Supplementary Materials

A Bispecific Nanobody with High Sensitivity/Efficiency for Simultaneous Determination of Carbaryl and its Metabolite 1-Naphthol in the Soil and Rice Samples

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1. Materials and Instruments

Carbaryl and its metabolites, 1-naphthol, were obtained from Energy Chemical Co. Ltd. (Shanghai, China). The high-specificity carbaryl-VHH plasmid without any crossover rate was kindly presented by China Agricultural University (He at al., 2019). The high-specificity 1-naphthol-VHH plasmid without any crossover rate was prepared in the early experiment of our laboratory (Chen at al., 2022). Bovine serum albumin (BSA) was purchased from Merck Co. Ltd. (Shanghai, China). The *E. coli* DH5a and *E. coli* BL21 (DE3) was purchased from TransGen Biotech Co. Ltd. (Beijing, China). The *Sfi*I restriction enzymes and T_4 DNA ligase were supplied by Thermo Fisher (Shanghai, China). 3,3',5,5'-tetramethylbenzidine (TMB) was supplied by Aladdin Chemical Technology Co., Ltd. (Shanghai, China). The DNA double strands and recombinant plasmid were amplified and constructed using a PCR instrument (TOne 96G, Biometra GmbH, Germany). The results of amplification were verified by a Nucleic acid electrophoresis apparatus (JY600C, Beijing). High-speed centrifugation (SORVALL LYNX 4000) and ultraviolet-visible spectrum (NanoDrop 2000C) were purchased from Thermo Fisher (Shanghai, China). The ELISA plates were washed in a WellwashTM microplate washer (HBS-4009, DeTie, Nanjing, China).

2. Expression and purification of BsNb

To express the target protein, we used chemically competent cells BL21 (DE3) that were transformed with the recombinant plasmid. The transformation was achieved through a heat shock at 42 °C for 90 seconds, followed by overnight incubation on LB plates at 37 °C. Subsequently, we selected positive clones for sequence identification. The clone with the correct

sequence was then induced to express the protein. It was cultured in LB medium supplemented with 100 μg/mL ampicillin at 37 °C until reaching an OD_{600} value of approximately 0.4-0.8. At this point, we added 1 mmol/L of IPTG to initiate protein expression. The bacterial culture was incubated overnight at 37 °C with shaking at 250 rpm. To harvest the protein, the bacteria were pelleted by centrifugation at 13,523 g for 20 minutes at 4 °C. The fusion protein was extracted from the cell periplasmic fraction using the cold osmotic shock method. Subsequently, purification was performed using Ni-NTA resin. The purification process involved eluting different impurities with PBS (40 mL), followed by elution with 10 mM imidazole (40 mL) and 20 mM imidazole (20 mL), respectively. The target protein was then obtained by eluting with 200 mmol/L imidazole in 0.01 mol/L PBS. After purification, the protein was dialyzed against 0.01 mol/L PBS and stored at -20 °C for further use.

3. Development of anti-carbaryl and anti-1-naphthol ELISA

To prepare the enzyme plate for the assay, the coating hapten carbaryl-BSA/1-naphthol-BSA was diluted with coating fluid to a concentration of 1 μg/mL. A volume of 100 μL per well was added to the enzyme plate, followed by incubation at 37 °C for 12 hours. The plate was then covered with blocking fluid and incubated at 37 °C for an additional 3 hours. After drying at 37 °C for 1 hour, the plate was ready for use.

The first step involved testing the antigen binding activity of C-N-VHH and N-C-VHH. BsNbs, including C-N-VHH and N-C-VHH, were prepared at a range of concentrations using a two-fold dilution method. The initial concentration of BsNbs was 1 μg/mL. Following the addition of BsNbs to the plate and a 30-minute incubation at 37 °C, 100 μL of rabbit anti-VHH

[HRP] (diluted at 1:5000) was added and incubated for another 30 minutes at 37 °C. Subsequently, 100 μL of TMB substrate was added to the plate, and the culture was placed in a constant temperature water bath at 37 °C for 10 minutes. The reaction was stopped by adding 50 μL of stop solution (10% H_2SO_4) per well. The absorbance values at A450 nm were then measured using an enzyme-labeled Analyzer.

For the ic-ELISA, the initial concentrations of carbaryl and 1-naphthol were set at 10 μg/mL, and the procedure was identical to the one described above. Following the assay, calibration curves were fitted using Origin 2022 software, with A450 nm as the ordinate and the logarithm of the drug concentration as the abscissa. Finally, the obtained IC_{50} values of BsNbs were compared.

4. Sample preparation and recovery test

For soil sample preparation, the impurities of 10.0 g soil in farmland were removed and sieved through 100-mesh screen. Rice sample (20.0 g) was prepared through crushing and ultra-centrifugating. Then all samples were shaken (soil sample: 260 rpm, 25 °C, 30 min by shaking table; rice sample: 2 min each time, 3 times by homogenate machine), extracted by 80 mL of 70% methanol (water to extract carbaryl and methanol to extract 1-naphthol) and filtered with a 0.45 μm filter paper. After fully air (carbaryl)/nitrogen (1-naphthol) drying, corresponding blank samples matrix solution were obtained. Subsequently, the sample matrix influence was detected by diluting the solutions with 0.01M PBS solution at various ratios (5, 10, 20, 40 times). For the recovery test, different concentrations of carbaryl and 1-naphthol (0.02 mg/kg, 0.10 mg/kg, 0.50 mg/kg, 2.50 mg/kg) were added to soil or rice samples. After thorough mixing and shaking, the

