

# Versatile Application of Nanobodies for Food Allergen Detection and Allergy Immunotherapy

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**ABSTRACT:** The unique characteristics of camelid heavy-chain only antibody (HCAb) derived nanobodies (Nbs) have facilitated their employment as tools for research and application in extensive fields including food safety inspection, diagnosis and therapy of diseases, etc., to develop immune detecting techniques or alternative candidates of conventional antibodies as diagnostic and therapeutic reagents. The wide application in the fields of food allergen inspection and immunotherapy has not been addressed as not much results published in the literature. The robust properties and straightforward selecting strategy of Nbs impel the advantageous employment compared with monoclonal antibodies (mAbs) to establish immunoassay and serve as blocking antibodies to compete immunoglobulin E (IgE) binding epitopes on food allergens. More and more efforts have been invested to develop specific Nbs against food allergen proteins, such as macadamia allergen of Mac i 1, peanut allergen of Ara h 3, and lupine allergen of Lup an 1, which demonstrated the potential of Nbs for research and application in food allergen surveillance. Meanwhile, the paratopes of Nbs preferably targeting the unique epitopes of food allergens can provide more possibilities to serve as blocking antibodies to shield IgE binding epitopes for food allergy immunotherapy. Regardless, the research and application of Nbs in the field of food allergen and allergic reactions are expected to attract dramatic focus and produce promising research outputs.

**KEYWORDS:** nanobody, food allergen, food allergy, immunoassay, immunotherapy

## INTRODUCTION

Food allergens are generally nominated as the protein components in food that cause immunoglobulin E (IgE)-mediated hypersensitivity disease, which also coined Type I allergy and has become an important health concern worldwide with symptoms from minor digestive disorders to a more severe reaction that can even lead to life threatening complications.<sup>1–4</sup> Food allergens can be identified from different resources of raw materials including milk, egg, peanut, wheat, soy, tree nut, and shellfish.<sup>5</sup> The general collection of food allergens can reach more than 380 different types on the basis of the index of WHO/IUIS (<http://www.allergen.org>), and the number is constantly increasing with the deep exploration of allergenic proteins.

The risk of food allergy for sensitized individuals originates from the ingestion of intentionally supplemented food allergens, or incidentally introduced trace allergens in food-stuffs, which are identified as unintentionally contaminated allergen components during the production or storage of raw materials or the food products and pose a greater threat to consumers.<sup>6–8</sup> Especially, the unintentional contamination of food allergens poses great economic losses to enterprises and health related threats to the public. Thus, the focused aspects of food allergen related allergic diseases rely on the avoidance of food allergen ingestion and the curative strategy after unintentional intake, among which the allergen avoidance on the basis of the precise detecting techniques is well-accepted as the most important strategy,<sup>9,10</sup> as no causal curative strategy has been applied in clinical application for food allergy.

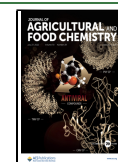
It is of great significance to provide all of the potential allergenic ingredients in food products for sensitized consumers to avoid ingestion of sensitizing components. To address and realize the protection of the health of certain consumers through allergen management, the application of reliable food labels with information on the presence of allergens has been obligated in legislation in some countries.<sup>11,12</sup> The Food and Drug Administration (FDA) of United States, as well as the European Union (EU), has required enterprises to label all the potential allergens since 1996, with the requirements to declare and indicate priority allergens on food labels. Many other countries also specify the indication of food allergens through labeling, though the regulatory provisions have dissimilarities and variations.<sup>13,14</sup> Herein, reliable and precise detecting techniques are of great importance for food manufacturers to clear the presence of intentionally supplemented or unintentionally contaminated allergens to ensure compliance with such legislation and avoid economical loss since the unexpected allergen is obscured.<sup>15,16</sup> The frequently applied detecting strategies against food allergens mainly include protein-based immunoassay of enzyme-linked immunosorbent assay (ELISA) and DNA-

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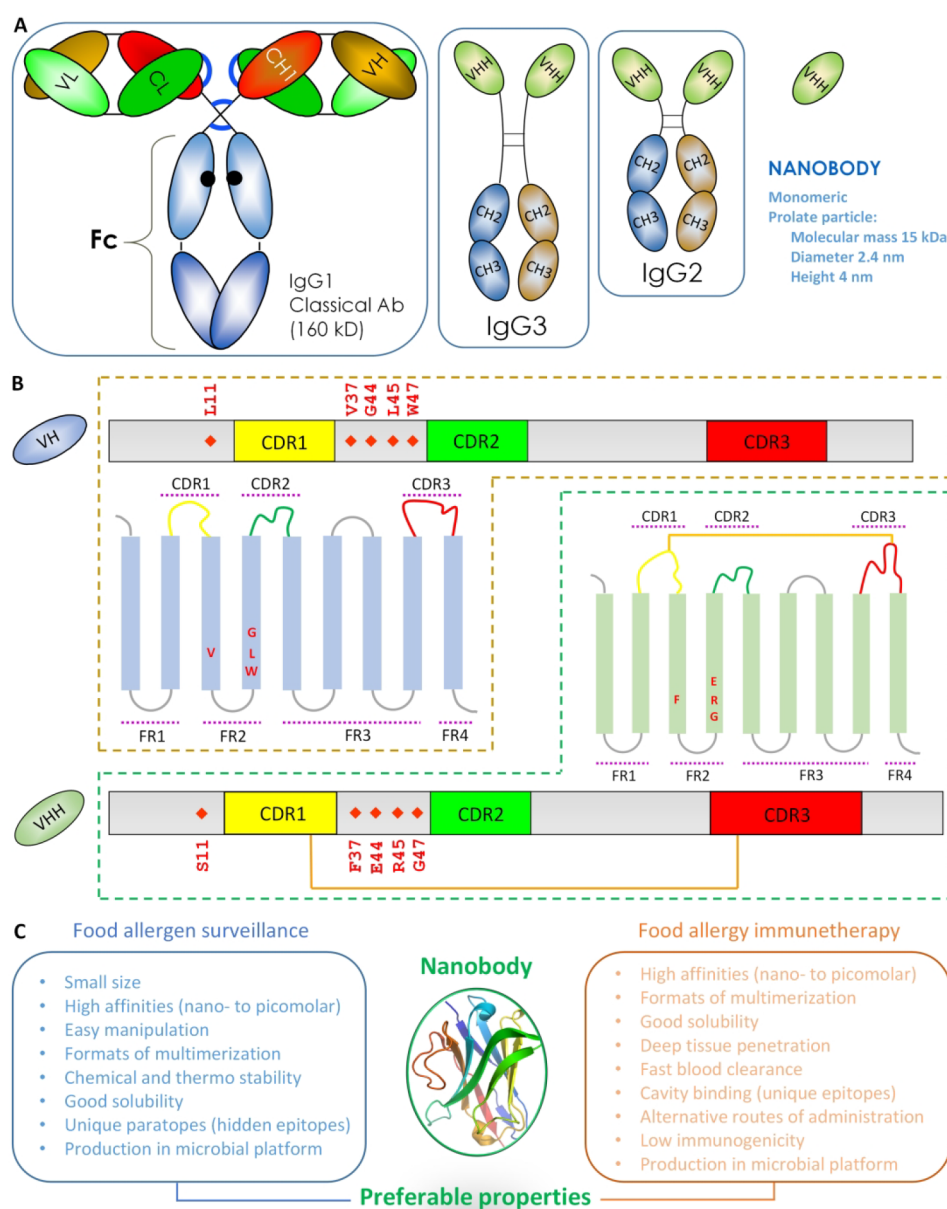


