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Review

Nanobody-based food allergen surveillance: current status and prospects

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Abstract

The incidence of food allergy has increased in recent decades, posing drastic risks to sensitive individuals, leading to mild to severe allergic symptoms. There is still no effective immune therapeutic strategy for food allergy that addresses accurate analytical methods to indicate the presence of allergens to prevent exposure of sensitive individuals. Currently, the most commonly applied detection method is immunoassay developed with food allergen-specific antibodies, especially the conventional formats of monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs), which serve as dominant detection reagents for food allergen analysis, although with the disadvantages of being labor-intensive, costly, batch differences, and significant cross-reaction, etc. Camelid-derived nanobodies (Nbs) have attracted tremendous attention to explore their application in food hazard analysis because of robust characteristics like unique paratopes, high stability and affinity that strongly contribute to the beneficial effect. However, extensive analysis is needed to validate the potential use of Nbs as detection reagents and the advantages for food allergen surveillance. Previous reports have demonstrated the potential of Nbs for immunoassay development against food allergens, such as macadamia allergen Mac i 1, peanut allergens Ara h 1 and Ara h 3, lupin allergen Lup an 1, milk allergen β-lactoglobulin, etc. In this review, we comprehensively summarize the structural and biochemical properties of Nbs that benefit the application of Nb-based immunoassay, as well as the representative detection strategies, to provide research data for newly developed Nb formats for food allergen analysis, and investigate the future establishment of Nb-based surveillance against major food allergens.

Keywords: Food allergy; food allergen; nanobody; immunoassay.

Introduction

Food allergy is a type of hypersensitivity mediated by specific immunoglobulin E (IgE) specific to food protein ingredients to cause symptoms ranging from mild hives and gastrointestinal disturbances to more severe reactions such as anaphylactic shock or even life-threatening illness. Food allergy affects an estimated 8% of children under age 5 and up to 4% of adults, with raised prevalence in the past two decades, and is currently classified as a serious public health problem (Alves *et al.*, 2016; Aquino and Conte, 2020; Barni *et al.*, 2020). The clear classification between food allergy and food intolerance is fundamentally defined by sensitized immune reactions to food allergy, and intestinal disorders or insufficiency, such as lactose malabsorption for food intolerance (Wang *et al.*, 2011; Yu *et al.*, 2016). Major allergens have been identified as proteins mainly from eight types of food including egg, milk, tree nuts, etc., with sesame newly identified as the ninth major food allergen resource, which covers almost 90% of all serious food allergies. Biased food allergy can be observed in sensitive individuals from different regions, such as severe reaction to peanut in North America (Chafen *et al.*, 2010; Weiss and Smith, 2023).

The anaphylaxis of sensitized individuals occurs upon exposure to certain food allergen proteins and subsequent IgE-mediated degranulation of effector cells (e.g. mast cells and basophils). Epitopes of food allergens interact with the binding domains of IgEs to trigger conformational variation to complementarily integrate with the FcεRI receptor on the surface of these effector cells, as a result provoking the release of histamine and other inflammatory mediators to initiate an immediate allergic reaction upon re-exposure to the sensitized food ingredients (Stone *et al.*, 2010; Robison, 2014). Therefore, the avoidance of food allergen exposure is considered the most effective strategy to prevent food allergy occurrence. Legislation concerning food ingredient surveillance and food allergen labeling has been implemented to indicate the presence of certain food allergens for consumers. This has been implemented to clearly label the common allergen ingredients required by the Food Allergen Labeling and Consumer Protection Act in the USA, and the act approved by the European Union (EU) requires similar principles with more comprehensive disclosure of allergenic food ingredients. In addition, voluntary advisory statements like 'may contain peanuts' allows the food industry to provide consumers with information about the unavoidable presence of allergen residues, sometimes at very low levels (Taylor and Hefle, 2006; Ford *et al.*, 2010). Unexpected exposure to food allergens can result from accidental contamination during food processing

or transportation, food adulteration, cross-contamination and even fraud, which can dramatically raise the risk of allergic reaction for sensitive individuals and significant economic losses for the food industry (Röder *et al.*, 2008; Prado *et al.*, 2016; Campuzano *et al.*, 2020). The above-mentioned principles address the effective strategy to avoid trace allergens exposure based on sensitive detection methods. Thus, it is of great significance to establish sensitive and reliable food allergen detection methods, with emphasis on the exploration of novel detection reagents or advanced detection methods with combinational techniques (Robison and Singh, 2019; Hu *et al.*, 2022b).

Various detection methods have been developed for application under different circumstances, including polymerase chain reaction (PCR), chromatography, and antibody-based enzyme-linked immunosorbent assay (ELISA) (Picariello *et al.*, 2011; McGrath *et al.*, 2012). PCR-based surveillance generally provides precise results, albeit hampered by false positives arising from unspecific amplification of irrelevant fragments (Sin *et al.*, 2014). Chromatography is often considered the standard validation, with the drawbacks of being time-consuming and labor-intensive and the requirements for skilled operators and expensive equipment (Heick *et al.*, 2011). Antibody-based immunoassay is the most frequently employed analytical method against food hazards in complex matrices, with the advantages of *in situ* manipulation and cost-effective application. The result output can be realized after conjugating food allergen-specific antibodies with various substrates for distinct signal development or chromogenic, fluorescence, electrochemical signals, etc. (Su *et al.*, 2013; Sharma *et al.*, 2016; Wu *et al.*, 2016). Currently, most of the immunoassays are generated based on conventional monoclonal or polyclonal antibodies and lack lot-to-lot variation. Furthermore, the preferable targeting of conventional antibodies on linear epitopes distributed on surface of food allergens potentially raises the possibility of cross-reaction with other allergens, which cause significant false detection (Niemi *et al.*, 2007; Orcajo *et al.*, 2019). Thus, further study is required to explore and identify the detection reagents or methods to provide more precise or sensitive detection of food allergens.

