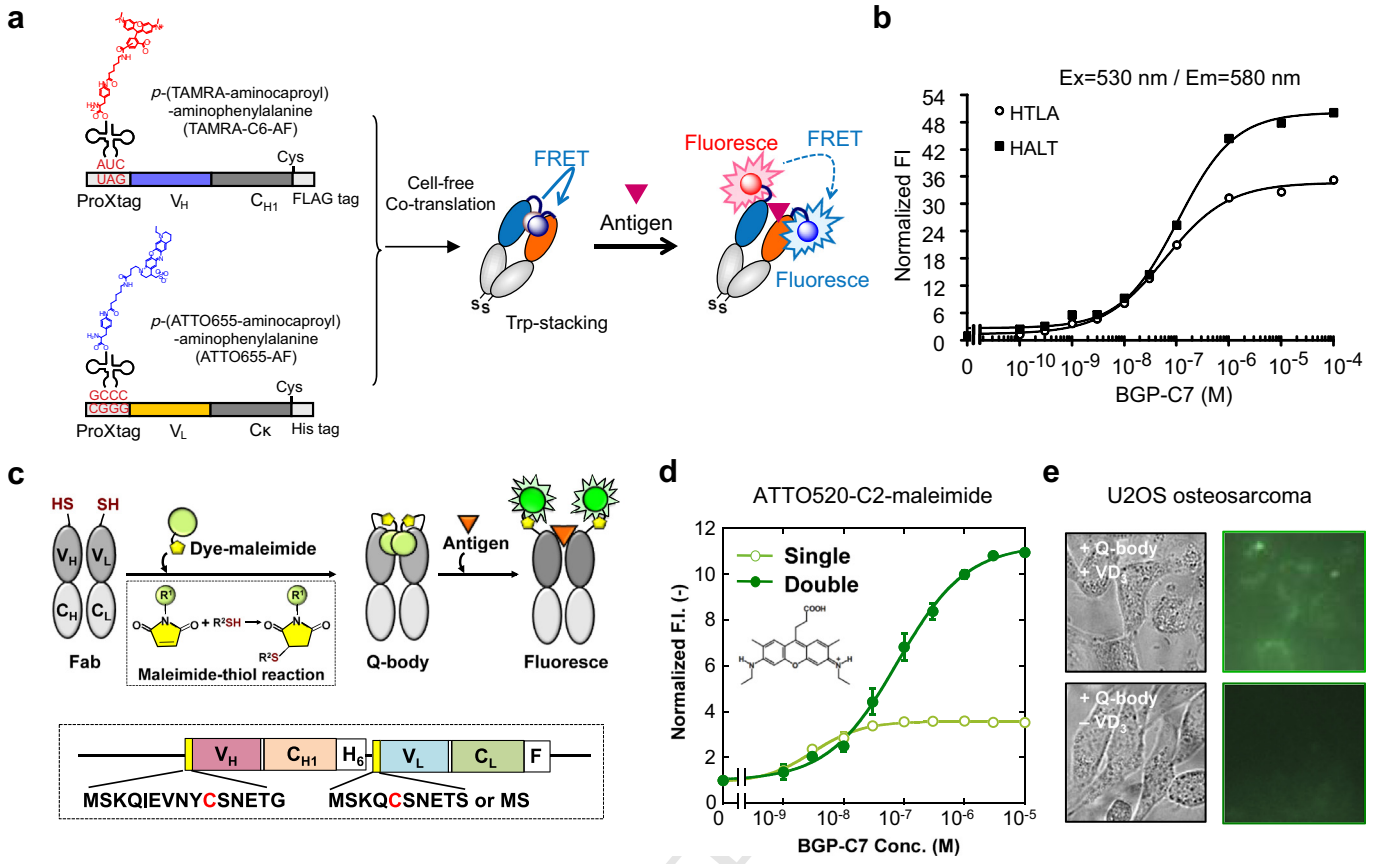

Fig. 4. Enhanced fluorescent response by Fab fragments incorporated with multiple dyes (Ultra Q-bodies, UQ-bodies). Modified from [77]. (a) Scheme to prepare hetero-double-labeled UQ-bodies in vitro. During translation reaction, TAMRA and ATTO655 dyes were incorporated into the N-terminal region of heavy (Fd) and light chains, respectively, using specific aminoacyl tRNAs. (b) The dose–response of the UQ-bodies. HTLA denotes the TAMRA-labeled H chain and ATTO655-labeled L chain, while HALT denotes vice versa. The donor-derived fluorescence is shown, each normalized by the value without antigen. (c) Scheme to prepare Fab-type Q-bodies from recombinant Fab fragment expressed in *E. coli*. The schematic construct of the expression vector is shown below, in which two types of N-terminal tag sequences at the L chain are shown. H_6 and F denote His_6 and Flag tags, respectively. (d) Dose–responses of ATTO520-labeled Q-bodies. Single and double denote the number of dyes attached to the Fab. (e) Visualization of BGP produced by osteoblast-like cells by double-ATTO520 UQ-body. U2OS cells were added for inducing differentiation with vitamin D3 (VD_3) for 48 h to produce BGP. Only when the cells were added with VD_3, fluorescence of the cells was observed.

when the thermostability of two proteins was compared by temperature-dependent fluorescence recovery (thermal shift assay), the Fab showed higher melting temperature (73 °C) than the scFv (61 °C). Probably, more stable structure of Fab fragment resulted in deeper quenching of TAMRA dye and higher antigen-dependent fluorescence recovery.

In addition, dual-labeling the two chains with the same or different dyes resulted in generally higher fluorescent response, probably due to additional quenching induced by dye–dye interaction. When two different dyes such as TAMRA and ATTO655 were used, efficient FRET between them was observed, resulting