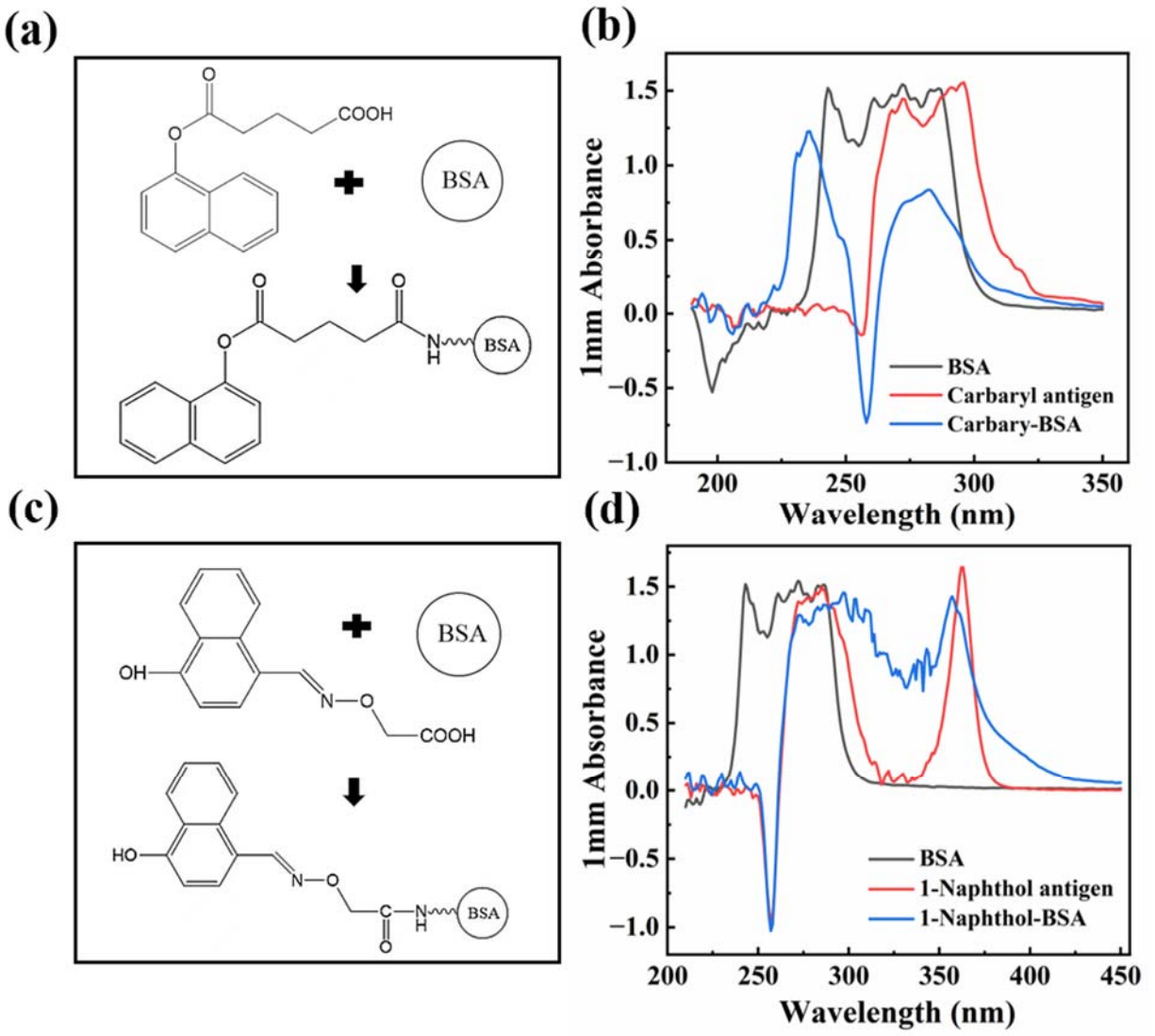
sample solutions were subjected to the sample pretreatment method specified for testing. Detection was then performed to analyze the samples.

To verify the accuracy and reliability of the proposed ELISA method, LC-MS/MS (Liquid Chromatography-Tandem Mass Spectrometry) was employed as a reference method. The chromatography process was conducted according to the specific conditions outlined in Table S2, adhering to the Chinese national standards.

References

He, J., Tao, X., Wang, K., Ding, G., Li, J., Li, Q., Gee, S.J., Hammock, B.D., Xu, T. 2019. One-step immunoassay for the insecticide carbaryl using a chicken single-chain variable fragment (scFv) fused to alkaline phosphatase. Anal. Biochem. 572, 9-15. https://doi.org/10.1016/j.ab.2019.02.022.

Chen, Z., Wu, H., Shen, Y., Wang, H., Zhang, Y., Hammock, B.D., Li, Z., Luo, L., Lei, H., Xu, Z. 2022. Phosphate-triggered ratiometric fluoroimmunoassay based on nanobody-alkaline phosphatase fusion for sensitive detection of 1-naphthol for the exposure assessment of pesticide carbaryl. J. Hazard. Mater. 424(Pt C), 127411. https://doi.org/10.1016/j.jhazmat.2021.127411.