Camelid-derived single domain antibodies are antibody fragments engineered from camelid heavy chain-only antibodies (HCAbs), which are naturally devoid of light chains and the first constant domain of the heavy chains (CH1). The derived variable domain of HCAbs is termed nanobodies (Nbs) because of their small size in the nanometer range (2.5 nm×4 nm×3 nm; Jindal *et al.*, 2020; Liu *et al.*, 2021). As the smallest antigen-binding unit, Nbs have been characterized with a variety of loop structures and lengths in the variable binding domains, with an affinity comparable to that of conventional antibodies (Roovers *et al.*, 2007; Muyldermans, 2013). Nbs have been identified with excellent properties including good solubility, high stability, ease of manipulation, and binding potential to unique epitopes with high affinity, which fundamentally contribute to their application as detection reagents against different food hazards such as mycotoxins, pesticides, and macromolecules of proteins (Sun *et al.*, 2021). The excellent characteristics and application of Nbs in food safety analysis have been summarized in published literature to investigate their potential as detection reagents for the construction of newly defined methods (Hu *et al.*, 2017; Moradi *et al.*, 2020; Hu *et al.*, 2022b). The robustness of Nbs

has been demonstrated in different studies with the straightforward selection of specific Nbs against the target of interest from the pre-immunized library through phage display technology (Muyldermans, 2021). Numerous Nbs have been prepared for the detection of proteins, mycotoxins, pesticides, environmental pollutants, etc. (Bever *et al.*, 2016; Chen *et al.*, 2019). The successful application of Nbs for the diagnostic detection of disease-related biomolecules or the analysis of food hazards has emphasized their advantages as detection reagents to specifically identify the targets with high affinity and selectivity, and more importantly, to preferably interact with conformational epitopes, thus potentially increasing the precision of the detection (Hu *et al.*, 2022b; Wu *et al.*, 2023). The unique properties or preparation strategies of Nbs have evoked their capability as detection reagents for food allergen analysis, which has not been addressed in previous reports since our group made efforts to investigate the application of Nbs in food allergen analysis (Li *et al.*, 2023; Yao *et al.*, 2024). The potential application of Nb-based detection methods has been validated in serial investigation, as well as their unique properties for food allergen detection. In this review, we discuss the structural and biochemical properties of Nbs and review the research progress of Nbs in food allergy applications, as well as the unique characteristics that contributes to the advanced application of Nb-based detection on food allergens.

The Biochemical Properties of Nbs

Antibodies or immunoglobulin G (IgG) are serum glycoproteins with four polypeptide chains that typically form a Y-shaped structure, which is involved in immune reaction when stimulated by external antigens. Several fragments can be engineered from this general architecture, such as the antigen-binding fragment (Fab) and the single-chain variable fragment (scFv), which contains a biometric region but lacks a constant moiety (Figure 1A; Salvador *et al.*, 2019). Camelid-derived Nbs originating from HCAbs represent the only derived antibody fragment with complete antigen recognition ability (Figure 1B). In general, the structure of Nbs is similar to that of the corresponding variable domain (VH) of conventional antibodies with four conserved frame regions (FR1–FR4) encircled by three highly variable antigen-binding loops (complementarity-determining regions, CDR1–CDR3) (Wang *et al.*, 2016b; Li *et al.*, 2018). As summarized in Figure 1C, CDR1 and CDR2 regions of Nbs are partially involved in antigen interactions, with CDR3 as the region that dominantly contributes to antigen recognition and binding. The longer CDR3 facilitates the formation of convex finger-like paratopes to identify hidden epitopes, which are conducive to epitope binding to the grooves or cracks on the surface of the antigen, such as the catalytic sites of enzymes located in the grooves. This produces the regulatory effect of Nbs on catalytic activity, which illuminated the advantages of Nbs on enhanced binding specificity by targeting the unique epitopes of antigens (Muyldermans *et al.*, 2009; Steeland *et al.*, 2016; Wang *et al.*, 2018). The different epitope preferences of Nbs and conventional antibodies have been commonly observed in previous studies to indicate binding to conformational or unique sites of Nbs rather than the linear or surface epitopes of conventional antibodies in most cases (De Genst *et al.*, 2006; Uchanski *et al.*, 2020). The cross-reaction of conventional antibodies significantly aroused from the commonly
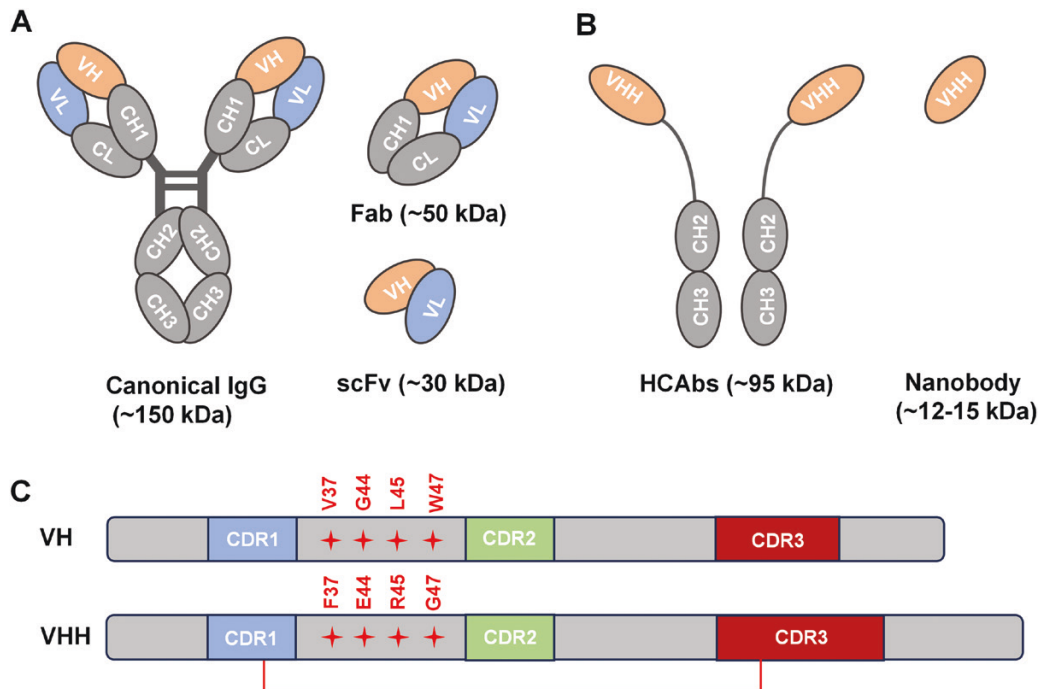
Figure 1. Schematic representation of antibody fragments and the unique structure of Nbs. (A) Conventional antibodies comprising two light chains (L) and two heavy chains (H) to form the typical Y-shape structure, with the derived antibody fragments including Fab and scFv; (B) HCAbs identified in camelid animals are naturally devoid of light chains and the first constant domain (CH1) with the derived antigen binding domain coined Nbs with molecular mass approximately 15 kDa; and (C) The similar molecular structure between VH and VHH domains is summarized to include the framework regions (FR1–4) and the complementarity determining regions (CDR1–3), with the distinctive longer CDR1 and CDR3, and hydrophilic amino acids (F42 or Y42, E49, R50, and G52) distribution for VHH. The disulfide bond between CDR1 and CDR3 could be observed generally for Nbs.