**Figure 1.** Schematic representation of antibody fragments and the unique properties of Nbs. (A) IgGs presented in camelids can be classified as conventional antibodies comprising two light chains (L) and two heavy chains (H) coupled to each other by different disulfide bonds and heavy chain antibodies (HCAbs) that are naturally devoid of light chains and the first constant domain (CH1). The corresponding fragment VHH of HCAb is commonly named nanobodies (Nbs) with a molecular mass around 15 kDa and a size of  $2.4 \times 4 \text{ nm}^2$ . 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). A longer CDR1 and CDR3 for VHH is generally observed by comparing with VH. (B) Mutation of hydrophobic amino acids (V42, G49, L50, and W52) in VH to the more hydrophilic amino acids (F42 or Y42, E49, R50, and G52) can be observed. The joining of CDR1/2 with CDR3 is commonly realized by an interloop disulfide bond to increase the conformational stability of Nbs. Nbs are characterized with unique properties by comparing with conventional antibodies or the derived antibody fragments including small size, high affinity, good solubility, and so on. (C) Unique characteristics facilitate Nbs as the perfect candidates for research and application in the fields of food allergen surveillance and food allergy immunotherapy.

based real-time polymerase chain reaction (RT-PCR).<sup>17</sup> DNA-based assay can provide high precision but is relatively inefficient or inconvenient for in situ application and cannot be applied for the surveillance of certain allergens.<sup>18,19</sup> The immunoassay based on allergen-specific antibodies is detecting approaches with extensive application, and varied techniques with the characteristics of rapid, multiplex for food allergen detection have been developed.<sup>20–22</sup> Although the immunoassay is commercially available for the detection of certain allergens, the limitation remains to restrict the application and

detection of certain allergens including narrow detection limits, insufficient specificity and sensitivity, significant cross-reaction of conventional antibodies, and insufficient reproducibility. These drawbacks dominantly rely on the employed conventional monoclonal antibodies (mAbs), and the alternative candidates for the development of immunoassay with comparable advantages can be explored and investigated.

Upon unexpected ingestion of food allergens for sensitized individuals, the most frequently applied curative strategies mainly depend on anti-inflammatory approaches to reduce

allergic syndrome.<sup>23</sup> The causative therapeutic strategy represents immunotherapy against allergic reaction to specific allergens through inducing a protective immunity in patients.<sup>24,25</sup> Research activities mainly focus on the approaches to raise the immune tolerance to certain allergens or identification and development of inhibitory molecules to block or compete the binding epitopes of IgE.<sup>26,27</sup> Meanwhile, the development of immune tolerance against certain food allergens also contains the variation of an immune response such as a shift from T helper 2 (Th 2) to Th 1, as well as the modulation of cytokine secretion to suppress the IgE related response or the induction of allergen tolerance.<sup>28–30</sup> The employment of peptide carrier vaccines or hypoallergenic allergens can induce the inhibitory effect on IgE binding mediated by the developed IgG response. Induced IgG is accepted to effectively block IgE binding epitopes and coin blocking antibodies.<sup>31,32</sup> Successful application depends on the identification and preparation of allergen-specific antibodies, and the efficacy of such blocking strategies was well-accepted as a complicated cause of the cumbersome and high cost to isolate mAbs with the capacity to inhibit IgE-mediated reactions.<sup>33,34</sup> Therefore, potential alternatives and candidates are extensively explored to ensure the straightforward preparation and cost-effective validation.

