Minimum Distance Between Two Epitopes in Sandwich Immunoassays for Small Molecules

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Abstract

The pursuit of the limit between dimensionalities is a scientific goal with high applicability. Sandwich immunoassay, usually based on two antibodies binding two epitopes, is one of the most popular mainstay tools in both academic and industrial fields. Herein, we determined and evaluated the minimum distance of two epitopes in sandwich immunoassays for small molecules. Briefly, nine model analytes comprising two hapten epitopes, i.e., melamine (MEL) and p-nitroaniline (NIA), were designed by increasing the linear chain linkers brick by brick. Two groups of monoclonal antibodies (mAbs) were produced with different recognition properties toward MEL and NIA using 12 new hapteins with different spacer arms. The results indicated that two epitopes of the analyte with a distance of only 2.4 Å could be simultaneously bound by two mAbs, which is the known limit of epitope distance in sandwich immunoassays thus far. We further found that an epitope distance of below 8.8 Å for the analyte generally induces noticeable steric hindrance of antibodies, preventing a sandwich immunoassay with high probability. These observations were investigated and evaluated by molecular docking, molecular dynamics, and surface plasmon resonance and using model and real analytes. Altogether, we determined the minimum distance of two epitopes and explored the molecular mechanism of the antibody–analyte–antibody ternary complex in sandwich immunoassays, providing a theoretical basis for hapten design, antibody discovery and development, and sandwich immunoassay establishment for small molecules.

Key word: sandwich immunoassay, small molecule, epitope distance limit, antibody–analyte-antibody ternary, molecular recognition mechanism
INTRODUCTION

The detection of small molecules, such as pharmaceuticals, environmental pollutants, and chemical contaminants, is an important subject in clinical diagnostics, environmental monitoring, and food analysis.\textsuperscript{1–2} For decades, the immunoassay, as a sensitive, straightforward, and cost-effective system, has been a mainstay for the quantifiable measurement of target analytes.\textsuperscript{3–4} It is reported that the global immunoassay market was 20.93 billion dollars in 2020 and expected to reach 22.32 billion dollars in 2021 with an annual growth rate of 7.03\%\textsuperscript{5}. Immunoassays generally fall into two broad formats, competitive and noncompetitive assays, where noncompetitive assays are generally referred to as sandwich immunoassays. They essentially differ in the amount of reagent used in the immunological reaction. In a competitive immunoassay, the analyte competes with the labeled (or immobilized) analyte for the limited specific antibody.\textsuperscript{6–7} In contrast, a sandwich immunoassay can bind an analyte based on two distinct antibodies present in excess, granting a higher level of sensitivity, precision, and specificity.\textsuperscript{8–9} However, sandwich immunoassays require large analyte molecules to allow at least two epitopes to be bounded by two antibodies. Small molecules are generally not assayed by sandwich immunoassay, as they cannot be simultaneously bound by two antibodies due to steric hindrance.\textsuperscript{10–11} Since the binding pocket of the antibody against the small molecule is mostly cavity-like, the small molecule is largely buried after binding to an antibody, resulting in an inaccessible binding site for the second antibody and the failure of the sandwich immunoassay. This assumption has been reinforced by the X-ray structural analysis of hapten–antibody complexes that show how small molecules become buried deeply in the cavity-like binding site, with contacting surface areas of 200–400 Å\textsuperscript{2}.\textsuperscript{12–13} Typical small molecules, such as drugs of abuse, antibiotics, and pesticides, possess a surface
area of only 100–800 Å², which theoretically is not large enough to simultaneously bind two antibodies.\(^\text{14-15}\) Therefore, sandwich immunoassays are widely used to detect macromolecules such as proteins and polypeptides, while competitive immunoassays are overwhelmingly popular for the detection of small molecules.\(^\text{16}\)

Considering the advantages provided by sandwich immunoassays, researchers have developed alternatives for small molecule detection such as noncompetitive immunoassays based on anti-metatype or anti-idiotype antibodies, solid-phase covalent epitope immobilization, and open sandwich immunoassays.\(^\text{17-21}\) However, these strategies are greatly hindered by unconventional antibody preparation procedures and complex operation procedures, limiting the practical application of such assays. Direct sandwich immunoassays for small molecules by two distinct antibodies are rarely reported. To the best of our knowledge, true sandwich immunoassays have been reported only for a few small molecules, i.e., ciguatoxin (1023 Da), tacrolimus (804 Da), naringin (580 Da), ponatinib (533 Da), oxytetracycline (496 Da), and imatinib (494 Da); details are provided in Supplementary Table 1.\(^\text{22-28}\) Given one small molecule, an approach to estimate the possibility of establishing a sandwich immunoassay is currently not known. Determining the epitope distance of the analyte required to allow the binding of two antibodies can provide useful clues for the sandwich immunoassay of small molecules. Furthermore, the molecular recognition mechanism of antibody–analyte–antibody ternary complexes in sandwich immunoassays for small molecules has not yet been explored, which is necessary structural information for hapten design, antibody discovery and development, and sandwich immunoassay establishment.

In the present study, we address these questions using two typical hapten epitopes, melamine (MEL) and $p$-nitroaniline (NIA), that were connected by linkers with an increasing linear alkane length of 1–14 methylenes to create nine model analytes. Two
groups of monoclonal antibodies (mAbs) against MEL and NIA were separately produced based on 12 newly designed haptens, including six MEL haptens and six NIA haptens. Numerous mAb pairings were studied to investigate combinatorial associations and determine the minimum two-epitope distance of the analyte that could still be simultaneously bound by two distinct mAbs. The molecular mechanism of antibody–analyte–antibody recognition in sandwich immunoassay was further investigated by molecular docking, molecular dynamics, and surface plasmon resonance (SPR). Finally, model analytes, i.e., sulfabenzamide–norflxacin (SBA-NOR) conjugates, and a real analyte, namely abamectin (ABM), were used to evaluate the conclusions of this study.