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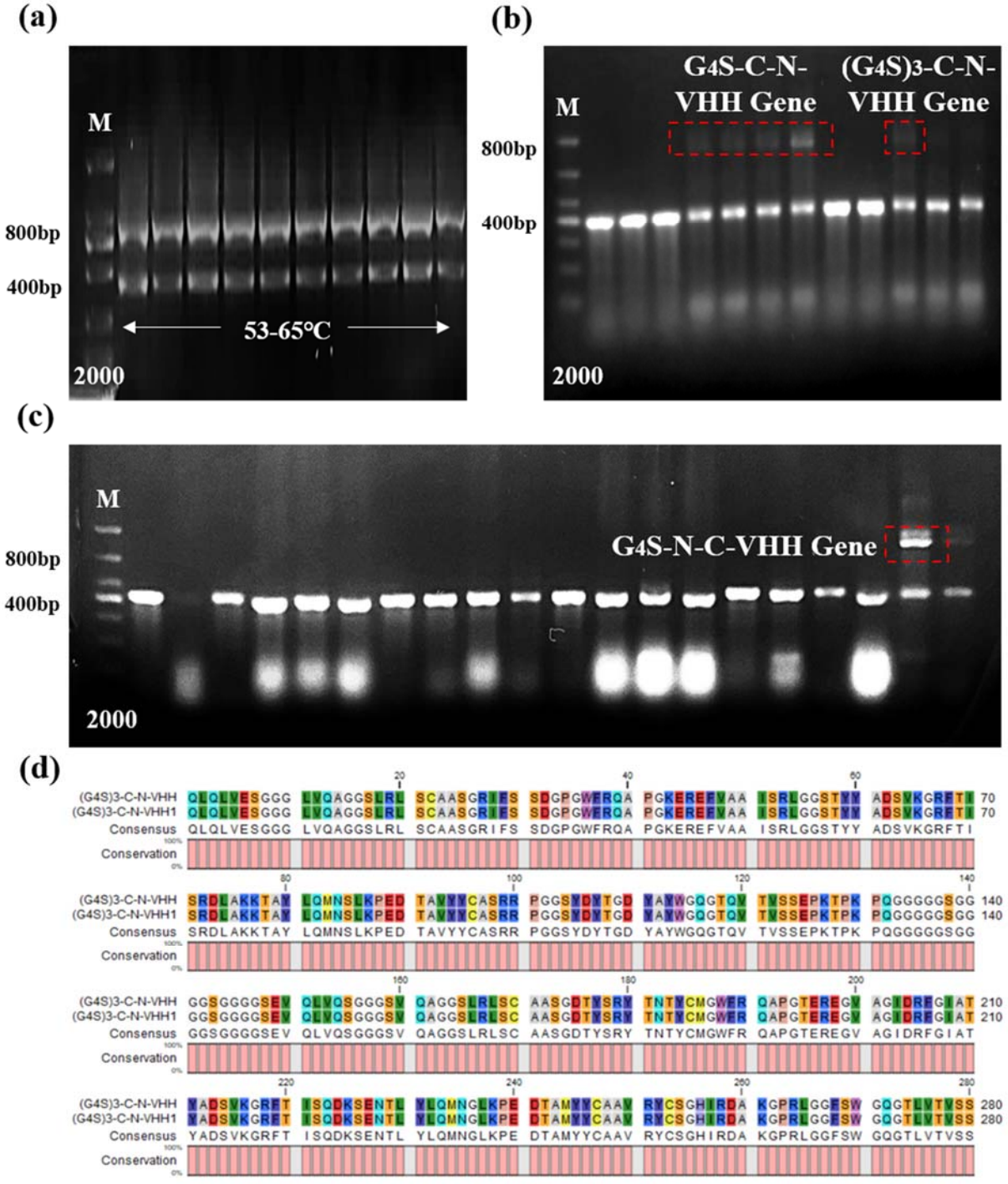
102 **Figure S1.** Coating antigen of carbaryl and 1-napthol. (a) Antigen structure of carbaryl and

103 coating hapten; (b) The UV wavelength of carbaryl antigen and coating hapten; (c)1-naphthol

104 complete antigen and coating hapten; (d) The UV wavelength of 1-naphthol antigen and coating

105 hapten.



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107 **Figure S2.** Verification of target vector construction. (a) The verification results of the PCR

108 reaction at different annealing temperatures; (b) Colony PCR nucleic acid electrophoresis

109 verification results (C-N-VHH); (c) Colony PCR nucleic acid electrophoresis verification results

110 (N-C-VHH); (d) Sequencing results of $(G_4S)_3$-C-N-VHH.