On the basis of the requirements of the alternative candidate of conventional mAbs to realize not only the sensitive detection of trace allergens in food but also application as blocking antibodies for allergy immune therapy, the emphasized antibody fragment of camelid derived nanobodies (Nbs) is proposed to fulfill both of the demands addressed. Nbs are a type of potent antibody fragments engineered from the variable domains of heavy chain-only antibodies (HCAbs), which are naturally devoid of light chains, as well as the first constant domain (CH1) in the heavy chain, and were discovered by serendipity in camelids by Hamers-Casterman et al.<sup>35</sup> The unique type of HCAbs exhibits comparable affinity with conventional antibodies against cognate antigen, despite the single variable domain (VHH) of an antigen-binding site.<sup>36</sup> This variable domain of these heavy-chain antibodies comprises full antigen binding potential, with a strong affinity to its cognate antigen, and so it is considered to be the smallest, naturally occurring, intact antigen binding fragment.<sup>36–38</sup> The lower molecular mass usually between 12 and 14 kDa and smaller size in dimension (~2.5 nm in diameter and ~4 nm in height) facilitated the alternative trademark of a nanobody registered by a pioneer corporation of Ablynx (now Sanofi) focused on the development and application of Nbs for diagnosis and therapy (Figure 1A). Continuous efforts have been invested in the field of Nb research, and remarkable output has been obtained to eventually stimulate the foundation of more industries such as Chromotek, Agrosavfe, QvQ, Camel-IDS, Hybrigenics, Confo Therapeutics, and so on. The robust characteristics of Nbs have been demonstrated under most of the conditions employed in the research. The straightforward selection strategy of specific Nbs from the preimmunized or naïve library based on the display technology could facilitate the preparation of Nbs against almost any interested target.<sup>39</sup> The application of Nbs on the research of food related science and engineering has covered broad food components from protein to chemicals,<sup>40,41</sup> whereas food safety detection based on specific Nbs against food allergens has drawn remarkable focus to develop varied types of detection strategies, as well as

extensive application to reduce allergenicity.<sup>42–44</sup> Here, in the current report, the beneficial characteristics of Nbs will be summarized to elucidate the application of Nbs on immunodetection against food allergens and the immunotherapy through IgE epitope blocking. The general immunization and selecting strategies will be discussed on the basis of the published reports and personal experiences to facilitate the proposed experimental design for specific Nb preparation against food allergens. The potential application of isolated allergen-specific Nbs for epitope blocking will also be discussed to provide prospective insights. Generally, it is anticipated to provide base knowledge associated with immunization, Nb selection, and applied allergen surveillance to benefit food allergen related research and technique development based on Nbs.

**Unique Properties of Nbs.** As illustrated in Figure 1B, the similar molecular structure of Nbs with VH domains from conventional antibodies is summarized to include the framework regions 1–4 (FR1–4) and the complementarity determining regions 1–3 (CDR1–3). All of the architectures of Nbs are involved in the antigen interaction with the CDR3 as the main participants of antigen binding.<sup>36,45</sup> A longer CDR3 of Nbs is generally observed to process on average 18 amino acids by comparing with the 12–14 amino acids in CDR3 of VH domains, which is supposed to facilitate the formation of a flexible paratopes of Nbs to identify and bind to unique epitopes on the antigens, especially the capacity to fold as a finger-like paratope to bind the small cavities of the antigen.<sup>46–48</sup> Herein, Nbs are the perfect candidates to process the paratopes to target the catalytic sites of enzymes to fundamentally modulate the enzymatic activities.<sup>49,50</sup> Meanwhile, the different epitope preferences of Nbs and conventional antibodies can ensure their binding to different domains of the same antigen, which can facilitate the capture and detection of antigens with mAbs and Nbs, respectively, if no Nb-pair is obtained for the sandwiched immunoassay. The substitution and mutation of hydrophobic amino acids (V42, G49, L50, and W52) in VH to the more hydrophilic amino acids (F42 or Y42, E49, R50m, and G52) in Nbs can increase the solubility in aqueous solutions.<sup>51</sup> Furthermore, the joint of CDR1/2 with CDR3 by an interloop disulfide bond can potentially increase the conformational stability of nanobodies, which also elucidates the high stability of Nbs under harsh conditions as high temperature or the organic solvent.<sup>52</sup>

Nbs can be selected to target different protein isoforms, which is well-accepted as an important property to establish the detecting strategies against food allergens, as the presence of the precursor and the modified allergen proteins with different sizes or conformational structures. Moreover, the extended life under either lower (from –20 to +4 °C) or higher temperatures (60–80 °C), proteolytic conditions, nonphysiological pH (pH 3.0–9.0), elevated pressure (500–750 MPa), and chemical denaturants (2–3 M guanidinium, 6–8 M urea) can facilitate the convenient application of Nb-based immunoassay against food allergens under different condition with rapidness, replicability, and accuracy.<sup>53,54</sup> Nbs are generally expressed as the monomeric formats in microbial expression platforms including *Escherichia coli* (*E. coli*) or yeasts (*Saccharomyces cerevisiae* and *Pichia pastoris*) to ensure the low-cost preparation with the yield of milligram per liter of culture medium even in shake flasks, which indicates the efficient preparation of Nbs with consistent properties.<sup>55,56</sup> Though the small size of Nbs is a beneficial property in certain