MATERIALS AND METHODS

Reagents and apparatus

MEL, NIA, SBA, NOR, and ABM standards were bought from J&K Scientific Co., Ltd. (Beijing, China). Bovine serum albumin (BSA), ovalbumin (OVA), N-hydroxy succinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC), keyhole limpet hemocyanin (KLH), horseradish peroxidase (HRP) labeled goat antimouse IgG, and casein were acquired from Sigma-Aldrich (St. Louis, MO, USA). Incomplete Freund’s adjuvant (IFA), complete Freund’s adjuvant (CFA), cell culture medium, hypoxanthine–aminopterin–thymidine (HAT) medium, polyethylene glycol (PEG) 1500, and fetal calf serum were obtained from Gibco BRL (Carlsbad, CA, USA). Microtiter plates and cell culture plates were purchased from Corning Inc. (New York, NY, USA). The mAbs were conjugated with HRP by using an HRP conjugation kit from
Abcam (Cambridge, MA, USA). The buffer and solution used in this work can be found in the Supplementary Information.

The optical density (OD) of the enzyme-linked immunosorbent assay (ELISA) at 450 nm was recorded on a SpectraMax M5 (Downingtown, PA, USA). SPR experiments were conducted on an OpenSPRTM (Nicoya, CA, USA). A HiTrap Mabselect SuRe column was purchased from GE Healthcare (Chicago, IL, USA).

**Synthesis and characterization of analytes and haptens**

Nine model analytes, one analog, six MEL haptens, six NIA haptens, two SBA-NOR conjugates, and one ABM hapten were fully synthesized in 2–10 steps. The detailed synthetic procedures are provided in the Supplementary Information. The chemical structures of all analytes were confirmed by nuclear magnetic resonance spectrometry DRX-300 from Bruker (Billerica, MA, USA) and high-resolution mass spectrometry from Agilent Technologies (Santa Clara, CA, USA).

The three-dimensional structures of analytes were built in GaussView 5.0 (Gaussian, Wallingford, CT, USA). The analytes were then optimized by density functional theory (DFT) calculations at the TVZP functional level with an M06-2X basis set using the Gaussian 09 program (Wallingford, CT, USA). The epitope distances of the analytes were measured and extracted from the Gaussian formchk file in GaussView 5.0.
Production and characterization of monoclonal antibodies

The detailed synthesis of hapten–BSA immunogens, hapten–KLH immunogens, and hapten–OVA coating antigens is provided in the Supplementary Information. The hapten–BSA conjugates were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF–MS, Bruker, Bremen, Germany). Ten eight-week-old female BALB/c mice per immunogen were immunized according to our previously described procedure. Briefly, the first injection was 100 µg of the immunogen emulsified with CFA, followed by two subcutaneous injections of 100 µg of the immunogen with IFA. From the second immunization, mice antisera were collected on the 7th day after each immunization to monitor antibody titer and affinity by noncompetitive ELISA (ncELISA) and competitive indirect ELISA (ciELISA), as described in the Supplementary Information. After the third immunization, the mice exhibiting the highest affinity from each group were killed for subsequent cell fusion. The SP2/0 myeloma cells and splenocytes from the selected mice were mixed for cell fusion with the assistance of PEG 1500. The fused cells were propagated in HAT medium, and the hybridoma cells were screened for antibody production, followed by subcloning based on the limiting dilution method. Clones with high affinity were cloned three times before ascite production. The above experimental procedures were previously described by our group. The obtained mAbs were purified from ascites for immunoassay establishment using a protein-A affinity column, as described in the Supplementary Information.
Development of competitive indirect immunoassays for model analytes

Each well of the microtiter plates was first coated with 100 μL of the coating antigen in coating buffer and then incubated at 4°C overnight. Blocking buffer was then added to the plates (150 μL/well) and incubated for 2 h at 37°C. After washing, 50 μL of the analyte with serial dilutions was added to the wells as well as 50 μL of optimal diluted mAb. After incubation for 30 min at 37°C, the plates were washed two times and patted dry. Then, 100 μL of HRP labeled goat anti-mouse IgG (1:5000) was added, followed by incubation for 2 h at 37°C. After further washing, 100 μL of TMB substrate was added to each well, which was incubated for 15 min at 37°C. Finally, the chromogenic reaction was inhibited with 2 M H₂SO₄ (50 μL/well), and the OD values at 450 nm were measured.

The standard curves of competitive indirect immunoassay were fitted by the following equation using OriginPro 7.5 software (Northampton, MA, USA):

\[ Y = \frac{(A - B)}{[1 + (X/C) D]} + B \]  

(Eq. 1)

where A and B are the responses at high and low asymptotes of the curve, respectively, C is the concentration of the targets that results in 50% inhibition (IC₅₀), D is the slope at the inflection point of the sigmoid, and X is the calibration concentration.

The limit of detection (LOD) of the competitive immunoassay was defined as the concentration of analyte that provided a 10% reduction of the OD max (IC₁₀). The detection range was established between the values of IC₂₀ and IC₈₀.
Screening of monoclonal antibody pairs for model analytes

Microtiter plates were coated with purified mAb (capture antibody, 4 μg/mL, 100 μL/well) in coating buffer and incubated at 4°C overnight. The plates were subsequently washed and blocked with 150 μL of blocking buffer. Then, 100 μL of the model analyte (10 μM) was added and incubated at 37°C for 1 h, followed by another washing procedure. Next, 100 μL of diluted HRP-labeled mAb (detecting antibody, 0.2 μg/mL) was added to each well, which was incubated for 1 h at 37°C. The TMB substrate solution was added after washing, 2 M H$_2$SO$_4$ was added to stop the reaction, and the OD values were measured at 450 nm. The measured OD values were used to represent the formation efficiency of the sandwich immunoassay by different mAb pairs. Specifically, an OD value below 0.1 was considered to be indistinguishable from the background, i.e., a signal to noise (S/N) ratio below 2.1, indicating that sandwich immunoassay establishment had failed, while OD values of 0.5–1.0 and above 1.0 represented medium and high formation efficiencies of sandwich immunoassays, respectively.

Development of sandwich immunoassays for model and real analytes

The microtiter plates were coated with diluted capture mAb at 4°C. After blocking and washing steps, serial dilutions of the analytes were added to the wells, and the plates were incubated for 1 h at 37°C. Then, 100 μL of diluted HRP-labeled mAb was added to each well, followed by incubation for 1 h at 37°C. The solution was incubated for 15 min at 37°C before the enzymatic reaction was stopped with 50 μL of 2 M H$_2$SO$_4$. The
OD value of each well was measured at 450 nm. These values were plotted against the analyte concentration on a logarithmic scale, and the generated sigmoidal curve was mathematically fitted to Eq. 1. The LOD and the detection range of the sandwich immunoassay were the same as those of the competitive immunoassay.