observed similar linear or surface located epitopes of different food allergens. Whereas, Nbs have the potential to target either conformational or unique epitopes distributed in concave conformation of food allergens that the conventional antibody cannot reach, which potentially contributes to specific interaction with target food allergens rather than the allergens classified into the same families or with similar epitopes, or refer to the avoidance of cross-reaction that commonly occurs with conventional antibody-based detection (Vu *et al.*, 1997; Salvador *et al.*, 2019). The disulfide bond between the CDR1 and CDR3 regions of Nbs may have maximized the topology of the binding surface and facilitated the orientation of antigens by CDR3 to increase the stability of the structure (Siontorou, 2013; Mitchell and Colwell, 2018). The robust properties of Nbs were verified to possess approximately 100% binding activity after long-term storage at 4 °C, or even 37 °C, whereas the recommended stock condition is under –20 °C or –80 °C to maintain complete binding capability. Robustness has been classified under extreme conditions like high temperatures, extreme pH, and even chemical denaturants, presumably because of the completely reversible characteristics of Nbs under these conditions (Arbabi Ghahroudi *et al.*, 1997; Dumoulin *et al.*, 2002). The four highly conserved hydrophobic residues (V42, G49, L50, and W52) in the VH are replaced by smaller and/or hydrophilic residues in the Variable domain of heavy chain of HCAb (VHH), mostly Phe42, Glu49, Arg50, and Gly52, respectively, which potentially contribute to the improved solubility and the decreased probability of aggregation of VHH (Govaert *et al.*, 2012; Wang *et al.*, 2016b; Hu *et al.*, 2017). The small size and solubility of the Nbs may also guarantee

antigen affinity, with kinetic binding constants from picomole to nanomole (Muyldermans, 2013; Sockolosky *et al.*, 2016; Debie *et al.*, 2020). Moreover, Nbs can be easily formatted or diversified by genetic engineering to fuse newly introduced moieties to produce homologous or heterologous formats such as multi-specific and multi-valent Nbs to improve affinity, or conjugates with fluorescent proteins such as green fluorescent proteins to achieve multi-signal output (Robert *et al.*, 1999; Fridy *et al.*, 2014). In addition, the conserved sequence constitution of VHH has produced the semi-design or synthetic preparation to ensure batch selection and application, and the low immunogenicity supported by data from the Belgian Phase I trial of Ablynx NV (Vincke *et al.*, 2009; Kijanka *et al.*, 2013). Crucially, Nbs can be easily obtained, usually after a brief protein immunization of camelid animals to raise a strong immune response to ensure the straightforward acquirement of the complete repertoires of Nbs from peripheral blood mononuclear cells after sequential panning and screening processes (Pardon *et al.*, 2014; Romao *et al.*, 2016). The periplasmic expression of Nbs can be achieved by using prokaryotic expression systems (such as *Escherichia coli*). The oxidative environment and folding chaperones within the periplasm contribute to the correct folding of the VHH domain and the formation of disulfide bonds. The extraction of periplasmic proteins by osmotic shock can typically yield enough Nbs for research. Moreover, expression of Nbs in eukaryotic expression platforms such as yeast or mammalian cells can be accomplished to ensure clinical application if necessary (Liu and Huang, 2018). Moreover, the injection of multiple antigens can facilitate the simultaneous screening of specific Nbs against various antigens to ensure

cost-effective production, which allows the application of extensive research studies to fulfill requirements such as low cost, batch preparation, and customization (Liu *et al.*, 2018).

Beneficial Properties of Nbs for Food Allergen Surveillance

Food allergen exposure can occur anywhere in the food chain from manufacturing to transportation with unexpected contamination or intentional supplementation (Figure 2A). The establishment of Nb-based immunoassay has been proposed for food allergen analysis with certain properties potentially benefitting the application, and it is expected to enable comprehensive detection against most of the major allergens to serve as alternative detection strategies in addition to the mAb-based immunoassays currently employed (Figure 2B). Compared with those of the most commonly employed conventional antibodies, certain unique properties of Nbs could facilitate the preferred utilization as modules for detection method establishment. The preparation of food allergen-specific Nbs was realized after the immunization process to allow the *in vivo* maturation of target specific binders, which facilitates the straightforward selection and recombinant expression of Nbs by following well-established procedures (Khodabakhsh *et al.*, 2018). In contrast, mAbs are large multimeric proteins that typically require a post-translational maturation process like glycosylation to form complete capability of antigen identification, which requires expression in a eukaryotic expression platform, potentially raising manufacturing costs (Kijanka *et al.*, 2015; Salvador *et al.*, 2019). The straightforward preparation of Nbs allows immunization with complex food allergens to facilitate the simultaneous selection of specific Nbs against various allergens to significantly accelerate the research progress. More importantly, the utilization of crude extract protein for unbiased immunization and the selection of allergen-specific Nbs could greatly prevent the restriction of the purification of certain food allergens (He *et al.*, 2014; Hu *et al.*, 2021a).