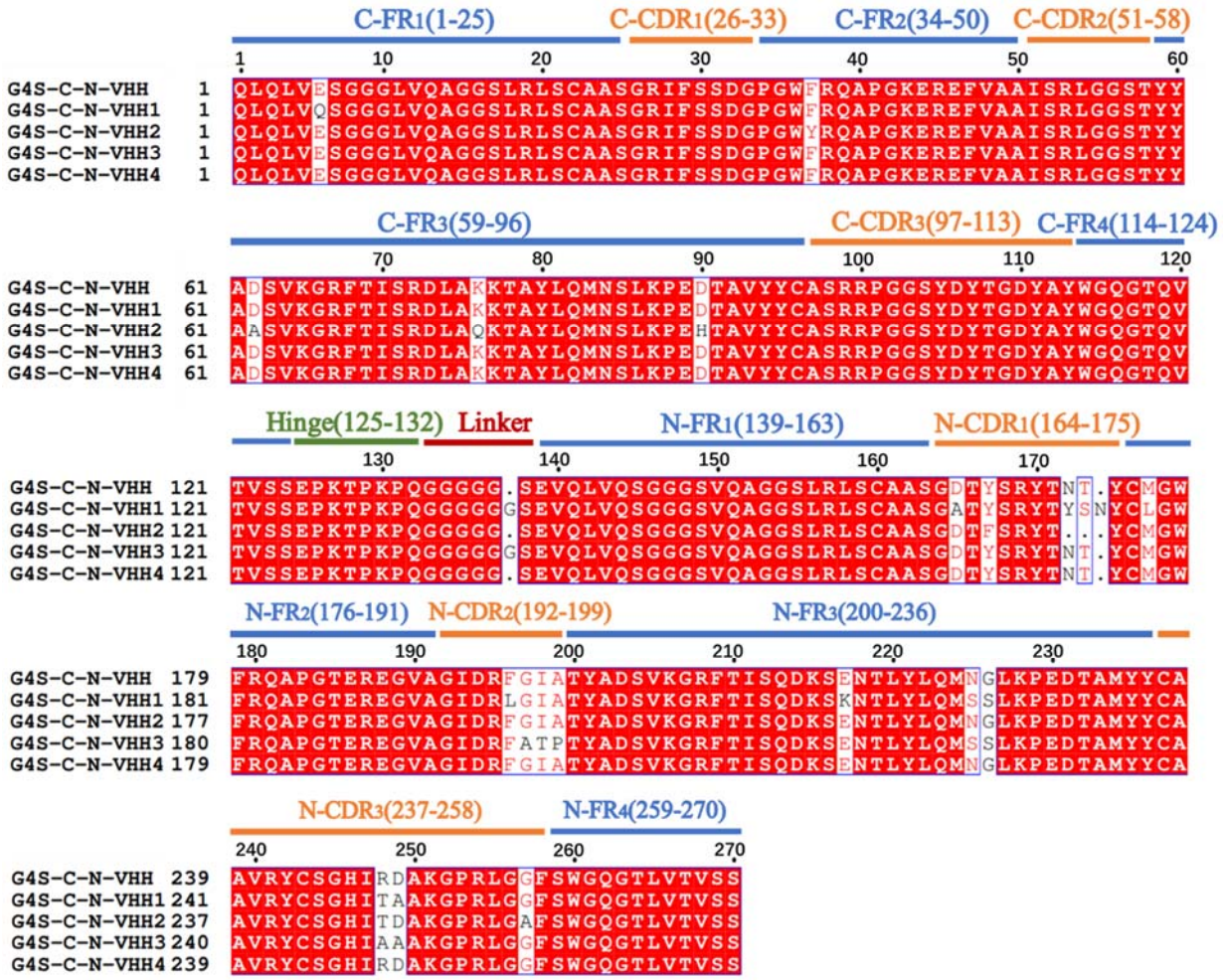
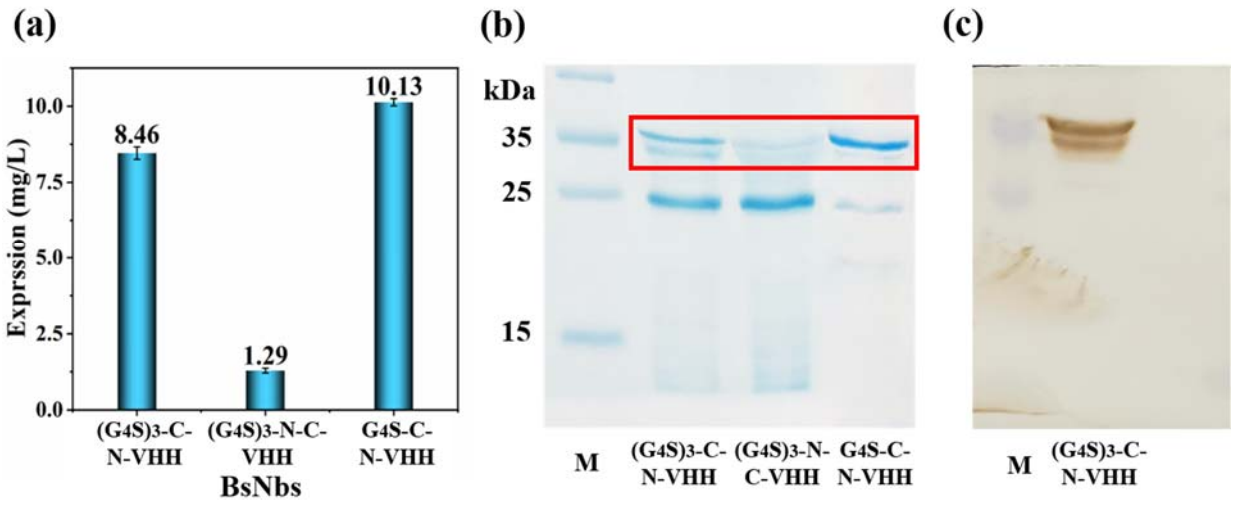


Figure S3. Sequence results of BsNbs (G4S-C-N-VHH1~4).



113

114 **Figure S4.** The results of expression, SDS-PAGE and Western blot of BsNb. (a) Expression

115 amount of BsNbs; (b) SDS-PAGE; (c) Western blot of $(G_4S)_3$-C-N-VHH.