**Antibody sequencing, molecular docking, and molecular dynamics analysis**

The cloning of the single-chain variable fragment antibody (scFv) was performed as described previously by our group. Three-dimensional structure predictions for scFv were generated using the Model Antibody of Discovery Studio from BIOVIA Co., Ltd. (San Diego, CA, USA) based on the primary structure of mAbs. After loading the amino sequences, the framework templates for the variable fragment (Fv) of the antibody sequence were identified for homology modeling. According to the sequence similarity and identity, the best matching overall templates were used to model the antibody framework for the antibodies, which determined the relative spatial orientations of the light and heavy chains. The binding between antibodies and epitopes (MEL or NIA) was performed with the CDOCKER docking program of Discovery Studio. After docking the epitopes into the top-ranked cavity of the antibody, the binding position of each complex with the highest score was selected to provide a starting orientation of each antibody–epitope moiety. Rosetta protein–protein docking protocol was then used for the antibody–antibody docking calculations to provide the relative positions of both antibodies. The antibody–epitope complex was then superimposed onto each antibody in the antibody–antibody complex, and a shared
binding cavity was formed between the two antibodies. The model analytes MN1–MN9 were subsequently positioned at the shared binding cavity, thereby forming an initial configuration of the entire antibody–analyte–antibody ternary complex. To minimize the production of sterically clashing ternary complexes, the conformations of the model analytes were automatically adjusted to adopt an extended conformation. Simulations described herein were carried out with the SANDER module of the AMBER 4.1 molecular dynamics simulation package (San Francisco, CA, USA). First, the generated ternary complex structure was solvated and neutralized in a box with TIP3P water with a minimum of 10 Å between the model and a wall of the box. The structure was initially subjected to energy minimization (1000-time steps) to remove unreasonable atomic contacts. Afterward, the prepared system was equilibrated using a canonical (NVT) ensemble, followed by an isothermal–isobaric (NPT) ensemble. Finally, 100 ns molecular dynamics simulations were carried out at normal temperature and pressure (300 K and 1.01325 bar, respectively), wherein a time step of 2 fs was applied using the SHAKE algorithm to fix the bonds involving hydrogen atoms. A brief protocol of the antibody–analyte–antibody ternary complex construction is provided and schematized in the Supplementary Information.

**SPR assay for antibody–analyte–antibody ternary complexes**

As a powerful instrument that provides in-depth, label-free binding kinetics for a variety of different molecular interactions, OpenSPR was selected to further evaluate the antibody–analyte–antibody ternary complex. SPR experiments were performed
using a Nicoya Lifesciences OpenSPR system equipped with a carboxyl modified chip. Initially, a maximum flow rate of 150 μL/min for the detection buffer (PBS, pH 7.4, 1% DMSO) was used to reach the signal baseline. Afterward, 200 μL of isopropanol was run for 10 s to remove bubbles. After reaching the baseline, the flow rate of the detection buffer was adjusted to 20 μL/min. Following surface activation by EDC/NHS within a contact time of 420 s at a flow rate of 10 μL/min, the capture mAb prepared at 2 mg/mL in 10 mM sodium acetate coupling buffer (pH 5.0) was immobilized at 25°C for 240 s. The surface was blocked with an injection of 200 μL of 1 M ethanolamine, and 100 μL of the diluted model analyte at a proper concentration was injected at a speed of 20 μL/min to interact for 240 s (Step I). After dissociation and stabilization (Step II), the detecting mAb at 2 mg/mL was sampled to observe the binding signal of the antibody–analyte–antibody ternary complex (Step III). The surface was regenerated with an injection of regeneration buffer (10 mM HCl, pH 2.0) at a speed of 150 μL/min between each analyte injection, and the chip was washed in 10 mM HCl (pH 2.0) to remove impurities. Data were collected at a rate of 5 Hz, single referenced with blank injections, and fitted to a 1:1 interaction model using Trace Drawer analysis software.

RESULTS AND DISCUSSION

Synthesis and characterization of model analytes, haptens, and conjugates

The objective of the present study was to explore the minimum epitope distance of the analyte in the sandwich immunoassay for small molecules. For this purpose, two hapten epitopes, MEL and NIA, were selected and separated by linear chain linkers with varied lengths to mimic epitope distance. Therefore, a library of model analytes,
including nine compounds with linkers of 1–14 methylenes with the MEL and NIA epitopes on each end, was designed and named MN1–MN9 with increasing length (Fig. 1a).

Antibodies with different recognition properties are of considerable importance for antibody pair screening to successfully establish sandwich immunoassays, significantly affecting the determination of minimum distance of the two epitopes. To obtain antibodies against MEL and NIA epitopes with recognition properties and as much diversity as possible, a hapten spacer arm between the epitope and carrier protein should be first considered, since the full exposure of the target analyte to the immune system is critical to induce highly specific antibodies. Thus, two sets of novel haptens were designed and named M1–M6 and N1–N6 (Fig. 1b and 1c). These haptens were characterized by the equably increased spacer arm length of two methylenes to produce antibody populations with various recognition properties toward MEL or NIA epitopes.

Before total synthesis, we conducted computational chemistry to obtain the size of all designed model analytes. The computational results indicated that the smallest structure MN1 with a minimum epitope distance of 2.4 Å had been obtained, and the epitope distances of the model analytes gradually increased to reach a maximum of 19.0 Å for the largest structure MN9 (Fig. 1a). It was observed that the sizes of most model analytes in the study were inferior to those that had been assayed by sandwich immunoassay (Supplementary Table 1). The detailed synthesis and characterization of the model analytes and haptens are provided in the Supplementary Information (Supplementary Figs. 1 and 2).