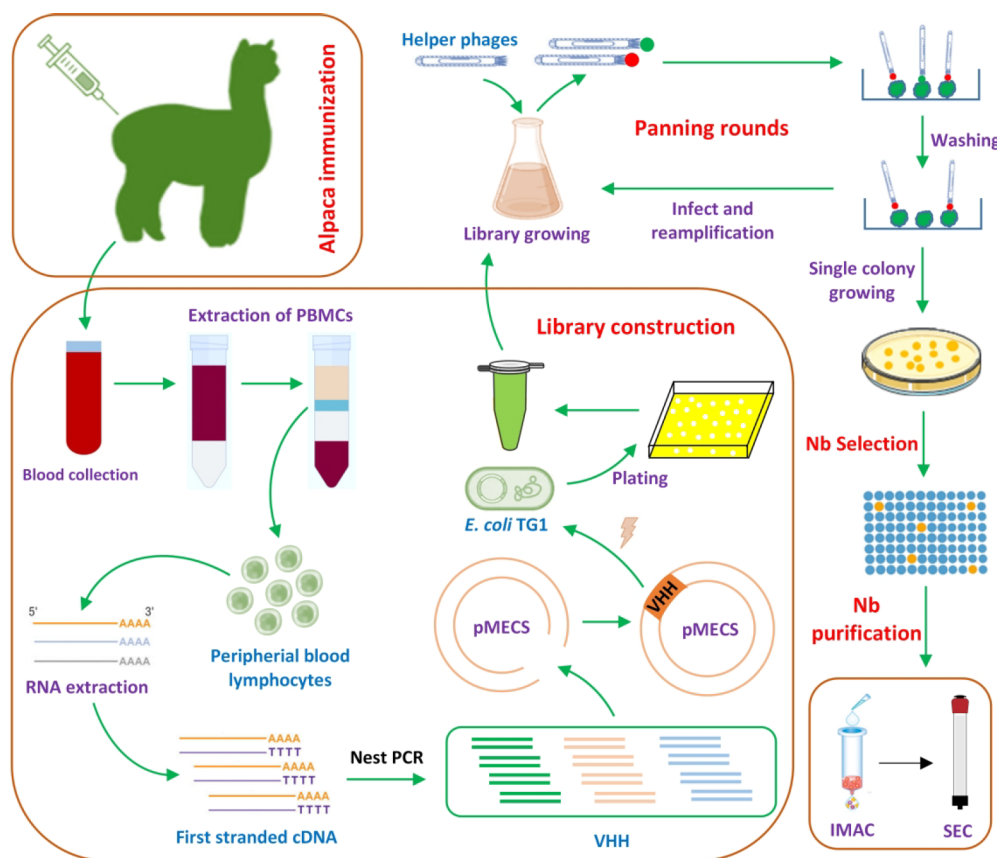


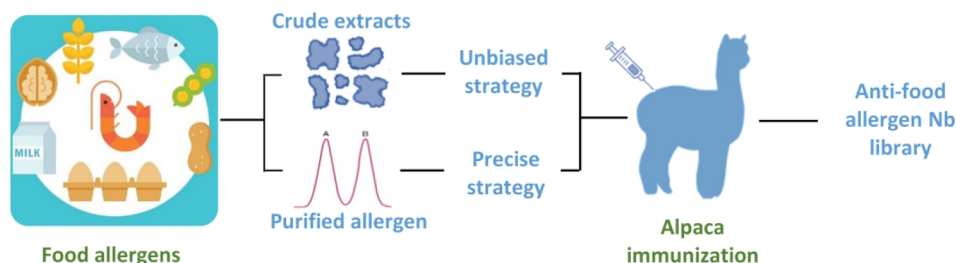
**Figure 2.** General process of Nb selection and production. Selection and production of Nbs were initiated by injecting the antigen of interest into a young alpaca for the process of “alpaca immunization”. After immunization, blood was drawn for peripheral blood lymphocytes preparation. Total RNA was extracted from the obtained lymphocytes and subjected to prepare cDNA for the following rounds of nest PCR to amplify encoding fragments of VHH. VHH fragments were subcloned into the phagemid of pMECS for electro-transforming into *E. coli* TG1 competent cells, and the Nb library was constructed after collecting all the colonies on the LB agar plates. After the growing of a helper phage-rescued Nb library, phages displayed with Nbs were precipitated for the enrichment of antigen binding phage particles through three rounds of biopanning. The positive colonies were obtained for the selection of specific Nbs after performing PE-ELISA. The obtained positive Nbs were transformed into *E. coli* WK6 cells for production and purification through IMAC and SEC.

applications, it can be an unfavored characteristic for immune assay against food allergens as the requirement to increase amount to ensure the sensitivity and affinity. Alternatively, the easy manipulation to construct multimeric or multispecific Nbs could ensure the targeting to the food allergens with high affinity and simultaneous potential of targeting to multiple targets.<sup>57,58</sup>

**Advantages of Nbs Compared with Conventional Antibodies for Food Allergen Related Research and Application.** The significant weakness of conventional antibodies is their fragility, which allows only for application in aqueous phase. Moreover, due to the complex heterotetrameric structure and posttranslational modifications, mAbs are dominantly expressed in mammalian cells, which results in high expenses and attendance during selection and large-scale production.<sup>59,60</sup> Whereas, Nbs can be easily selected from varied libraries by phage display technology and produced in prokaryotic expression platform with considerable scale. For the selection of binders from the immunized library, the VHH repertoire from peripheral blood cells of the immunized camelid is cloned and Nbs could be retrieved with highest affinity and specificity to the target.<sup>61</sup> An identical procedure has been adapted to select binders from the large nonimmune (naive) or synthetic Nb libraries. Naive libraries could be constructed from the VHH repertoire of nonimmunized

camelid members. Whereas, the synthetic libraries are constructed based on the rational design and optimization of the codons in the antigen binding loop regions based on a relative robust scaffold.<sup>62</sup> Regardless, the selected Nbs can be produced easily and properly in microorganisms, mammalian cells, or plants.<sup>55</sup> The Nb holds great promises in the field of immune assay, mainly due to the unique paratope architecture, monomeric and robust behavior, and favorable solubility (Figure 1C).<sup>52,54</sup> The unique property facilitates Nbs to possess a long CDR3 to form a finger-like structure that penetrates into cavities on the antigen surface.<sup>50</sup> For those VHHs that do not have a long CDR3, the prolate shape of the Nb creates a convex paratope that interacts with antigen concave surfaces. Consequently, Nbs are directed against unique antigen epitopes that are low or not antigenic for classical antibodies, which makes Nbs perfect candidates for the labeling and detection of food allergens with special flat structure or multimeric properties. The small size and monomeric single domain nature form the basis for the flexible engineering of Nbs. The engineering of Nbs facilitates the conjugation of additional proteins, reporter molecules, or fluorophores for the development of derived detection strategies against food allergens. Generally, chemical conjugation through the free amino on constituted amino acids, such as the lysins, can ensure conjugation with a ligate of