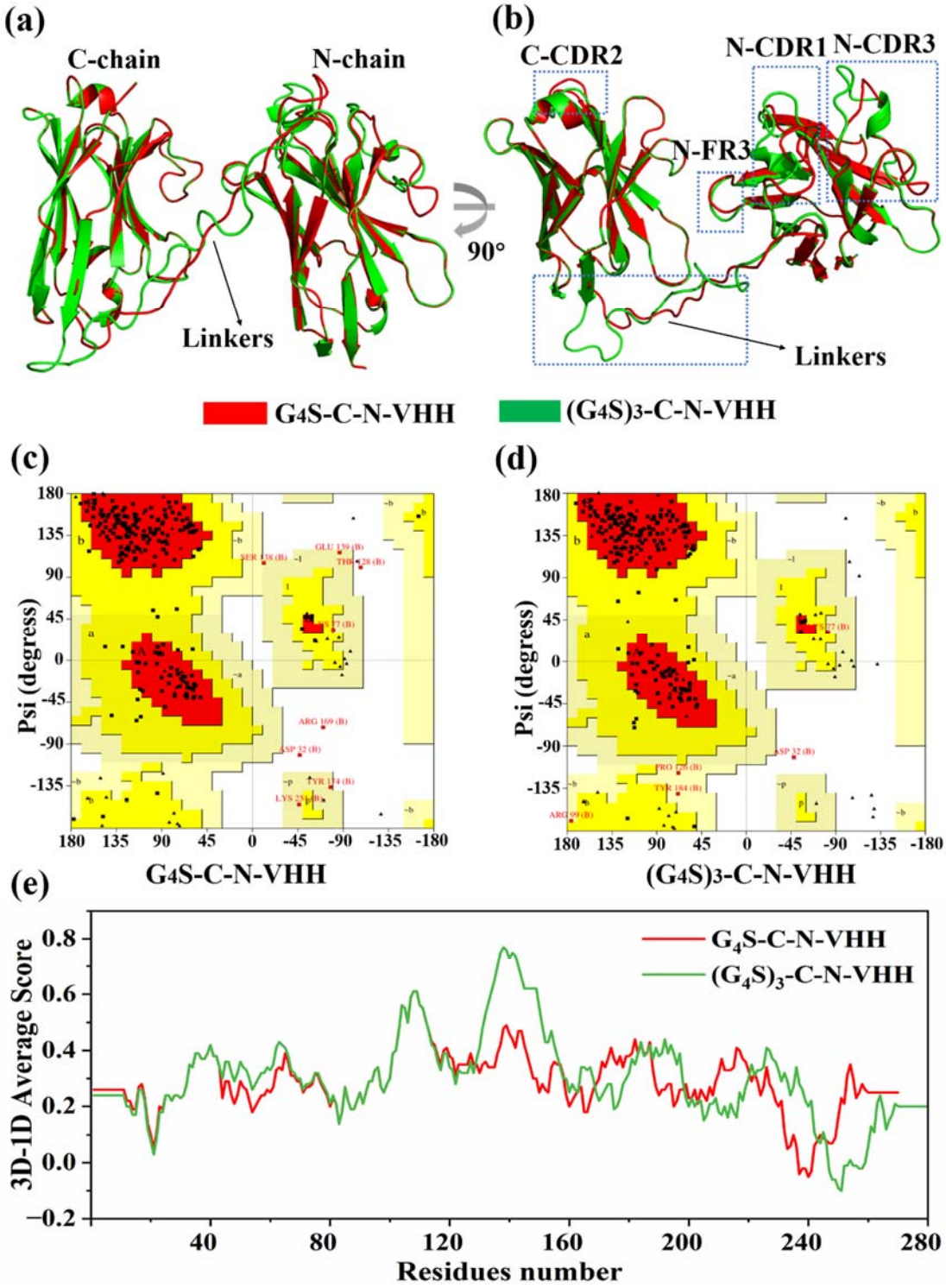


Figure S5. 3-D modeling and model evaluation of G_4S-C-N-VHH and $(G_4S)_3$-C-N-VHH. (a-b) The 3-D structure of G_4S-C-N-VHH and $(G_4S)_3$-C-N-VHH; (c-d) Ramachandran map of G_4S-C-N-VHH and $(G_4S)_3$-C-N-VHH; (e) The 3D–1D averaged score of G_4S-C-N-VHH and $(G_4S)_3$-C-N-VHH.