Since a hapten cannot be effectively recognized by the immune system to induce
subsequent antibody response, it should be first conjugated to a carrier protein.\textsuperscript{31} Thus, the carboxyl groups of all haptens were conjugated to BSA as immunogens and OVA as coating antigens to screen antisera and produce mAbs. The preparation steps of these hapten–protein conjugates are described in the Supplementary Information. Since the hapten-carrier ratio could influence the level of antibody response of the host animals,\textsuperscript{39–40} it was carefully controlled in the range of 19.3–23.6 for MEL haptens–BSA and in the range of 9.0–13.2 for NIA haptens–BSA and successfully characterized by MALDI–TOF–MS (Supplementary Fig. 3 and Supplementary Table 2).

Production and characterization of monoclonal antibodies

Each hapten–BSA was used to immunize a set of 10 BALB/c mice three times at 21-day intervals. The mice antisera of 12 groups respectively immunized with haptens M1–M6 and N1–N6 were monitored from the second immunization. MEL or NIA was used to determine the antibody titer and affinity by ncELISA and ciELISA. In the study, the antibody titer is defined as the antisera dilution that furnished an $\text{OD}_{\text{max}}$ between 1.5 and 2.0, while antibody affinity is expressed as the inhibition ratio calculated according to Eq. S2 at a MEL or NIA concentration of 10 $\mu$g/mL as described in the Supplementary Information. In short, a higher antibody titer or antibody affinity indicates a stronger antibody response. All haptens with various spacer arm lengths generated specific antibody responses to MEL or NIA in all mice. In addition, the levels of antibody response varied significantly depending on the degree of exposure of the epitope to the immune system via the spacer arm (Supplementary Table 3). The study
aimed to produce mAbs that maximally recognize the two epitopes (MEL and NIA) with as much diversity as possible to facilitate the development of sandwich immunoassays for the model analytes. Thus, mice with the highest affinity to MEL or NIA from all groups were chosen to produce mAbs (Supplementary Table 4). Notably, the mAbs prepared from haptens M1, M5, and M6 failed due to their low titer and poor affinity. In total, 17 mAbs were obtained, including 6 anti-MEL mAbs and 11 anti-NIA mAbs (Supplementary Table 4). Specifically, mAbs induced by haptens M3 and M4, possessing a moderate spacer arm length, showed comparable affinities to MEL with IC$_{50}$ values ranging from 16 to 79 ng/mL. mAbs from haptens N3 and N4 showed high affinities toward NIA, with IC$_{50}$ values ranging from 81 to 601 ng/mL, which were nearly 2–67 times better than those of mAbs from haptens N1, N2, N5, and N6 with short or long spacer arm lengths.

Since the recognition property of the antibody could affect the detected minimum epitope distance of the analyte in the sandwich immunoassay, the recognition properties of 17 mAbs for model analytes were evaluated using ciELISA (Supplementary Tables 5 and 6). For mAbs from haptens M3, M4, N3, and N4, a clear increase in mAb affinity for MN1–MN7 was observed as the epitope distance of the model analyte increased from 2.4 Å to 13.9 Å but decreased for MN8 and MN9 with longer epitope distances of 16.4 Å and 19.0 Å, respectively. The trends of mAbs 5A3, 10A9, and 4E1 from haptens N1 and N2 were not obvious, comparably recognizing all tested model analytes; however, mAbs 7F2, 1E2, and 2F5 from haptens N5 and N6 showed higher affinities to MN6–MN9 with longer epitope distances. These results
indicated that the spacer arm length of the haptens had a clear influence on the induced antibody recognition property, as shown by the affinity of the antibody toward the tested model analytes.

Screening of monoclonal antibody pairs for model analytes

To establish a sandwich immunoassay for the model analytes, six anti-MEL mAbs and 11 anti-NIA mAbs with different recognition properties were tested for association combinations, leading to 132 mAb pairs for each model analyte and a total of 1188 pairs for the nine model analytes. Owing to the higher OD values provided by anti-MEL mAbs as the capture antibodies in the sandwich immunoassays, anti-NIA mAbs were served as the detecting antibody for subsequent sandwich establishment. The results of the mAb pairings when the model analytes MN1–MN9 were used at 100 μM are shown in Fig. 2a and 2b. Remarkably low success rates for sandwich immunoassay of model analytes with shorter epitope distances were clearly observed; almost all mAb pairs provided very low OD values for MN1–MN4 compared to MN5 with an epitope distance of 8.8 Å (Fig. 2b and 2c). The results suggest that steric hindrance occurs between the capture and detecting antibodies. Stable antibody–analyte–antibody ternary complexes could not form when the epitope distance for an analyte was below 8.8 Å, resulting in the failure of sandwich immunoassay establishment. Still, very few mAb pairs remained functional for MN1–MN4 proofed with an S/N > 2.1, such as 7A3 and 17B3 for MN1 (S/N = 2.9), representing a sandwich immunoassay for the smallest analyze ever described (Fig. 2a and Supplementary Table 1). The successful sandwich
immunoassay for MN1, with an epitope distance of 2.4 Å and a molecular weight of 304 Da, demonstrated that the simultaneous binding of two mAbs to an extremely small analyte could be achieved through the careful selection and extensive pairing of mAbs. Over 90% of mAb pairs allowed for the efficient sandwich immunoassays of analytes MN7–MN9 with an epitope distance of at least 13.9 Å, indicating successful simultaneous binding for most mAb pairs. Notably, unsuccessful mAb pairs were observed in all model analytes, showing that the production of mAbs with diverse recognition properties is important and necessary to establish sandwich immunoassays for small molecules.

mAbs from haptens with long spacer arms should possess deep binding cavities, recognizing not only the analyte epitope but also the spacer arm in most cases. The mAbs of 7F2, 1E2, and 2F5 from hapten N5 and N6 largely prevented sandwich immunoassays in all mAb pairs, regardless of the capture mAbs used, suggesting that mAbs with deep binding cavities prevent the simultaneous binding of two mAbs for analytes (Fig. 2b). On the contrary, mAbs induced by haptens with short spacer arms should have shallow binding cavities, mostly recognizing the fractional epitopes of MEL or NIA due to the partial exposure of the target analyte. The mAb pairs of 10A9 from hapten N1 with the shortest spacer arm and MEL mAb 6A8 can simultaneously bind to MN4–MN9, showing an advantage over mAbs with deep binding cavities. Despite this, the overall sandwich formation rates of mAbs from haptens N1 and N2 are much lower than those of mAbs from haptens N3 and N4 with moderate spacer arm lengths. The results further implied that haptens with extra-long or short spacer arms
should be avoided to produce a suitable antibody and establish efficient sandwich immunoassays for small molecules.