in the deeper quenching of donor dye (in this case TAMRA) (Fig. 4b). In the case of anti-BGP antibody, this resulted in greater antigen-dependent fluorescence increase as high as 50-fold. In the case of anti-serum albumin Q-body that showed a modest response of 1.5-fold as TAMRA-scFv, higher responses up to 9-fold were obtained with hetero-dual labeled UQ-bodies. Possibly, the UQ-bodies based on dye–dye quenching will be more advantageous for protein detection, because such dimers will be more susceptible to the binding of large proteins than that of small molecules.

Lastly, construction of Fab-type Q-body was successfully achieved using recombinant Fab fragments expressed in *E. coli*. In this strategy, the amber codon in the N-terminal tag was substituted with a codon encoding cysteine (Fig. 4c). The labeled Fabs by dye-maleimide showed a similar antigen (BGP-C7) dose–response to that observed by the UQ-bodies prepared in vitro (Fig. 4d). As a demonstration of its utility in cellular imaging, double ATTO520-labeled UQ-body was used to image BGP protein produced by osteoblastic cells. As a result, only the osteosarcoma cells induced for osteoblast differentiation show distinct fluorescence upon the addition of the UQ-body without any washing steps (Fig. 4e).

5. Conclusion

We showed that the merits of fluorescent reagentless biosensor approach over conventional heterogeneous immunoassays, which include faster detection with less handlings. These are especially advantageous in view of application to on-site detection devices, or to point of care (POC) devices. Also, two different principles to produce fluorescent reagentless biosensors, namely, the use of environment-sensitive dyes and the quenching of conventional organic dyes by the residues in natural polypeptide and by additional dyes (for Quenchbodies and UQ-bodies), were compared. The merits and possible demerits of each method are summarized in Table 1.