As illustrated in Figure 2C, a distinctive feature of Nbs relies on the generally longer CDR3 in comparison with that of conventional mAbs, which facilitates the formation of flexible paratopes to potentially target unique epitopes, such as the concave catalytic sites in enzymes or the conformational epitopes on food allergens. This could dramatically benefit the detection of certain allergen proteins in the food matrix by avoiding the cross-reaction of current detection methods. Moreover, the longer CDR3 region of Nbs can ensure interaction with a wide range of epitopes in nanomolar or even picomolar ranges of affinity. This means that the affinity of Nbs is equal to or better than that of conventional antibodies, potentially contributing to a higher sensitivity for detection (Salvador *et al.*, 2019). Meanwhile, targeting unique epitopes allows the identification of trace allergens in foods by interacting with conserved or preserved fragments even after food processing under certain conditions. Thus, it is expected to enhance the precision and sensitivity of food allergen analysis by using Nbs as the modules for detection (Aline Desmyter *et al.*, 1996; Muyldermans *et al.*, 2009). However, the preferable binding to conformational epitopes of Nbs could result in the limitation that specific Nbs can no longer identify target allergens upon food processing-induced structural variation. For analysis of a food sample with organic reagents such as *n*-hexane, the organic reagent residue may affect the subsequent immunoassays, and antibodies with high tolerance to organic solvents or pH are preferred (Wang *et al.*, 2022; Hu *et al.*, 2022b; Wu *et al.*, 2023). Thus, the robustness of Nbs under the abovem-mentioned harsh conditions is preferred and should benefit the development of detection methods for the following analysis in real samples. The evolution of hydrophilic amino acids within FR2 contributes to the high aqueous solubility of Nbs (Maass *et al.*, 2007), and Nbs have good stability to maintain their complete
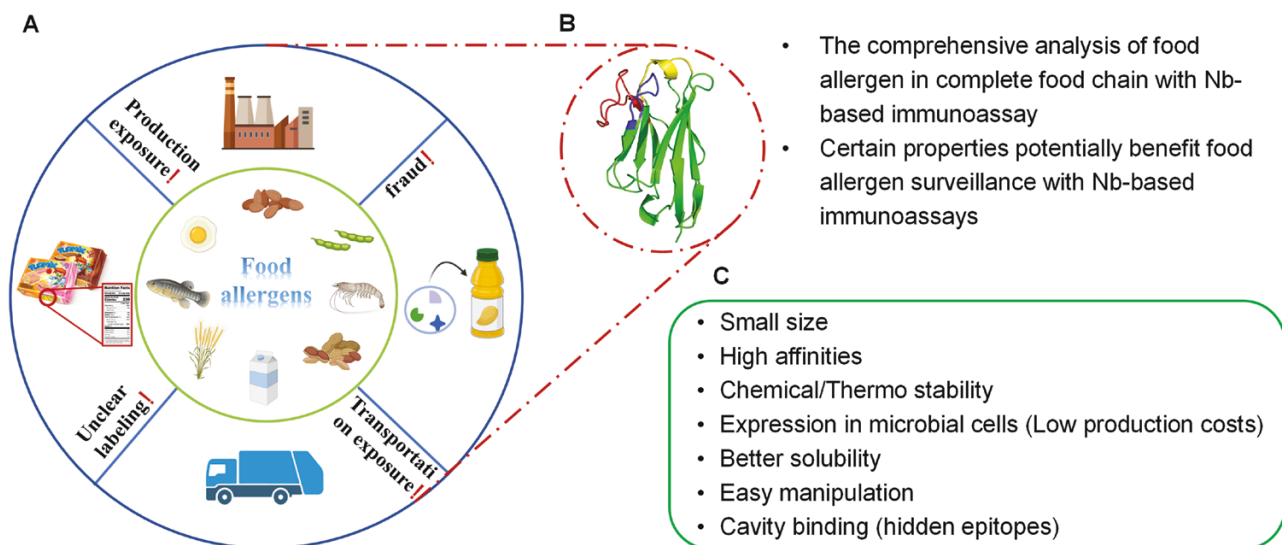

Figure 2. The beneficial characteristics of Nbs for food allergen surveillance. (A) Almost 90% of all serious food allergies are related to proteins (allergens) in nine foods including egg, milk, peanut, and tree nuts. The incidence of food allergen exposure occures during the complete food chain from manufacturing to transportation with unexpected contamination or intentional supplementation; (B) The comprehensive analysis of food allergen in the complete food chain is proposed with Nb-based immunoassay, and certain properties of Nbs potentially benefit food allergen surveillance with Nb-based immunoassays; and (C) The unique characteristics facilitate Nbs as the perfect candidates for research and application in the field of food allergen surveillance.

antigen binding ability after long-term storage under recommended conditions. When exposed to chemical denaturants (2–3 mol/L guanidine chloride, 6–8 mol/L urea), proteases and non-physiological pH (pH range 3.0–9.0), or organic solvents such as methanol and acetonitrile, Nbs can maintain antigen binding capacity, which emphasizes the application of Nbs or Nb-based detection strategies in general or hostile conditions (Dumoulin *et al.*, 2002; De Vos *et al.*, 2013), whereas mAbs are widely accepted to maintain binding capacity in the aqueous phase or more hospitable surroundings (He *et al.*, 2014; Liu *et al.*, 2017).

Furthermore, the strict modularity and monomeric characteristics allow easy manipulation to label Nbs with proposed conjugates through either *in vivo* fused expression or *in vitro* decoration to form multi-valent or multi-specific Nbs for improved affinity or applicability, although appropriate labelling strategies need to be utilized to guarantee the binding capacity of Nbs after conjugation. Significantly, *in vivo* decoration or fusion of Nbs with signal-developing modules can ensure the consistency of detection methods to avoid batch-to-batch differences resulting from the variation of antibody conjugation, which are often identified as the bottleneck drawbacks of the current mAb-based immunoassay (Cortez-Retamozo *et al.*, 2004; Behdani *et al.*, 2013; Huet *et al.*, 2014). Alternatively, the conjugation of Nbs with signal molecules can also be achieved by an *in vitro* method through chemical reaction with lateral amino acid residues like lysine, although the randomly distributed amino acids could potentially result in the shield of binding ability if conjugation occurred in the loops responsible for antigen targeting (Pleiner *et al.*, 2015). Exogenous cysteine can be introduced into the C-terminus of Nbs through genetic manipulation to artificially control the conjugation without significant interference on the binding capability of Nbs (Brooks *et al.*, 2002; Massa *et al.*, 2014). Site-specific chemical enzyme protein functionalization is a suitable coupling strategy in which a specific active peptide tag, such as Sortase A or transglutaminase, is introduced at the C-terminus of Nb and the compound of interest can be attached to the activated peptide tag through a biorthogonal reaction (Stephanopoulos and Francis, 2011; Rashidian *et al.*, 2016). In conclusion, various strategies can be applied for the coupling of Nbs with ligands of interest for signal development, which could significantly benefit the construction and application of Nb-based detection by following the purpose of research and industrial applications.