**Development of competitive and sandwich immunoassay for model analytes**

The pair that exhibited the best sandwich efficacy, i.e., mAbs 7A3 from hapten M4 and 17B3 from hapten N4, was selected to perform competitive and sandwich immunoassays for MN1–MN9 (Fig. 3a and 3b). The performances of competitive and sandwich immunoassays were compared by their LOD, detection range, and specificity.

The LOD values of the competitive immunoassays based on 7A3 for MN1–MN7 gradually decreased from 0.2 to 0.001 nM as the epitope distance of model analytes increased from 2.4 to 13.9 Å, and increased LOD values were observed for the longest model analytes, MN8 and MN9, with epitope distances of 16.4 and 19.0 Å, respectively (Fig. 3c and Table 1). Similarly, the LOD values of the competitive immunoassays based on 17B3 gradually increased and then decreased for MN1–MN9 with epitope distances of 2.4–19.6 Å (Fig. 3d and Table 1).

For the sandwich immunoassay of the model analytes, as expected, the sandwich immunoassays based on the 7A3–17B3 pair provided significantly dose-dependent signals for all model analytes (Fig. 3e and Table 1). These results also showed that the sensitivities of both competitive and sandwich immunoassays for model analytes are dependent on the length of the molecule. Therefore, an appropriate molecule length may be beneficial to achieving high sensitivity. Both competitive and sandwich immunoassays for MN1–MN4 with short epitope distances of ~6.3 Å showed low
sensitivity, with competitive immunoassays being more susceptible to the short epitope
distance of the analyte (Fig. 3 and Table 1). Only weak signals were detected for MN1–
MN4 in sandwich immunoassay even with high concentrations of the analyte (100 µM).
The LODs of the sandwich immunoassays for MN1–MN5 are at least three orders of
magnitudes lower than those of competitive immunoassays, suggesting that small
molecules with an epitope distance below 8.8 Å (MN5) are unlikely to be sensitively
detected by sandwich immunoassay due to steric hindrance. In contrast, sandwich
immunoassays exhibited very low LODs of 0.00035–0.9 nM and a wider detection
range for MN6–MN9 than those of the corresponding competitive immunoassays with
LODs of 0.001–38.8 nM (Table 1 and Supplementary Table 7). These results showed
that the sandwich immunoassay for small molecules afforded superior sensitivity and
detection range for MN6–MN9 with an epitope distance of 11.6–19.0 Å. In the case of
avoiding steric hindrance as much as possible, these findings suggest the preferential
use of sandwich immunoassays for the determination of small molecules due to its
higher sensitivity and wider detection range.

Furthermore, to evaluate the specificity of both immunoassay formats, a new
structural analog of the analyte was synthesized based on MN3 by introducing one
additional methyl group to the NIA end, as shown in the Supplementary Information.
The results in Table 1 show that the competitive immunoassay based on two single
mAbs could detect the analog with a sensitivity comparable to that of MN3, while the
sandwich immunoassay did not recognize the analog even at 100 µM. The sandwich
immunoassay, which used two distinct mAbs, provided high specificity toward the
target analyte, and it demonstrates potential high accuracy for subsequent practical
application.

Molecular recognition mechanism by molecular docking and molecular dynamics

To further explain the scientific basis of antibody–analyte–antibody recognition
utilized by the sandwich immunoassay for small molecules in this study, structural
studies on mAb pairs 7A3–17B3, 7A3–10A9, and 7A3–7F2 in the complex with typical
model analytes were conducted by molecular docking and molecular dynamics after
antibody sequencing (Supplementary Table 8). Unlike the docking of the antibody–
antigen binary complex, there are few protocols for the construction of antibody–
analyte–antibody ternary complexes. According to prior experiences in proteolysis–
targeting chimeras (PROTAC), we first studied the antibody–analyte–antibody ternary
complex in sandwich immunoassays for small molecules (Supplementary Fig. 4).42–43

Briefly, taking 7A3–MN7–17B3 as an example, the starting orientations of the 7A3–
MEL and 17B3–NIA binding moieties should be first provided to form an initial
configuration. After 7A3 and 17B3 protein–protein docking to determine the relative
position of the two mAbs, the 7A3–MEL and 17B3–NIA binding moieties were
superimposed onto their respective mAbs in the 7A3–17B3 complex to form a shared
binding cavity. Finally, after removing the MEL and NIA, MN7 was placed in the
shared binding cavity, and molecular dynamics was carried out to obtain the stable
conformation of 7A3–MN7–17B3.

We first measured the distance between MEL and NIA in the shared binding cavity
of 7A3–17B3, represented as a white surface (Fig. 4a). The epitope distance of 9.2 Å from the shared binding cavity of 7A3–17B3 was theoretically determined, which meant that steric hindrance between the two mAbs could be avoided in this situation. Thereafter, MN1–MN9 with various epitope distances were superimposed onto the shared binding cavity of 7A3–17B3 with extended conformations. The results showed that MN1–MN4 with epitope distances of 2.4–6.3 Å were too short to be superimposed onto the shared binding cavity, meaning that stable antibody–analyte–antibody ternary complexes could not be formed using these model analytes (Fig. 4a). Steric hindrance occurs when MN1–MN4 bind to two mAbs at the same time, and only weak sandwich immunoassays could be achieved as a result (Fig. 2b). MN5 with an epitope distance of 8.8 Å, comparable to the distance of the shared binding cavity of 7A3–17B3, was then superimposed onto the shared binding cavity, and the initial ternary complex was successfully generated as expected. Molecular dynamics study provided the stable conformation of the 7A3–MN5–17B3 ternary complex, with a root mean square deviation (RMSD) of 0.6 Å in 60–100 ns (Fig. 4b and Supplementary Fig. 5A). Thus, the epitope distance of ~8.8 Å for the analyte was considered the shortest for establishing sandwich immunoassays with high success rates for small molecules, as demonstrated both experimentally and through molecular dynamics study.