## 1. Food allergen preparation and Nb library construction



## 2. Selection of Nbs against Food allergen and development of immunoassay

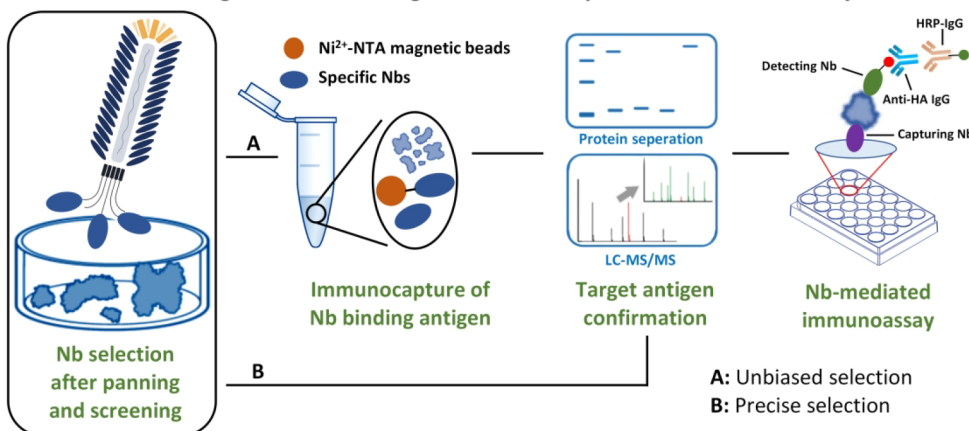


**Figure 3.** Schematic illustration of strategies for selection of food allergen-specific Nbs. Strategies for the selection of food allergen-specific Nbs can be classified as unbiased or precise approaches according to the applied antigen as crude protein extract or the purified allergens, respectively. The immune library was constructed by immunizing animals with allergen components, as well as the selection of specific Nbs. After obtaining the Nbs through unbiased strategy, the steps of immune-capture and antigen identification were performed to confirmed the target (A). The Nbs obtained by precise strategy can be proceeded to the antigen confirmation and antibody evaluation processes for the following studies (B).

interest. However, the random conjugation can result in the loss of antigen binding capacity of Nbs upon the presence of certain amino acids with free amino groups in antigen binding regions. Another option is to introduce purposely the extra cysteine far from the antigen binding domain to ensure the conjugation with effects on the binding capacity of Nbs.<sup>63</sup> Furthermore, the more specific conjugation through the peptide-mediated manipulation is more applicable, such as the Sortag-mediated ligation after reaction with Sortase A to facilitate the conjugation with any probe of interest.<sup>64,65</sup> Another comparable advantage for Nbs is that it is resistant to the stringent conditions mentioned above, which can ensure the full antigen-binding capacity to facilitate the extensive application under different conditions. Whereas, the application of classical antibodies is strictly defined to ensure the functionality and activity of employed antibodies. Taken together, Nbs process prominent characteristics such as the high stability against high temperature and pressure or extensive applicability under chemical solvents for the surveillance of trace food allergens.<sup>22,44</sup>

**General Strategies for the Selection of Nbs against Food Allergens.** Generally, antigen-specific Nbs are selected on the basis of phage display technology to obtain the positive clones with protein-based phenotype and phagemid included genotype simultaneously. The general procedures for immunization, construction of Nb library, selection of positive binders, and production of Nbs are detailed illustrated in Figure 2. The selection of antigen-specific Nbs were accomplished on the basis of phage-display technology after

a biopanning process to enrich positive binders for the following selection. Generally, the initial step is to construct a library with sufficient Nb reservoir, and different libraries can be applied according to the antigens of interest and the following application.<sup>61</sup> The immune Nb library is the most recommended and applied format as it provides the highest possibility to select specific binders of the antigens. For the construction of an immune library, immunization of 5–7 weeks by subcutaneous injection of antigens into camelid family members, such as dromedary, camel, llama, or alpaca, is required to guarantee the raised titer of B cells producing antigen-specific HCAs in the blood. Therefore, the peripheral blood lymphocytes can be prepared from a blood sample of 50–100 mL to retrieve the *in vivo* affinity-matured Nbs.<sup>66</sup> The technique steps are to extract RNA to prepare cDNA first and then perform nested PCR to amplify the Nb gene fragments. Nb gene fragments were then ligated into a phagemid of pMECS and transformed into *E. coli* to construct a library with a diversity of at least 10<sup>7</sup> cfu/mL. The constructed immune library can ensure the raised titer of antigen-specific Nbs, which is helpful to select positive binders after a few rounds of biopanning based on phage display technology. Whereas, the other types of Nb libraries has been employed for the selection of specific binders, including the naïve library and artificial synthetic library. For the application of Nbs on food safety detection, it is not applicable to immunize the animals with certain toxins or food hazards with a more severe toxic reaction under certain cases. The alternative strategy is to construct the naïve libraries from the animals without undergoing the