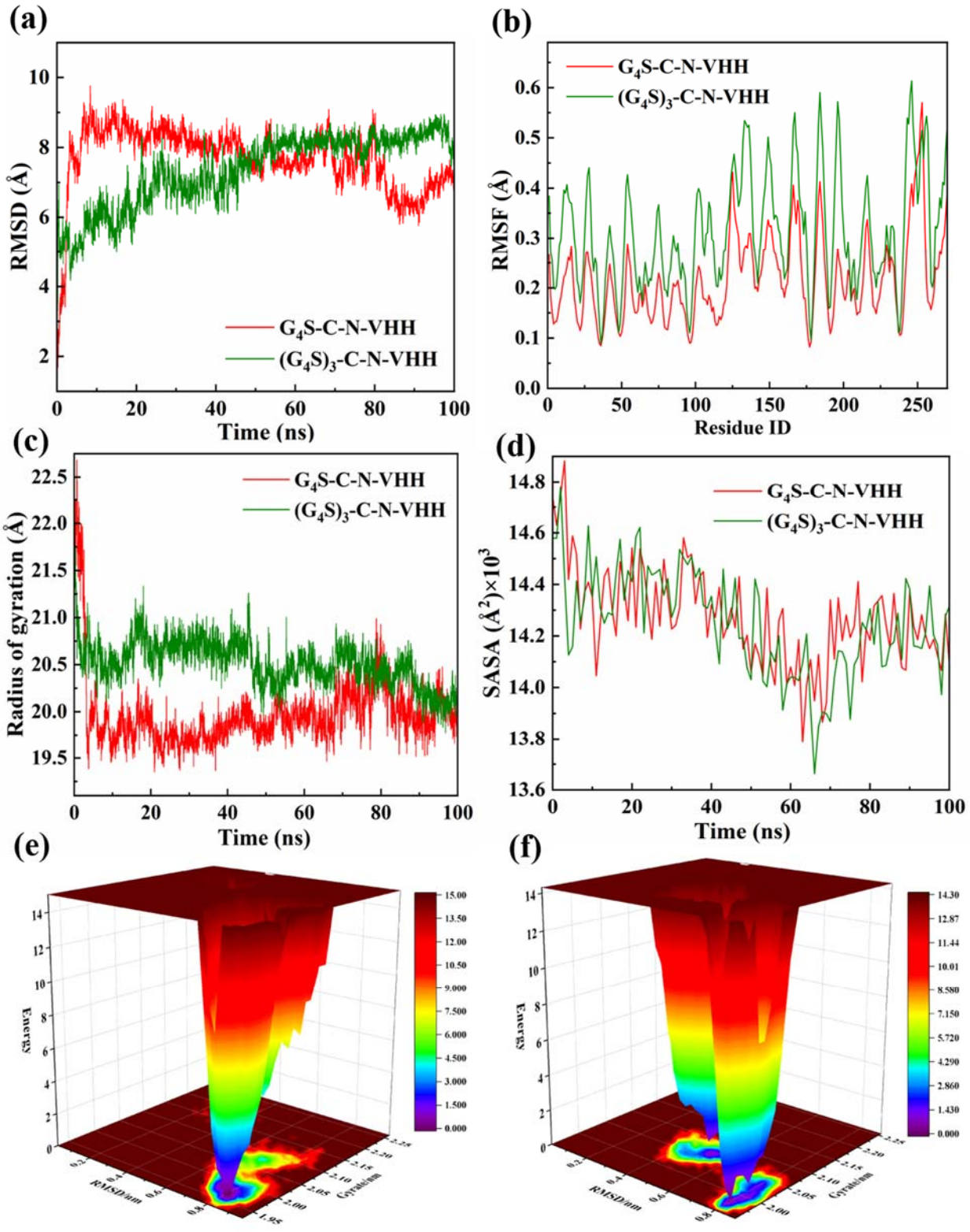


Figure S6. Results of molecular dynamics. (a) Root-mean-square deviation (RMSD); (b) Root-mean-square fluctuation (RMSF); (c) Radius of gyration; (d) Solvent accessible surface area (SASA) in the simulation process; (e-f) The Free Energy Landscapes.

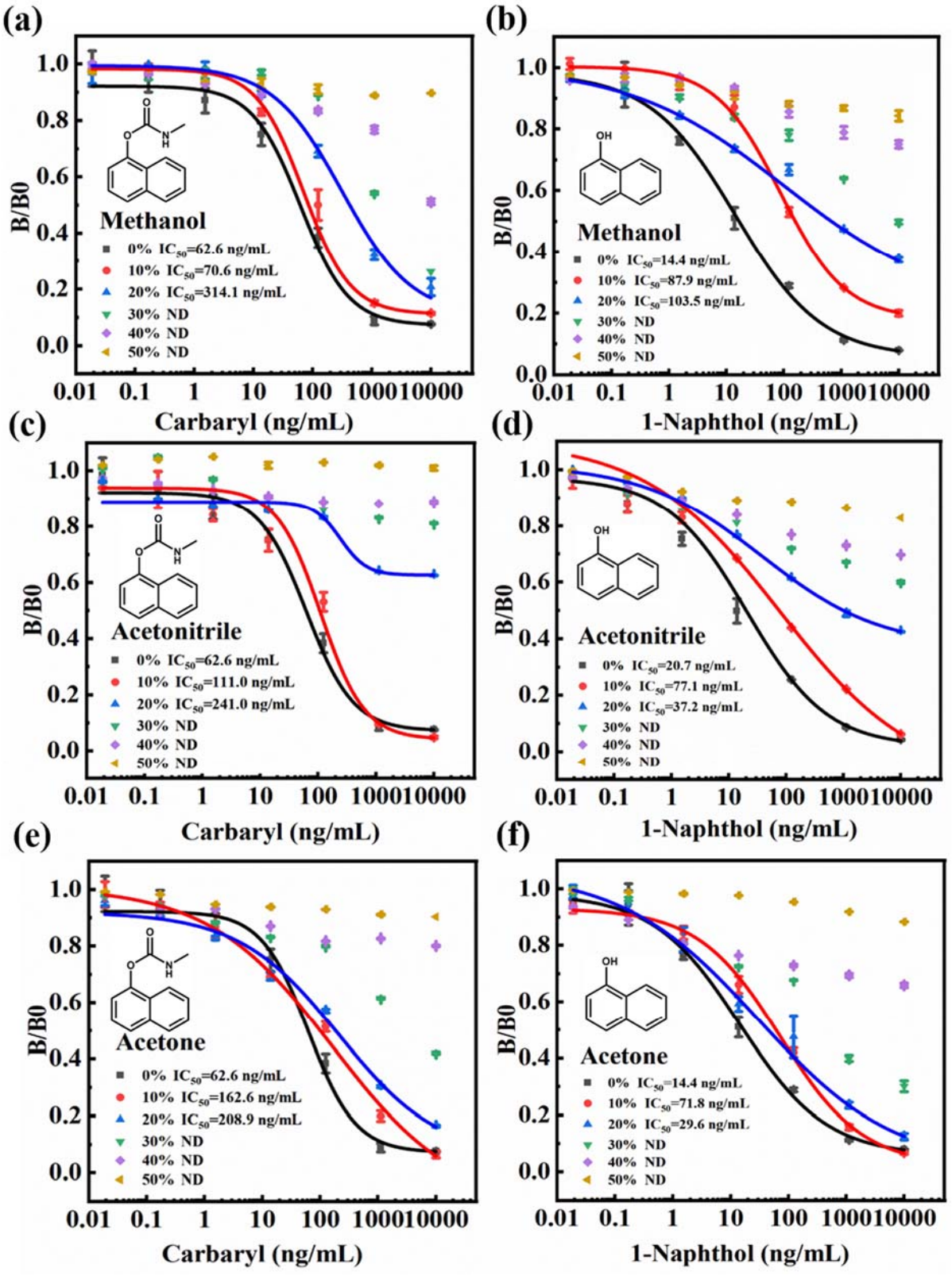
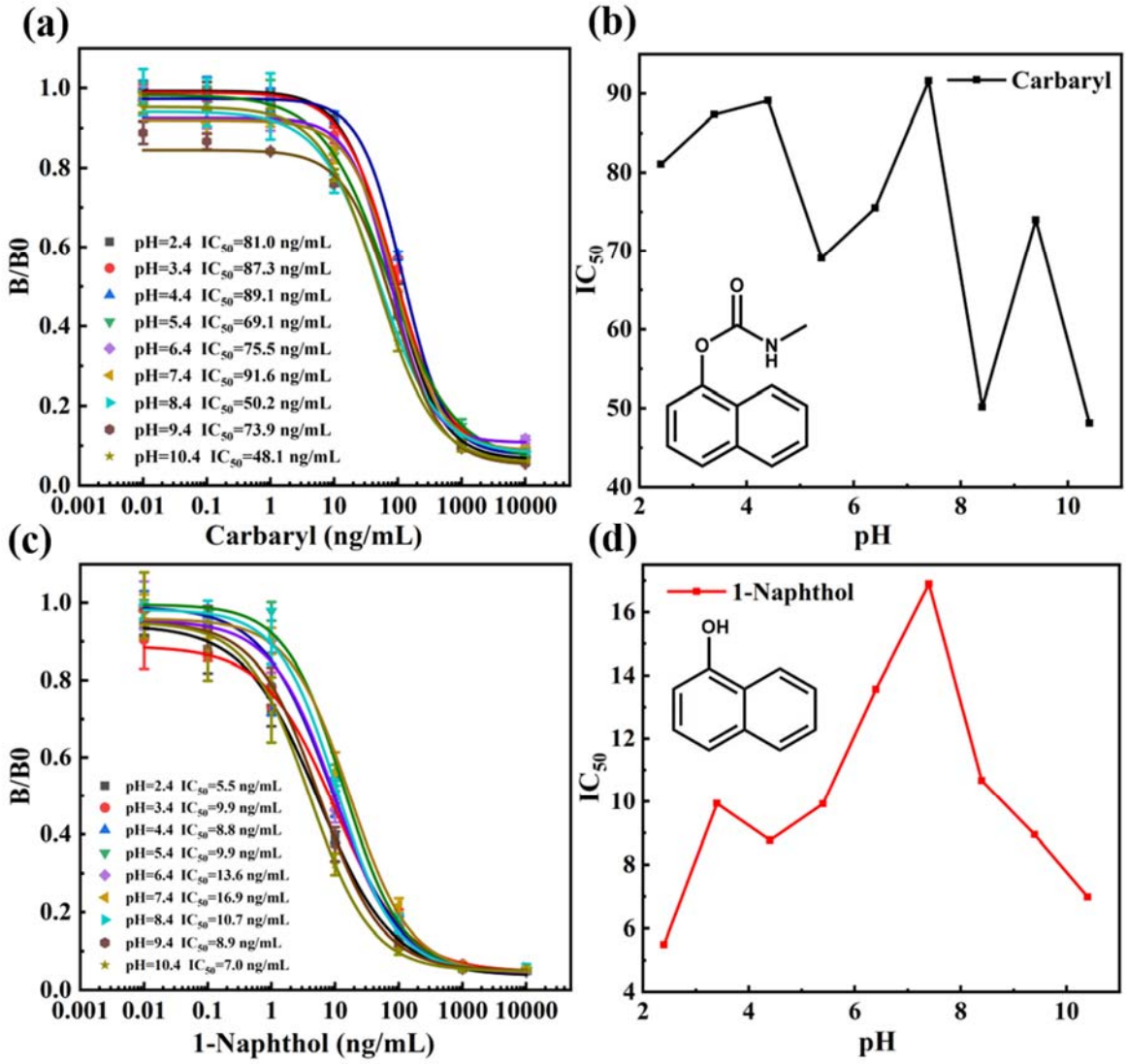