In the above sandwich immunoassay experiment (Figs. 2 and 3), the sensitivity of the sandwich immunoassay based on 7A3–17B3 for MN5 was significantly better than that for MN1–MN4 but far worse than that for MN6–MN9, indicating that the epitope distance of the analyte is critical for the sensitivity of the sandwich immunoassay. We
then studied the correlation between the analyte epitope distance and the sensitivity of the sandwich immunoassay using MN5, MN7, and MN9 with the same 7A3–17B3 mAb pair. Steric hindrance was observed when MN5 was clamped by 7A3 and 17B3 simultaneously in the real binding process, as indicated by the relatively lower sensitivity in the sandwich immunoassay with a LOD of 265.4 nM (Fig. 2). Thus, we analyzed the recognition mechanism of the 7A3–MN5–17B3 ternary complex to gain insight into the interactions. The binding cavity of the complex was mainly maintained by hydrogen bonds provided by G307 of 7A3 as well as R55 and N40 of 17B3. For MN7, the epitope distance of 13.9 Å prevents damage to the sensitivity of the sandwich immunoassay from steric hindrance to the greatest extent (Fig. 4b). The 7A3–MN7–17B3 ternary complex affording the most sensitivity sandwich immunoassay with a LOD of 0.00035 nM, was maintained by conventional hydrogen bonds and hydrophobic forces from Y379, D327, E319, L439, and S370 of 7A3 as well as Y210, Y212, and Y152 of 17B3 (Fig. 4c and Supplementary Fig. 5b). Surprisingly, a hydrogen bond formed between the NIA epitope and S370 of 7A3 from hapten M4, indicating that the 7A3–MN7 binding cavity was tighter than that of 7A3–MN5. Thus, increased interaction forces made the 7A3–MN7–17B3 complex more stable compared to the 7A3–MN5–17B3 complex, improving its sensitivity in sandwich immunoassays. MN9, with the longest epitope distance of 19.0 Å, provided a 2,500-fold worse sensitivity than that of MN7 in the sandwich immunoassay. Following molecular dynamics study, the expected stable ternary complex 7A3–MN9–17B3 was obtained at 20 ns. The lower sensitivity of the sandwich immunoassay for MN9, with a LOD of 0.9 nM, can be
attributed to the higher flexibility of MN9 due to its longer epitope distance, increasing the stretch degree between the ends of the two mAbs (Supplementary Fig. 4d, Fig. 5c, and video). The RMSD of 0.6 Å for the 7A3–MN9–17B3 complex was higher than the 0.4 Å for the 7A3–MN7–17B3 complex, further showing that long epitope distances of an analyte may not always be conducive to sandwich immunoassays with high sensitivity. The epitope folding of the analyte caused by long flexible chains should be considered to develop a robust sandwich immunoassay for small molecules. These results show that an analyte epitope distance of at least 8.8 Å can bind two mAbs with acceptable steric hindrance, and an epitope distance of ~13.9 Å was suggested to develop sandwich immunoassays with high sensitivity.

Compared to 7A3–17B3, with the best performing sandwich immunoassay, 7A3–10A9 and 7A3–7F2, which are representative of medium- and low-performing mAb pairs, exhibited lower success rates in the sandwich immunoassays for model analytes with varied epitope distances (Fig. 2). Besides the epitope distance, the success of sandwich immunoassay was found to heavily depend on the recognition property of the two mAbs employed. Thus, we chose 7A3–10A9 and 7A3–7F2 to address the importance of the selected mAbs for the performance of sandwich immunoassay and carried out docking and molecular dynamics of the ternary complexes with MN7. The mAbs 10A9 and 7F2 with different recognition properties were produced from MEL haptens N1 and N5, respectively. Similar to the 7A3–MN7–17B3 ternary construction, NIA was first docked to 10A9 and 7F2. The epitope distances of the 7A3–10A9 and 7A3–7F2 shared cavities were measured as 8.3 and 11.9 Å, respectively, indicating that
the 10A9 and NIA binding cavities were shallower than those of 7F2 and NIA (Supplementary Fig. 6). It can be assumed that 10A9 from hapten N1, possessing the shortest spacer arm, may only recognize the fractional epitope of NIA due to the shielding effect of the carrier and then forms a shallow binding cavity (Fig. 4e). In contrast, 7F2 from hapten N5, with a longer spacer arm, recognizes not only NIA epitopes but also part of the spacer arms to form a deep binding cavity. Subsequently, molecular dynamics analysis of the two mAb pairs, 7A3–10A9 and 7A3–7F2, was conducted, and stable ternary complexes were obtained (Supplementary Fig. 5d, Fig. 5e, and video). Interaction between 10A9 and NIA occurs only at the nitro group end in the 7A3–MN7–10A9 complex, as the shallow binding cavity of 10A9 causes the flexible linker of MN7 to become more exposed. This causes the analyte to fold and accounts for the medium performance of the sandwich immunoassay (Fig. 4e). For 7A3–MN7–7F2, the NIA epitope of MN7 is deeply inserted into the binding cavity of 7F2 and forms hydrogen bonds with D338, Q443, and Y445 in 7F2 (Fig. 4f). This is unfavorable to the simultaneous binding of the other mAbs to the analytes due to steric hindrance and leads to the poor performance of the sandwich immunoassay. The results showed that the recognition properties of mAbs significantly affect the performances of sandwich immunoassays for analytes and are closely related to the designed hapten structure. Compared to 17B3 from hapten N4, which performed the best in sandwich immunoassay, 10A9 and 7F2 from haptens with too short or long spacer arms usually formed shallow or deep binding cavities and induced steric hindrance, which is not suitable for the sandwich immunoassay of small molecules. The study provides
significant reference value for the selection of paired mAbs that may be useful to create sandwich immunoassays with less steric hindrance.

It is worth noting that chain-like model analytes were selected herein to more intuitively investigate the distance limit of two epitopes in sandwich immunoassays. Thus, the conclusions obtained from our study may not be fully applicable to every kind of small molecule for establishing successful sandwich immunoassays and will be further evaluated using a real analyte at the end of the study.