Selection Strategies for Specific Nbs Against Food Allergens

Obtaining a library containing the required genetic information is essential for the preparation of Nbs with high specificity and affinity against food allergens, which can be generated from immune, naïve or synthetic libraries (Liu *et al.*, 2018). Immune libraries are the most recommended and commonly used option, and can be more convenient to screen efficient binders against targets of interest. The general steps for immunization and selection of specific Nbs are extensively illustrated in Figure 3. First, a variety of preliminarily purified or single allergen proteins are usually used for immunization after subcutaneous injection into alpacas, camels, etc. during a period of 5–7 immunizations, to ensure that the titers of B cells produce antigen-specific HCAbs in the blood (Pardon *et al.*, 2014). After immunization, 50–100 mL of blood was collected for peripheral blood lymphocytes isolation and mRNA extraction. Because the entire antigen-binding fragment of HCAb consists of a VHH domain encoded by a gene fragment of only approximately 400 bp, it is easily amplified by PCR in a single amplicon. Therefore, the VHH gene region was amplified by two-step nested PCR using cDNA as template. In the first PCR, primers were used to amplify
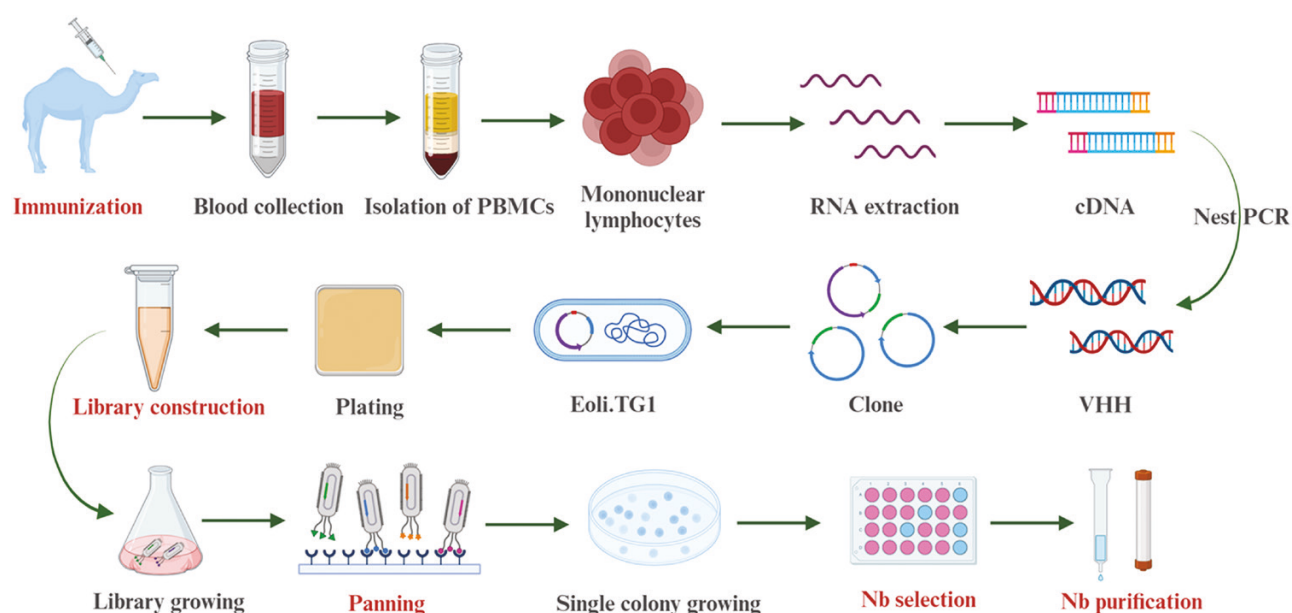
Figure 3. Schematic illustration of the strategies for food allergen immunization and Nb selection. Injection of mixed food allergens into young alpaca is to raise immune response for enrichment of allergen-specific B cells. After immunization, blood was collected for isolation of peripheral blood lymphocytes and ertraction of mRNA. VHH fragments will be amplified from the template originating from HACbs to allow insertion into a bacteriophage and sequential transformation into *Escherichia coli* to construct a library. The panning rounds were performed using phage display technology to retrieve specific Nbs for screening of positive clones. Specific Nbs were expressed in bacterial periplasm and purified through immobilized metal affinity chromatography and gel filtration.

the conserved region within the CH2 exon from all IgGs, resulting in an IgG1 heavy chain with a sequence of ~900 bp and a sequence of ~700 bp corresponding to IgG2 and IgG3 heavy chains. In the second step PCR, the VHH fragments are amplified from the template originating from HACbs in the first PCR to allow insertion into a bacteriophage, followed by sequential transformation into *E. coli* to construct a library (He *et al.*, 2017). Subsequently, the panning rounds were performed using phage display technology to retrieve specific Nbs displayed in phages and serve as the reservoir for the screening of positive clones that could potentially bind to the antigen of interest (Su *et al.*, 2022). Once positive clones have been screened, a microbial expression system is used to obtain specific Nbs with high yields (Arbabi-Ghahroudi *et al.*, 2005). For bacterial expression, a secretion signal is preferred to allow the expression of Nbs in periplasm for conformational maturation in the oxidative environment. Therefore, purification to specifically obtain the Nbs from the less periplasmic protein complex through immobilized metal affinity chromatography and gel filtration is straightforward. The main advantage of this type of library is that the specificity of the expected Nbs is determined by the antigenicity of the antigen, making it easier to retrieve Nbs with high affinity and specificity.

Alternatively, synthetic or naïve library can also be utilized to retrieve food allergen-specific Nbs under certain circumstance. For the application of the synthetic library, it is recommended to exhaustively analyze the binding repertoires of allergen-specific Nbs through machine learning to form the basic theoretical preference of amino acid distribution, which could provide guidelines for the design of an amino acid sequence in the synthetic library to potentially increase the possibility of acquiring specific binders (Muyldermans, 2021; Contreras *et al.*, 2023). However, the naïve library is not recommended to retrieve food allergen-specific Nbs because no advantages could be summarized by comparison with the defined immune library. The separation and purification process of food allergens is complex, often involving several chromatography runs and limited resolution, or a lack of biochemical background of the allergens (Bland and Lax, 2000). Therefore, it is recommended to adopt alternative strategies for the construction of the Nb library and the selection of specific binders, and the selection of Nbs for food allergens is generally divided into two types—one is the unbiased immunization strategy of crude allergen protein extract, another is the precision immunization strategy of single protein. The challenge of the unbiased immunization strategy lies in the identification of the antigens. The simplest way to do this is to use Nbs to capture the antigen for Nb:antigen complexes by His-tag of the Nbs on Ni^{2+}-NTA magnetic beads or other alternatives, then sodium dodecyl sulfate–polyacrylamide gel electrophophoresis (SDS-PAGE) and trypsinization of the captured proteins for finally identification by liquid chromatography–tanden mass spectrometry (LC-MS/MS). Hu and colleagues reported the establishment of an immunoassay for macadamia nut allergen Vicilin-like Protein using an unbiased immunization strategy, which has also been successfully applied in peanuts and lupins (Hu *et al.*, 2021a, 2021b, 2023b). This unbiased immunization and selection strategy is expected to facilitate the identification of potential allergens from general protein extracts, which can be used as biomarkers for the surveillance of allergen contamination in foods. The precision immunization strategy against a single

protein is a more straightforward method for the preparation of specific Nbs. Hu *et al.* (2022a) prepared and applied specific Nbs against bovine milk allergens β-lactoglobulin.