Figure S7. Effects of different organic reagents on BsNb characteristics. (a, c, e) Different concentrations of methanol/acetonitrile/acetone to carbaryl; (b, d, f) Different concentrations of methanol/acetonitrile/acetone to 1-naphthol.



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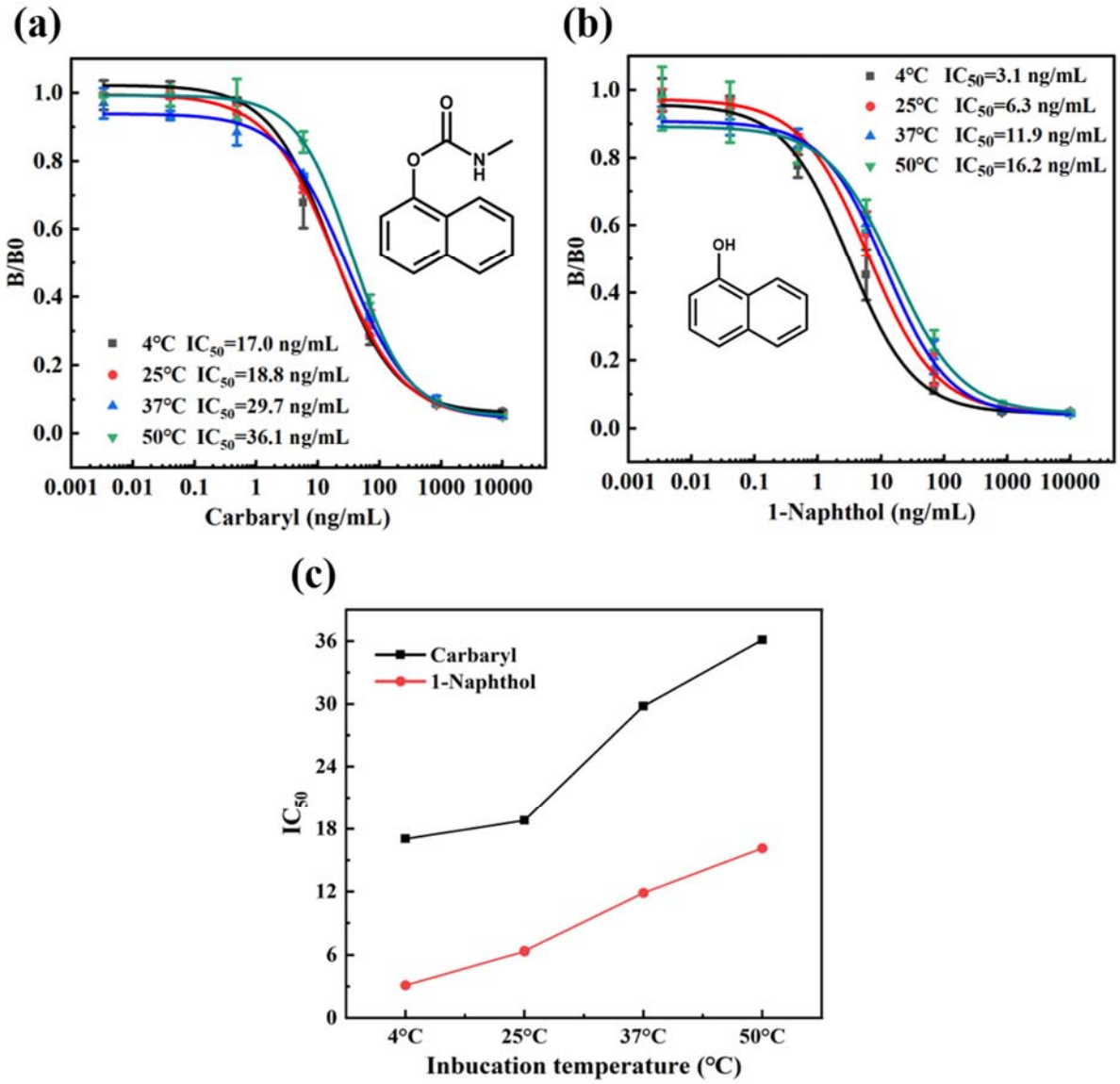
130 **Figure S8.** Effects of different pH values to carbaryl (a-b) and to 1-naphthol (c-d).