Validation of sandwich immunoassays for model analytes by surface plasmon resonance (SPR)

SPR analysis was further used to validate the specific binding of antibody–analyte–antibody ternary structures formed in the sandwich immunoassays based on the 7A3–17B3 pair. First, mAb 7A3 was immobilized on a chip, and then, each model analyte was added at 10 µM, except for MN1 that was added at 100 µM. It should be stated that MN3 and MN8 could not be analyzed by SPR due to their poor solubility in PBS. All tested model analytes demonstrated increased signals at step I, indicating successful binding between 7A3 and the model analytes (Fig. 5a). Different degrees of dissociation were then observed over time in step II as the 7A3–analyte complex gradually became stable. Afterward, mAb 17B3 was added to bind the 7A3–analyte complex, and the signal was expected to increase further. The signal still increased significantly during step III, including for the smallest analyte MN1. To confirm whether the increased signal was specific, we set up a control to verify the binding
between the two mAbs without adding any model analyte but PBS. The PBS signal was
maintained at the baseline through the analytical procedure, indicating that no specific
binding had occurred between the two mAbs and proving that the increased signal
observed in step III was caused by the binding of 17B3 to the 7A3–analyte complex
(Fig. 5a). For MN1, MN2, and MN4, the signals increased slightly by 10–20 units after
the addition of 17B3, while those for MN6, MN7, and MN9 increased more noticeably
by 20–40 units, which is consistent with the performance of the model analytes in the
established sandwich immunoassay. Furthermore, MN7, achieving the highest
sensitivity in the established sandwich immunoassay, was analyzed by SPR at
concentrations of 1–10 μM. The 7A3–MN7 signal gradually increased in step I,
followed by varying degrees of dissociation as the MN7 concentration increased during
step II (Fig. 5b). After the addition of 17B3 in step III, the elevated signal of the 7A3–
analyte–17B3 ternary complex was observed, again indicating the formation of a stable
antibody–analyte–antibody ternary complex.

Validation of sandwich immunoassay for small molecules by new analytes

To validate the epitope distance required by establishing a successful sandwich
immunoassay for small molecules, we designed and synthesized two new model
analytes using typical antibacterial agents, SBA and NOR, that are widely used in
human and veterinary clinics. The synthesized analytes of SBA–C2–NOR and SBA–
C10–NOR are composed of SBA and NOR, separated by epitope distances of 3.8 and
13.9 Å, respectively (Fig. 5c). The mAbs against SBA and NOR, i.e., 4D11 and
N2H3A8, were previously prepared by our group and used to establish sandwich immunoassays. Both model analytes worked successfully in the established sandwich immunoassays, with LODs of 0.30 and 0.02 nM for SBA–C2–NOR and SBA–C10–NOR, respectively (Fig. 5d). The results showed that successful sandwich immunoassays could be achieved for small molecules even with short epitope distances above 2.4 Å. The effect of epitope distance of analyte on the sensitivity of the sandwich immunoassay was also further observed. The LOD of the sandwich immunoassay for SBA–C10–NOR with the suggested epitope distance of 13.9 Å was more than 10-fold that of SBA–C2–NOR with a short epitope distance of 3.8 Å (Fig. 5d and Supplementary Table 9). Moreover, the sandwich immunoassays provide clear advantages in sensitivity, detection range, and specificity compared to competitive immunoassays for new model analytes, especially for SBA–C10–NOR, where the LOD improved nearly 30-fold (Supplementary Table 8). For assay specificity, the sandwich immunoassays showed extremely high specificities toward the target analyte due to the simultaneous use of two mAbs (Supplementary Table 10), while competitive immunoassays provided structural analogs with high cross-reactivities, such as sulfaguanidine and ciprofloxacin, with the potential risk of false positives.

We further verified sandwich immunoassays for small molecules using a circle-like real analyte of ABM, a macrocyclic lactone insecticide that is widely used in agriculture for pest control. The haptens ABM–A (prepared previously by our group) and ABM–B were designed for the preparation of mAbs by introducing spacer arms at the 4′–OH and 5′–OH of ABM, respectively, to couple with KLH (Fig. 5e,
Supplementary Fig. 7, and Supplementary Information). After the extensive screening and pairing of mAbs, 3A9 from hapten ABM–A and 2C5 from hapten ABM–B were combined to establish a sandwich immunoassay for ABM, affording a LOD of 1012.1 nM (Fig. 5f). The low sensitivity of the sandwich immunoassay for ABM was possibly due to steric hindrance caused by the overlap of the antibody binding sites, reducing the binding ability of each mAb to the ABM. The results of ABM demonstrated the need for careful hapten design and extensive mAb screening to establish highly sensitive sandwich immunoassays. It is worth noting that the study had not always witnessed the dominant performance of sandwich immunoassays for the detection of small molecules, which is heavily dependent on the structure of the target analyte and optimum antibody pairs. These results using a real analyte proved that small molecules with certain epitope distances can be detected by sandwich immunoassay.

Conclusion

Small molecules are generally not thought to be assayed by direct sandwich immunoassay, because they cannot be simultaneously bound by two antibodies. In the present study, we demonstrated that small molecules consisting of two epitopes separated by as little as 2.4 Å could be detected by sandwich immunoassay. Through experimental and theoretical study, the success of a sandwich immunoassay was found to be highly probable for two epitopes with an analyte distance above 8.8 Å. These results confirmed the feasible formation of ternary complexes by the association of two mAbs with a very short epitope distance. Notably, the high flexibility of extra-long
epitope distances may decrease the stability of these antibody–analyte–antibody complexes, resulting in the low sensitivity of the sandwich immunoassay. Besides, antibodies with deep or shallow binding cavities induced by haptens with extra-long or short spacer arms are detrimental to the validation of sandwich immunoassays for small molecules. We believe that the data presented here can be of general interest in the field of immunoassays, providing a reliable reference for the successful establishment of sandwich immunoassays for small molecules with improved sensitivity, detection range, and specificity.
Table 1 Comparison of sandwich immunoassays and competitive immunoassays for model analytes of LODs.