Nb-Based Immunoassays for Food Allergen Surveillance

Food allergy is an abnormal immune reaction that occurs in a reproducible manner after ingestion/exposure to a certain food component. Currently, the reported detection methods of food allergens are mainly based on gene-level and protein-level determination (Ivens *et al.*, 2016; Tsakali *et al.*, 2019). Gene level-based determination methods are mainly PCR and real-time fluorescence quantitative PCR, etc., which cannot directly reflect the presence or absence of allergen protein and do not seem to work with food allergens that contain a lot of proteins or a low level of DNA, and the results may be questionable upon processing (Campuzano *et al.*, 2020; Eischeid *et al.*, 2021). Detection methods against allergen proteins are mainly chromatography and immunoassay (Flicker *et al.*, 2020). The most applicable detection is realized with antibody-based immunoassays for *in situ* application, especially mAb-based detection methods (Sena-Torralba *et al.*, 2020). Various detection methods based on traditional antibodies have been developed for food allergen analysis, although with drawbacks including the significant cross-reaction on different food allergen proteins, intensive labor and cost, and batch difference during production or manipulation (Figure 4A).

Generally, almost 90% of all serious food allergies are related to proteins (allergens) in nine types of food including egg, milk, peanut, and tree nuts. The food allergens identified to date of major concern are summarized in Table 1, as well as the prepared conventional antibodies and Nbs for immunoassay development. Food allergens from different foods can be classified into the same families to exhibit similar epitopes, as a result contributing to the cross-reaction of detection methods, which potentially emphasizes the particularity of food allergen analysis. Researchers, including our group, have made tremendous efforts to prepare food allergen-specific Nbs, as well as to develop an Nb-based immunoassay for food allergen surveillance. The published literature has verified the successful application of alternative strategies for Nb preparation, and the detection of trace allergens with established methods. Moreover, Nb-based food allergy immune therapy has also been addressed, providing newly defined therapeutic strategies for research and clinical application (Figure 4B).

Wheat is an important crop that can cause allergies upon the presence of gluten or other relevant proteins in cereals, crackers, bread, beverages, and other foods. Gluten is a complex of more than 50 types of protein, which are divided into two categories: glutenins and gliadins (Chu *et al.*, 2012; Aquino and Conte, 2020). García-García *et al.* (2020) established an indirect ELISA using gliadin-specific Nb for the detection of wheat allergens in food, with a detection range of 0.1 to 10 µg/mL and a limit of detection of 0.12 µg/mL. Peanuts are a popular food resource for direct ingestion or supplementation in different prepared foods. Peanut allergy has become a major health problem worldwide, especially in developed countries (Burks, 2008). Ara h 1 and Ara h 3 belong to the Cupin superfamily and both are important peanut allergens in specific allergy populations (Flinterman *et al.*, 2008). Hu's team prepared specific Nbs against peanut Ara
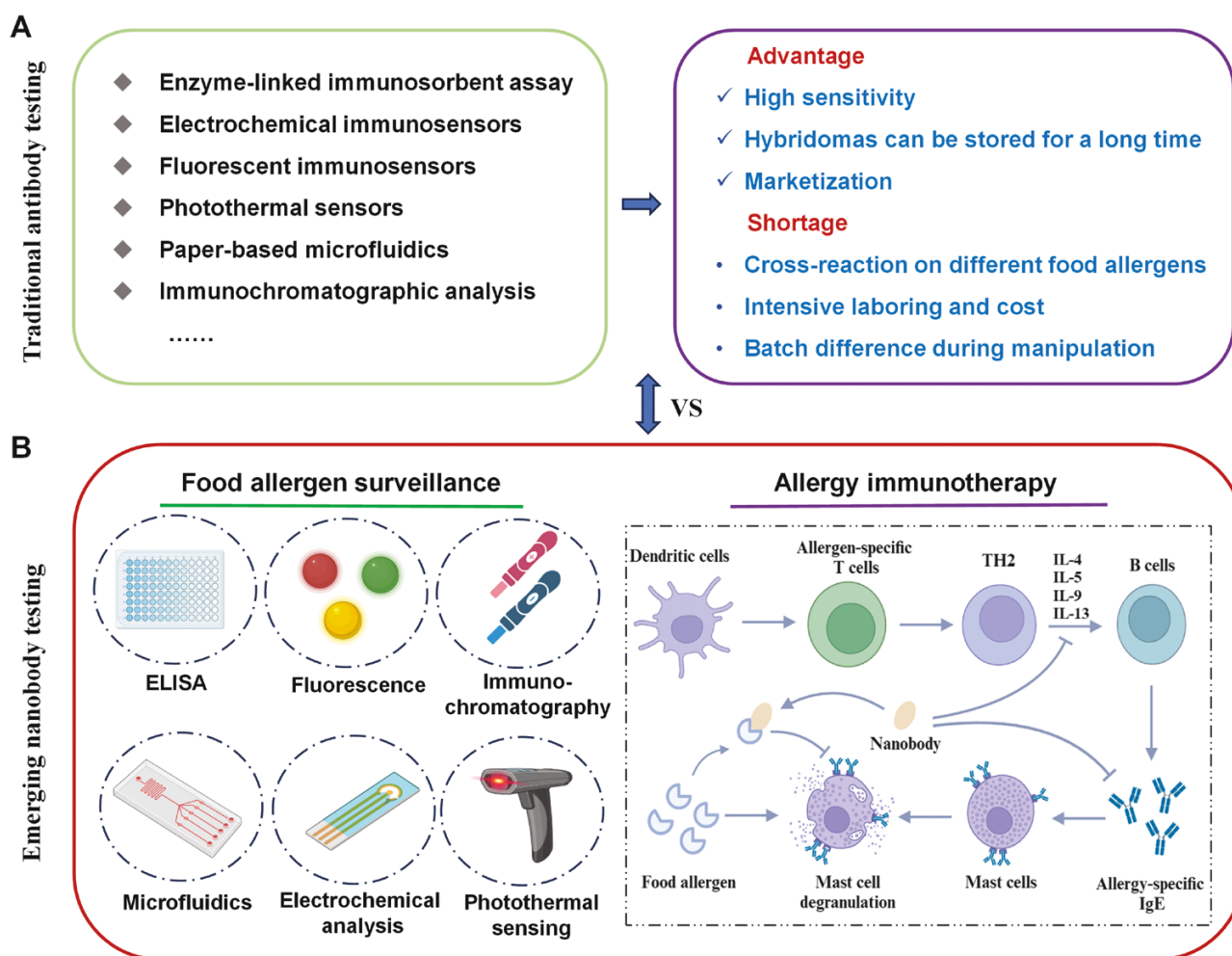
Figure 4. The proposed immunoassays with conventional antibodies or Nbs for food allergen analysis. (A) Various detection methods based on traditional antibodies have been developed for food allergen analysis including enzyme-linked immunosorbent assay (ELISA), electrochemical immunoassay, and fluorescence-based immunosensors. The significant drawbacks of traditional antibody-based detection is summarized including the significant cross-reaction of different food allergen proteins, intensive labor and cost, and batch difference during production or manipulation; (B) The development of Nb-based immunoassays for food allergen surveillance. Various detection methods have been established to validate the successful application of Nbs for the detection of trace allergens. Nb-based food allergy immune therapy has been addressed to provide newly defined therapeutic strategies.