immunization process. Anyhow, it is highly possible to have the binders with lower affinity from the naïve library, or even without retrieved Nbs after panning and screening. On the basis of the structure of unveiled Nbs and the targeted antigens, the well-designed and optimized synthetic library can also be utilized for the selection of specific binders against the targets of interest.<sup>67,68</sup> For the synthetic library, Nbs with poorer biochemical characteristics including the expression pattern and the stability could be expected under certain cases compared with the binders from the immune library. After obtaining positive binders, Nbs were produced in periplasm of *E. coli* WK6 cells for purification through a two-step purification strategy including immobilized metal affinity chromatograph (IMAC) and size exclusion chromatograph (SEC). Generally, a high yield can be concluded as milligrams per liter of cultural medium, which can drastically reduce the cost for Nb preparation and ensure the repeatability of application. The strategy for the selection of specific Nbs against food allergens can be different with other proteins of diagnostic and therapeutic molecular markers, which generally requires the preparation of purified antigen proteins. For food allergens, it is frequently visualized that it is difficult to prepare the purified formats or the deficiency of biochemical background for many of the food allergens. Thus, it is recommended to apply alternative strategy for the construction of the Nb library and selection of specific binders. The alternative strategies for the selection of binders against food allergens can be applied, to either employ general allergen protein extract or the purified allergen with the coined strategies of unbiased or precise selection, respectively (Figure 3).<sup>69</sup> The utilization of general protein or purified allergens for immunization mainly depends on the research purpose to either identify specific Nbs against certain allergens or identify noncharacterized allergens based on selected Nbs. Meanwhile, the high-cost and toughness to prepare purified food allergens are other factors needed to be considered. It has been reported to obtain Nbs through varied strategies based on the research targets of interest. Hu et al. reported the selection of specific Nbs against macadamia allergen of vicilin-like antimicrobial peptides by using an unbiased strategy for the immune surveillance of this tree nut component in foodstuff. The immune library was constructed by immunizing animals with crude protein extract, as well as the selection of specific Nbs. After obtaining the Nbs, the steps of immune-capture and antigen identification were performed to confirmed the target. This study demonstrated the successful application of an unbiased strategy for the selection of Nbs against food allergens.<sup>44</sup> Meanwhile, the researcher also validated the application of the unbiased strategy through selecting specific Nbs against peanut and lupine allergens.<sup>22</sup> Chen et al. screened specific Nbs against the peanut allergen of Ara h 3 through a precise strategy by preparing the purified Ara h 3 first.<sup>41</sup> It could be more straightforward by using homogeneous allergens for the selection of allergen-specific Nbs. But, it can be restricted by various factors including the obscure background of allergens, as well as the high-cost for allergen preparation. Thus, both of the mentioned strategies can be employed on the basis of the research targets and the application situation.

**Nb-mediated Surveillance of Food Allergens.** Generally, food allergens are the nutritional components from natural or processed food to supply the protein and amino acids to sustain daily activities. It should not be classified as a kind of food hazards under most cases, as it will not harm the

systemic health if no adverse immune response could be observed or, in another expression, no allergic reaction could be raised. However, it will be a kind of food hazard in the case of the unfavored allergic reaction, and any food protein may be considered a potential allergen. Currently, no personalized or specialized curative approaches are proposed; so, as to avoid the unfavored reaction against allergens, the primary strategy is to avoid the interaction and intake of the allergen by determining the presence or absence with the sensitive detection techniques.<sup>23,25</sup>

To date, various analytical methods have been explored and applied to detect allergens, such as DNA-based assays (e.g., RT-PCR) and immunoassays (e.g., ELISA).<sup>70,71</sup> DNA-based assays are used to determine the presence of certain allergens after amplification of the DNA fragment. It can be stable and sensitive as DNA is relatively more stable than protein during food processing or storage, as heat-induced modification or glycosylation of allergen protein will affect the detection of protein-based assay. Whereas, the trace DNA in food is always not consistent with the allergen protein format, and even the loss of DNA fragments results in a failed DNA-based assay.<sup>72</sup> Meanwhile, the requirement of operational equipment and professional skill limited the in situ application. Thus, DNA-based assay is not widely applied, and antibody-based immunoassays are the most developed and routinely applied detection methods in the food industry. Regular immunoassays are established on the basis of the preparation of specific monoclonal or polyclonal antibodies, which dramatically affected by the purified allergen proteins, and the modification of allergens will result in poor-quality reagents that attribute inevitably to the low detection efficacy. Almost all of the commercial ELISA kits are developed by using mAbs, as polyclonal antibodies regularly cross-react with different allergens, and the batch difference cannot ensure the consistency of the immunoassay. Furthermore, mAbs can always provide the cross-reaction of the immunoassay to different food allergens from the same families.<sup>43,73</sup> The selection of positive mAbs mainly depends on the hybridoma and selection of positive clones, which hold a lot of cost and attention and are not highly efficient to select specific antibodies. More importantly, you can just prepare the antibodies against purified food allergens, and it does not work by using an unbiased strategy. On the basis of the multiple types of food resources and the complex classification of food allergens, a high-throughput detection technique is emergently required by the food industry to serve the food safety inspection and quality control of food products. There is a bottleneck of monoclonal antibody-based immunoassay, including the instability, heterogeneity, and the inapplicability of high-through detection.

On the basis of the unique characteristics, Nb is an attractive immunoreagent and has been employed to develop immunoassay of food allergens extensively. In comparison to conventional mAbs, Nbs can be easily selected through phage display technology and produced in an *E. coli* expression platform with accepted yield and homogeneity. Especially, Nbs can be produced by using an unbiased strategy to yield the detecting techniques against food allergens with little background information. The longer CDR3 of Nbs facilitates the formation of unique paratopes to recognize the epitopes that are less accessible or even cryptic for mAbs on the cognate epitopes with high specificity and affinity, which is helpful to prepare Nbs with less or no cross-reaction between different