<table>
<thead>
<tr>
<th>Model analytes</th>
<th>Competitive(^a)</th>
<th>Competitive(^b)</th>
<th>Sandwich(^c)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>7A3</td>
<td>17B3</td>
<td>7A3+17B3</td>
</tr>
<tr>
<td>MN1</td>
<td>0.2</td>
<td>0.6</td>
<td>29944</td>
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<tr>
<td>MN2</td>
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<td>19202</td>
</tr>
<tr>
<td>MN4</td>
<td>0.7</td>
<td>0.02</td>
<td>15335</td>
</tr>
<tr>
<td>MN5</td>
<td>0.08</td>
<td>0.03</td>
<td>265.4</td>
</tr>
<tr>
<td>MN6</td>
<td>0.004</td>
<td>0.6</td>
<td>0.002</td>
</tr>
<tr>
<td>MN7</td>
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<td>0.7</td>
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</tr>
<tr>
<td>MN8</td>
<td>0.4</td>
<td>1.7</td>
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<tr>
<td>MN9</td>
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<td>33.7</td>
<td>0.9</td>
</tr>
<tr>
<td>MN3 analog(^d)</td>
<td>0.6</td>
<td>0.3</td>
<td>/(^e)</td>
</tr>
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</table>

\(^a\) Competitive immunoassay for model analytes by using mAbs 7A3. \(^b\) Competitive immunoassay for model analytes by using mAbs 17B3. \(^c\) Sandwich immunoassay for model analytes by using mAbs 7A3 and 17B3. \(^d\) The structure of MN3 analog is \[\text{structure image}\]. \(^e\) Negative detection results of MN3 analog was obtained in sandwich immunoassay.
**Fig. 1.** The chemical structures of model analytes and haptens. (a) Schematic of 9 model analytes consisting of MEL and NIA (Top) by varied epitope distance from 2.4 to 19.0 Å (Bottom). (b) The chemical structures of MEL haptens with varied spacer arm lengths. Hapten M1 (n=1), hapten M2 (n=3), hapten M3 (n=5), hapten M4 (n=7), hapten M5 (n=9), hapten M6 (n=11). (c) The chemical structures of NIA haptens with varied spacer arm lengths. Hapten N1 (n=0), hapten N2 (n=2), hapten N3 (n=4), hapten N4 (n=6), hapten N5 (n=8), hapten N6 (n=10).
**Fig. 2.** Combinatorial associations of mAbs in established sandwich immunoassays for MN1–MN9. (a) Schematic of the designed sandwich immunoassay (left) and OD values for the color boundary (right). (b) OD values obtained in established sandwich immunoassays for MN1–MN9 based on mAb pairings. The MEL mAbs listed in the columns from left to right and the NIA mAbs listed in the rows from top to bottom were arranged according to their hapten spacer arm length from shortest to longest. All experiments were conducted in triplicate (n = 3). (c) Percentages of mAb pairings that allow for sandwich immunoassays as a function of epitope distance.
**Fig. 3.** Schematic diagrams of the (a) competitive immunoassay for the model analyte based on mAbs against MEL (left) or NIA (right) and the (b) sandwich immunoassay based on two distinct mAbs for the model analyte. The sandwich immunoassays were performed using anti-MEL mAbs as the capture antibody for MN1–MN9 to ensure maximum efficiency with consideration of higher affinity. (c) Calibration curves of the competitive immunoassays for MEL, MN1–MN9, and the analog based on mAb 7A3 (n = 3). (d) Calibration curves of the competitive immunoassays for NIA, MN1–MN9, and the analog based on mAb 17B3 (n = 3). (e) Calibration curves of the sandwich immunoassays for MN1–MN9, MEL, NIA, and the analog using mAb 7A3 as the capture antibody and mAb 17B3 as the detecting antibody (n = 3).
**Fig. 4.** Molecular recognition mechanism in sandwich immunoassays explored by molecular docking and molecular dynamics. (a) After antibody–antibody docking, a shared binding cavity (represented as a white surface) forms between 7A3 and 17B3 based on the initial conformation of single-antibody docking with MEL or NIA (left). Overlap diagram of MN1, MN5, MN7, and MN9 in the shared binding cavity composed of 7A3 and 17B3. MN5 can be contained in the shared binding cavity, while MN1–MN4 are too short to enter the binding cavities of both mAbs simultaneously (right). Interactions of ternary complexes (b) 7A3–MN5–17B3, (c) 7A3–MN7–17B3, (d) 7A3–MN9–17B3, (e) 7A3–MN7–10A9, and (f) 7A3–MN7–7F2; the capture (left) and detecting (right) antibodies are respectively labeled in orange and blue.
**Fig. 5.** Validation of sandwich immunoassays by SPR and new analytes. (a) SPR analyses of 7A3–analyte–17B3 ternary structures using 10 µM of each analyte, except for MN1 (100 µM). Step I represents the binding process of analytes toward 7A3 that is immobilized on the chip, step II represents the dissociation and stabilization process of the analytes binding to 7A3, and step III represents the binding process of 17B3 and the binary complexes of 7A3 and analytes. (b) SPR analysis of 7A3–MN7–17B3 with various concentrations of MN7 (1–10 µM). (c) SBA–C2–NOR and SBA–C10–NOR structures; the SBA and NOR moieties are labeled in green and pink, respectively, and the linker is labeled in black. (d) Calibration curves of the SBA–C2–NOR and SBA–C10–NOR sandwich immunoassays for 4D11 and N2H3A8 (n = 3). (e) ABM structure; the hydroxyl groups at the 4’ and 5’ positions are labeled in cyan and purple,
respectively. (f) Calibration curves of the sandwich immunoassays for ABM by 3A9 and 2C5 (n = 3).
Graphical Abstract
Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.

Data availability
The data supporting the findings of this study are available within the paper and its Supplementary Information.

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Author contributions
B. Y. conceived and designed the overall project with W. Z. and S. J. providing guidance. F. J. and W.W. designed and performed the screening of mAbs. L. M. and S. S. carried out the computational studies. B. Y., D. L., Y. W., and W.K. wrote the manuscript with the input of all authors.

Competing interests
The authors declare no competing financial interest.
Additional information

Supplementary information The online version contains supplementary material available at

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Institutional Review Board Statement: The laboratory animals in our experiments were used in accordance with the relevant Chinese laws and according to the China Agriculture University regulations concerning protection of animals used for scientific purposes (2010-SYXK-0037). The use of mice in this study was approved by the Ethics Committee on Experimental Animals and Animal Tests of China Agricultural University under review number AW82701202-2-2.
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