h 1 and Ara h 3 using an unbiased immunization strategy and established a sandwich ELISA method. The detection range of Ara h 1 was between 162 ng/mL and 2200 ng/mL, and the detection limit was 1.95 ng/mL, which is more sensitive than mAb-based detection even without normalization against crude protein extract (Hnasko *et al.*, 2022; Hu *et al.*, 2023a). The reliability of the proposed method was evaluated by adding different concentrations of the peanut allergen Ara h 1 to skim milk and chocolate samples to simulate food matrix. The structural prediction of Nb by AlphaFold2 and molecular docking with antigen Ara h 1 revealed the binding of CDR1 and CDR3 of Nb to the α-helix and β-strand of Ara h 1. An electrochemical immunoassay was then developed on a screen-printed electrode using the obtained Nb pairs, with a detection range between 4.5 ng/mL and 55 ng/mL and a limit of detection of 0.17 ng/mL, which provided 11-fold higher sensitivity and shorter operation time than ELISA. More importantly, the captured electrode was universal to provide a reference for the development of Nb-based electrochemical detection methods against other allergens (Hu *et al.*, 2023a). Chen *et al.* (2019) constructed a random triplet (NNK) library

of CDR regions of camel Nb backbone and prepared Ara h 3-specific Nbs from them, and obtained Ara h 3-Nb combined crystals, which provided the necessary information for understanding the sensitization of this important peanut allergen. Hu *et al.* (2023b) prepared Nbs against Ara h 3 by using an unbiased immunization strategy, and established a sandwich ELISA method with a detection range of 0.2–10.6 μg/mL and a detection limit of 53.13 ng/mL. To improve the performance of peanut allergen Ara h 3 detection, Yao *et al.* (2024) constructed a proportional fluorescence and colorimetric dual-mode immunosensor using *o*-phenylenediamine (o-PD) as the peroxidase substrate, as well as boron and nitrogen co-doped carbon dots (B/N-CDs) sensing probe. The fluorescence signal of Ara h 3 had a good linear relationship between 10 ng/mL and 1200 ng/mL, the detection limit was 6.61 ng/mL, and the colorimetric detection signal was between 30 ng/mL and 1500 ng/mL, with detection limit of 9.79 ng/mL (Yao *et al.*, 2024). In addition, Hu *et al.* (2021a) validated the applicability of the unbiased strategy in macadamias and lupins by retrieving specific Nbs belonging to vicilin's Mac i 1, providing valuable information on potential macadamia

Table 1. Immunoassays against food allergens

Food	Allergen	Traditional antibodies	Detection range and detection limit	Nanobodies	Detection range and detection limit	Reference
Egg	Ovomucoid	mAb-based sELISA[a]	0.1–6.25 ng/mL and 0.041 ng/mL	–	–	Li *et al.*, 2008
	Ovalbumin	mAb-based sELISA[a]	1.95–500 ng/mL and 0.51 ng/mL	–	–	Peng *et al.*, 2012
	Ovotransferrin	mAb-based biosensors	50–3800 ng/mL and 23.55 ng/mL	–	–	Wang *et al.*, 2016a
	Lysozyme	mAb-based iELISA[b]	0.38–4.8 µg/mL and 0.264 µg/mL	Anti-lysozyme nanobody for enzymatic inhibition	–	Vidal *et al.*, 2005; Qiu *et al.*, 2017
Milk	α-Lactalbumin	mAb-based iELISA[b]	10–500 ng/mL and 0.1 ng/mL	–	–	Jeanson *et al.*, 2002
	β-Lactoglobulin	mAb-based cELISA[c]	78–10 000 ng/mL and 114 ng/mL	Nb-based cELISA[c]; Nb-based sELISA[a]	39–10 000 ng/mL and 4.55 ng/mL	Orcajo *et al.*, 2019; Hu *et al.*, 2022a
	Casein	mAb-based sELISA[a]; pAb-based sELISA[a]	0.1–10 µg/mL and 0.04 µg/mL	–	–	Zhou *et al.*, 2013
Peanuts	Ara h 1	mAb-based sELISA[a]	– and 10 ng/mL	Nb-based sELISA[a]; Nb-based biosensors	4.5–55 ng/mL and 0.86 ng/mL	Holden *et al.*, 2005; Hu *et al.*, 2023a
	Ara h 2	mAb-based cELISA[c]	– and 0.5 ng/mL	–	–	Schmitt *et al.*, 2004
	Ara h 3	mAb-based sELISA[a]	382–12 676 µg/g	Nb-based sELISA[a]	10–1200 ng/mL and 6.61 ng/mL	Pandey *et al.*, 2019; Hu *et al.*, 2023b
Shrimp	Tropomyosin, TM	mAb-based sELISA[a]; pAb-based sELISA[a]	0.08–512 ng/mL and 0.64 ng/mL	Nb-based biosensors	– and 0.02 µg/mL	Zhang *et al.*, 2014; Jiao *et al.*, 2024
	Arginine kinase, AK	mAb-based biosensors	1–1000 ng/mL and 0.11 ng/mL	–	–	Wang *et al.*, 2021
	Sarcoplasmic calcium binding protein, SCP	mAb-based biosensors	– and 0.5 µg/mL	–	–	Huang *et al.*, 2024
Tree nuts	Lupine protein (Lup an 1)	pAb-based sELISA[a]	– and 1 µg/g	Nb-based sELISA[a]	0.036–4.4 µg/mL and 1.15 ng/mL	Holden *et al.*, 2005; Hu *et al.*, 2021b
	Macadamia protein	–	–	Nb-based sELISA[a]	0.442–2800 µg/mL and 27.1 ng/mL	Hu *et al.*, 2021a
Soybean	β-Conglycinin, 7S	mAb-based sELISA[a]; pAb-based sELISA[a]	3–100 ng/mL and 1.63 ng/mL	–	–	Hei *et al.*, 2012
	Glycinin, 11S	mAb-based sELISA[a]; pAb-based sELISA[a]	3–200 ng/mL and 1.63 ng/mL	–	–	Chen *et al.*, 2014
	Gly m Bd 28K	pAb-based iELISA[b]	80–2000 ng/mL and –	–	–	Liu *et al.*, 2013
	Gly m Bd 30K	pAb-based sELISA[a]	– and 0.47 ng/mL	–	–	Morishita *et al.*, 2008
Fish	Parvalbumin protein	pAb-based cELISA[c]	0.59–150 µg/mL and 0.59 µg/mL	–	–	Jiang and Rao, 2023
Wheat	Gliadin	mAb-based sELISA[a]	4–40 ng/mL and 4 ng/mL	Nb-based sELISA[a]	0.1–10 µg/mL and 0.12 µg/mL	García-García *et al.*, 2020; Holden *et al.*, 2005
	Glutenin	mAb-based cELISA[c]	– and 25 ng/mL	–	–	Tranquet *et al.*, 2015