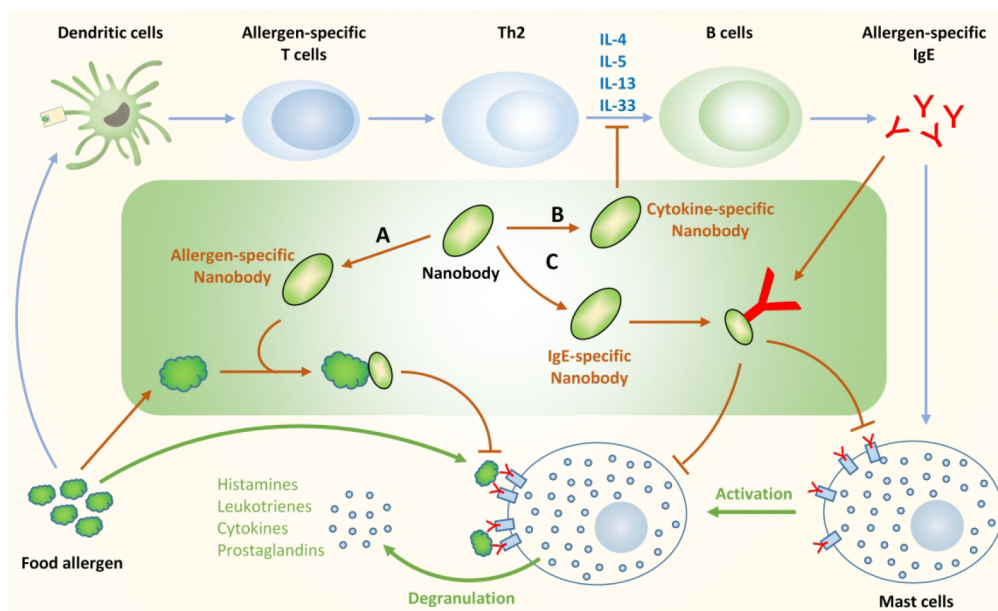


**Figure 4.** Food allergen related allergic reaction and Nb-mediated immunotherapy of food allergy. Food allergens and the digested peptides are presented to naïve T helper (Th) cells via by normally antigen presenting cells (APC) like dendritic cells, resulting in Th cell priming and activation. The activation of Th cells results in the secretion of cytokines that stimulate B cells to synthesize IgE antibodies specific for food allergen. T helper 2 (Th2) cells cause the secretion of various interleukins (ILs) including IL-4, IL-5, IL-9, and IL-13 to promote the secretion of IgE specifically to food allergens and then bound primarily on mast cells and basophils by the high affinity surface IgE receptors (FcεRI). Upon ingestion, the food allergen cross-links with the specific IgE on mast cells and basophils, releasing various inflammatory mediators including histamine, prostaglandins, leukotrienes, and platelet-activating factor to cause the symptoms of food allergy. Nb-mediated immunotherapy of food allergy comprises the strategies to generate specific Nbs against food allergens, in order to block the binding of IgE antibodies on food allergen epitopes and serve as blocking antibodies (A). Another strategy is to target the secreting cytokines to alleviate the symptoms of allergic reactions (B). Alternatively, IgE-specific Nbs can also be produced to interact with IgE to interfere the binding to Fcε receptors on related immune cells (C).

food allergens.<sup>36,42</sup> Hu et al. developed an immunoassay of heterologous sandwich ELISA against macadamia allergen of Mac i 1 based on the Nbs selected after unbiased immunization and selection. The method was established by providing a linear response in the range 0.442–2800  $\mu\text{g/mL}$  with a limit of detection of 27.1 ng/mL. The parameters of the developed ELISA can be more precise and sensitive if using the purified allergen for detection and validation. This immunoassay was verified by detecting the antigen spiked in milk samples, which provided evidence for the successful application of the unprejudiced strategy to retrieve Nbs against a priori undefined macadamia allergen and the design of highly reliable and effective immunoassay.<sup>44</sup> 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  $\mu\text{g/mL}$  with a detection limit of 1.15 ng/mL, which is relatively more sensitive than the mAb-based immunoassay.<sup>22</sup> The increasing research outputs are expected with the increasing interest to develop Nbs against different food allergens, which will provide the promising tools to not only develop detecting methods against food allergens but also serve as research reagents for structure determination and epitope mapping of food allergens.

#### Nb-Mediated Immunotherapy of Food Allergies.

Upon the unexpected ingestion of food allergens for sensitized individuals, the curative strategies especially causal approaches need to be highlighted for emergent treatment rather than food allergen avoidance.<sup>74</sup> Antibody-mediated immunotherapy of food allergy represents the updated focus of research or clinical application. Currently, most of the researches utilized human

or at least humanized mAbs to inhibit the binding of IgE to allergens or immune cells, as well as the resulting activation of immune cells related to airway or food allergies.<sup>73,76</sup> Orengo et al. reported the blocking effect of monoclonal IgG on airway allergy mediated by immunodominant cat allergen Fel d 1. Two potent allergen-blocking IgGs were selected, and it was demonstrated that even a single dose injection of blocking IgG could reduce clinical symptoms in response to nasal provocation, which implied the alleviated effect through simply augmenting the blocking IgG/IgE ratio.<sup>77</sup> The following clinical trial validated the reverse effect on allergic rhinitis after a single, passive-dose administration of Fel d 1-specific IgG antibodies, through the suppression of FcεRI, FcεRII, and T-helper cell type 2-mediated allergic responses.<sup>78</sup> Chinthrajah et al. reported a phase 2a clinical trial of a monoclonal antibody against IL-33, which is a pro-inflammatory cytokine that promotes B-class switching to IgE. A single administration of mAbs was able to induce immune tolerance in peanut sensitized individuals and reduce the symptoms in the enrolled patients.<sup>79</sup> IgE-specific humanized IgG1 monoclonal antibody was reported to block the binding of IgE with Fc receptors expressed on dendritic and mast cells either under research or clinical evaluation against individuals.<sup>80–82</sup> No matter of the inhibition mechanism, the efficacy of these blocking antibodies is continuously hampered by the cumbersome procedures to select effective antibody candidates and prepare a consistent antibody format with relatively high capacity to inhibit food allergen induced allergic reactions. Meanwhile, the functionality of mAbs to shield the IgE-related epitopes on food allergens is obscure as the inclination to

target linear epitopes, and the targeting specificity and affinity are crucial for their inhibitory efficiency on IgE binding and the following immune activation.<sup>83–85</sup> As mentioned at the beginning of this section, the employed mAbs are preferred as human origin or at least undergoing humanization, as it is not prevalent to using murine IgGs as the blocking reagents to compete with human IgE binding sites. Although the direct employment of murine IgG can help to avoid the complicated humanized procedures, the immunogenicity of this macromolecule can result in a dramatic harmful immune response to these IgGs.<sup>86,87</sup> The mitigation steps are mandatory to engineer murine IgGs as chimeric or humanized formats, which are questioned by researchers to invest endeavors for allergy immune therapy.<sup>88,89</sup> While the selection of mAbs is limited by hybridoma technology, it is generally unsuitable for a comprehensive screening of extensive antibodies due to the inefficient fusion and transformation, as well as the bottleneck to identify single positive B cell to produce an allergen-specific antibody.<sup>90,91</sup> The random combination of heavy chains and light chains can contribute to the formation of antibodies distinct with the natural antibody pairs and may hamper the efficient identification of inhibitory antibodies.<sup>92,93</sup>