The superiority of Quenchbody strategy over previous fluorescent reagentless biosensors can be depicted as follows. 1) Antibodies with high specificity and affinity can be obtained by a number of methods (conventional hybridoma technology, phage display or other in vitro selection technologies such as ribosome display) to a range of antigens from small molecule to large proteins, and all constitute a potential source of Q-bodies. In other words, the use of antibody instead of individual ligand-specific binding proteins allows the detection of a wider range of targets. 2) The original Quenchbodies with one dye utilizes

Table 1
Comparison of fluorescence-based homogeneous immunoassays.

Biosensor name	Principle and description	Merits	Reported sensitivity	Possible demerits
Fluorescence polarization immunoassay (FPIA)	Brownian motion; based on the relationship of the tumbling speed of analyte and the polarization of fluorescent dye	Simple mixing of sample and labeled Ag/Ab.	10 pM oligonucleotide [81]	Needs polarization filters. Not for large Ag like proteins. Suffers from fluorescence quenching
Fluorescence correlation spectroscopy (FCS)	Monitor fluorescence fluctuation derived from sample molecules moving in and out of focused laser beam.	Applicable to larger molecules than FPIA. Small analyte volume.	1.4 pM alpha fetoprotein [82]	Need of expensive equipment.
Fluorescence energy transfer immunoassays (FETI)	Ab-Ag or Ab-Ag-Ab complex is detected by FRET	Applicable to both small and large molecules. Higher sensitivity is attained if coupled with time-resolved measurement.	10 pM human chorionic gonadotropin [24]	Difficult to detect large Ags in sandwich format. Equipment costs.
Open sandwich fluoroimmunoassay (OS-FIA)	Antigen-dependent Fv stabilization is detected by FRET	Can detect both small and large antigens in noncompetitive mode.	70 nM hen egg lysozyme [28]	Not available for Abs with strong V_H/V_L interaction.
Ab-based reagentless biosensors	scFv labeled with environment-sensitive dye	Can detect protein targets by just mixing and measuring fluorescence.	10 nM dengue virus [46]	Needs special dye and the search of labeling position. Variable response.
Merobody	Reagentless biosensor based on merocyanine-labeled monobody	Applicable to imaging various targets in reductive intracellular environment	~300 nM Src-SH3 [50]	Limited fluorescent response. Ratio imaging needed.
DARPin-based biosensors	DARPin protein labeled with environment-sensitive dye	Works excellently in intracellular reducing milieu	0.3 nM maltose binding protein [63]	Only for proteins. Needs optimization of labeling position.
Quenchbody	De-quenching of Ab-attached fluorescent dye(s) due to Ag binding	Variety in detectable Ag size and Ab format.	0.3 nM human osteocalcin peptide [77]	Optimization of dye type and number might be necessary.

internal Trp residues as a major quencher. These Trp residues are conserved over a range of antibodies from various species, suggesting its versatility. 3) The labeling position is usually N-terminal, and there is no need to introduce mutations in the antibody itself. 4) It is not necessary to screen optimal labeling position, while optimization of linker and/or number and type of dyes might be necessary. 5) A possible combination of several quenching effects allows a wider range of measurable antigen size (molecular weight) and type (synthetic haptens to proteins). 6) Simultaneous detection of multiple antigens in the same sample liquid is potentially possible by using multiple Q-bodies labeled with a variety of dyes.

So how about shortcomings? A current 'must' include the development of a rapid and efficient methodology to select and optimize Quenchbodies. In other words, to obtain a good Q-body, selection of high-responder is necessary as well as the selection of good antibody with high affinity and specificity. A combination of affinity- and fluorescence-based selection methods is desired to further enlarge the scope of this method.

One of the R&D goals of Q-body approach in the near future will be the conversion of a natural antibody protein to a Q-body. Although it might not be an easy task to elucidate a general method, combinatorial use of N-terminal and/or V-region specific chemical labeling methods will potentially overcome this hurdle [78–80]. If it is realized, possibly, conventional time-consuming immunoassays like ELISA will be taken over especially for small molecule detection.

6. Uncited reference

[30]

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