[a]Sandwich ELISA.
[b]Indirect ELISA.
[c]Competitive ELISA.

allergens, with a detection range of 0.442–2800 µg/mL and a limit of detection of 27.1 ng/mL. In another study, specific Nbs against lupine allergen of Lup an 1 were selected, and a sandwich ELISA was developed, providing a linear range of 0.036–4.4 µg/mL with detection limit of 1.15 ng/mL, which is relatively more sensitive than mAb based immunoassay (Hu *et al.*, 2021b).

Cow's milk is considered the perfect source of protein to ensure the recommended daily intake due to its high protein content. However, the widespread consumption of cow's milk can greatly increase the likelihood of allergic reactions in sensitive individuals. As reported, milk allergy affects approximately 0.6%–2.5% of preschool children, 0.3% of adolescents, and 0.5% of adults (He *et al.*, 2018; Jaiswal and Worku, 2022). As one of the main allergens, β-lactoglobulin (BLG) has attracted tremendous attention from researchers for the generation of detection methods. Hu *et al.* (2022a) prepared specific Nbs against BLG using a precision immunization strategy with

the same epitope preference. A competitive ELISA (cELISA) was developed with a linear range from 39 to 10 000 ng/mL and a limit of detection (LOD) of 4.55 ng/mL, with a recovery of 86.30%–95.09% revealed by analysis of spiked samples. Meanwhile, a sandwich ELISA (sELISA) was established with Nb and BLG polyclonal antibody (pAb-BLG), providing a linear range from 29.7 to 1250 ng/mL and an LOD of 13.82 ng/mL with a recovery of 87.82%–103.97%. The interaction of selected Nbs with BLG-derived peptides was investigated by Nb structure modeling and BLG docking. No binding on hydrolytic peptides was revealed, which confirmed the precision of Nb-mediated immunoassays (Hu *et al.*, 2022a). Then, Li *et al.* (2024) established an sELISA method using BLG-specific Nb and mAb, with a detection range of 0.01 to 10 μg/mL and an LOD of 0.24 ng/mL, to accurately detect BLG in milk and food under different processing conditions. More importantly, the significant cross-reaction of BLG-specific mAb was observed by comparison with that of BLG-specific Nb in this study to validate the statement that Nb is expected to be employed as a detection reagent for immunoassay development. Nanobody-based 'fluorescence-photothermal' biosensors have also been developed for high-specificity and high-sensitivity BLG detection. In another work, two kinds of carbon dots, green and red, were synthesized, with Nb as the capture antibody and mAb as the detection antibody. Because the emission spectrum of the red carbon dots overlapped with the absorption spectrum of oxidized TMB (oxTMB), fluorescence quenching of the carbon dots was used to determine the fluorescence signal, and the detection range was 0.1 ng/mL to 0.1 μg/mL with a detection limit of 0.034 ng/mL. The high absorbance of oxTMB provided the basis for the stable output of the photothermal signal to provide a detection range of 0.1 ng/mL to 0.1 μg/mL in photothermal mode with a detection limit of 0.075 ng/mL. The applicability of the constructed immunosensor was verified in pasteurized milk, UHT milk, milk beverages, and green tea (Li *et al.*, 2024). With the increased occurrence of sensitization, and the emphasized focus on food safety, Nbs-based food allergen analysis is expected to be investigated extensively for the development of immunoassays with high sensitivity and applicability, or as alternative candidate to provide detection strategies for food allergen surveillance.

Conclusions

Food allergy is a pathological, potentially fatal immune response triggered by normally harmless food proteins. The prevalence of food allergies has raised the necessity of analytical method development to ensure the precise detection of trace food allergens. Nbs have attracted increasing attention as an alternative antibody format for the detection of food allergens. The unique characteristics of Nbs provide multiple advantages for food allergen analysis, such as high specificity, robust properties and ease of manipulation. The generation of allergen-specific Nbs undoubtedly represents another reasonable advance in the field of food allergen analysis. Previous studies have reported the preparation of specific Nbs against food allergens, including nuts, peanuts, legumes, and milk proteins. Corresponding immunoassays have been established such as electrochemical, fluorescence, and photothermal for sensitive detection. Moreover, employment as recognition

modules in conjugates facilitates the variation of Nb formats to be used in different research and applications, with the potential to be applied as reagents for immunotherapy for food allergies. In general, comprehensive Nbs against the representative food allergens should be prepared to support both research and industrial applications.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Some figures were created with BioRender.com.

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