The alternative candidates of Nbs are fundamentally expected and attracted the focus of researchers to extend their application as blocking reagents for the inhibition of IgE binding and allergic reaction. The unique characteristics of Nbs including the capacity to bind epitopes in convex, good tissue penetration and distribution, and robust properties of high stability and solubility can facilitate the employment of them as better blocking agents than conventional antibodies. Nanobody-mediated immunotherapy of food allergy comprises varied mechanisms based on the experimental design and selection of specific Nbs against different targets involved in allergic reactions. Generally, Nbs are generated to inhibit the IgE-mediated immune response through blocking the epitopes on food allergens to interfere with IgE binding on allergens, targeting certain cytokines to induce immune tolerance or interacting with IgE to interfere with the binding to Fc $\epsilon$  receptors on related immune cells (Figure 4).<sup>27</sup> The increasing reports are describing the selection of Nbs specific to different food allergens and application on the development of detecting methods for the surveillance of trace food allergens. It is an efficient strategy to generate specific Nbs with phage display technology, and generally a lot of Nb reservoirs can be obtained against the target of interest to bind extensively different epitopes on food allergen, which can facilitate the easy identification of Nbs with inhibitory effect on IgE binding. Further, the affinity of in vivo matured Nbs can be further augmented through varied approaches to improve the characteristics of selected binders.<sup>36,94,95</sup> The initial library construction of Nbs is completely different from the classic antibodies, as the immunization to camelid animals is preferred for most of the allergens, and alternatives can also be used including naïve and synthetic libraries if the antigen of interest is toxic, unstable, or nonimmunogenic.<sup>67,96,97</sup> The property of a longer CDR3 region facilitates Nbs to form flexible paratopes to bind unique epitopes on antigen that generally are inaccessible for conventional antibodies, which potentially provides extended opportunity to acquire Nbs with inhibitory effect.<sup>98,99</sup> To date, not much allergen-specific Nbs have been reported in the literature including peanut allergen Ara h 3 Nbs.<sup>41</sup> The complex of Ara h 3 with selected Nbs facilitated the formation of crystals for the identification of structural

information on Ara h 3. It is critical to evaluate the binding properties of selected Nbs against food allergens, in order to assess the IgE-blocking potential. Zettl et al. reported the selection of specific Nbs against the major birch pollen allergen of Bet v 1 and evaluated the inhibition on IgE binding.<sup>43</sup> This research just started the employment and evaluation of Nbs as blocking antibodies to alleviate airway allergic reactions. Three Nbs recognizing Bet v 1 were selected with high affinity and cross-reaction with Aln g 1 (alder) and Cor a 1 (hazel). The epitope mapping revealed the targeting to the  $\alpha$  helix at the C-terminus of Bet v 1, and the inhibition on allergic patients' polyclonal IgE binding to Bet v 1, Aln g 1, and Cor a 1 was validated. This research provided the first proof that allergen-specific Nbs are promising theranostic tools for the immune intervention of pollen allergy. The safety of Nbs for employment as theranostic reagents has been comprehensively validated in different research and clinical trials,<sup>100</sup> as well as the first therapeutic nanobody of Caplacizumab for the treatment of thrombotic thrombocytopenic purpura and thrombosis.<sup>101</sup> Though no research evidence on Nb-inhibition on food allergy has been published yet, it is expected that the first proof will come soon as the author is working to select Nbs against different food allergens to evaluate their inhibition on food allergic reactions.

## PERSPECTIVE

The increased incidence of food allergy caused by food allergen intolerance or unintentional ingestion for sensitive individuals has raised the focus on allergen inspection and immune therapy based on specific Nbs. The increased publications related to the exploitation of food allergen-specific Nbs as tools for research and application represent a reasonable progress for food allergen surveillance and allergy alleviation. The limited reports about the application of Nbs in the field of food allergen detection and immunotherapy could arise from the focus of Nbs as diagnostic and therapeutic reagents for various diseases. The investigation of allergen-specific Nbs was initiated just by employing varied immunization and selecting strategies like unbiased and precise approaches. The less exploration potentially originated from the toughness in the process of research and application rather than the intrinsic properties of Nbs. With the unique properties of Nbs including high affinity, solubility and stability, low immunogenicity, and the targeting to unique epitopes, researchers have input the attention to extend their application in the field of food allergic reaction and allergen inspection. Various evaluations have highlighted the role of Nbs, either to develop as the reagents for detecting food allergens or exploit the inhibitory potential on IgE binding and the resulted allergic reactions. The drawbacks of Nbs mainly originated from the single domain properties, and various strategies can be applied to overcome this issue, like production of multivalent or multispecific Nbs, grafting to humanized Fc domain, or reconstitute to HCABs to form multidomain binders. Whereas, this manipulation can also shield the favored characteristics of a single domain and need to be reconsidered comprehensively before engineering. The limited results have provided the evidence of Nbs as effective and easily manipulated candidates for research and application in the field of food allergen and allergic diseases. Moreover, the research on epitope mapping and structure analysis can also be proposed to further extend the application of Nbs in the field of food allergen investigation, to either utilize the properties of

Nbs for targeting to unique epitopes on allergen or play as a chaperone for allergen crystallization and precipitation. As the focus and publication increased with the endeavors invested, more evidence will be provided as the validation of Nbs for the suitability assessment in the research field of food allergens.

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### Notes

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