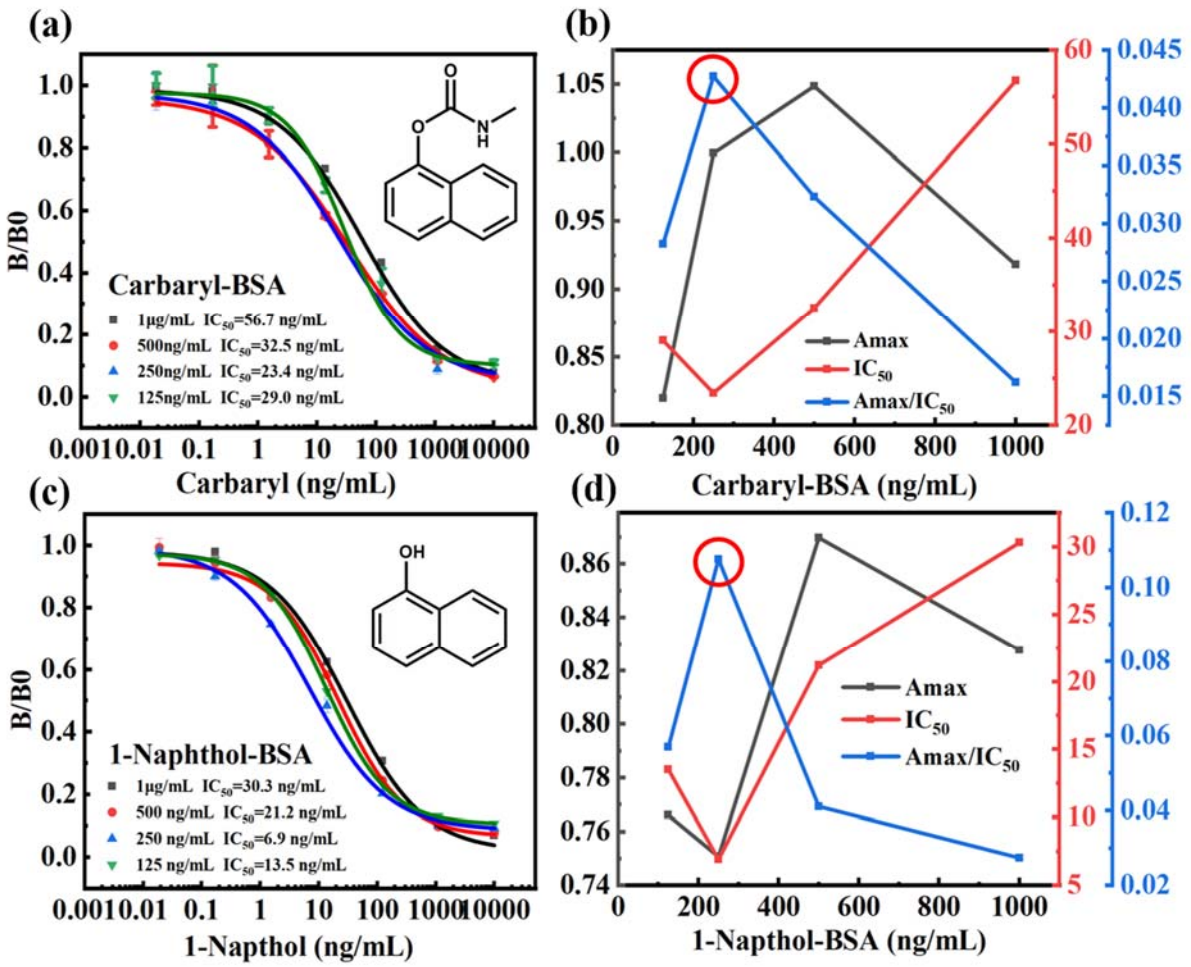


131

132 **Figure S9.** Effect of incubated temperature on the sensitivity of BsNb via ELISA. (a) Effect on

133 BsNb to carbaryl; (b) Effect on BsNb to 1-naphthol; (c) Variation of IC_{50} at different incubation

134 temperatures.



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136 **Figure S10.** Effects of different concentrations of coating hapten to BsNb against carbaryl (a-b)

137 and1-naphthol (c-d).

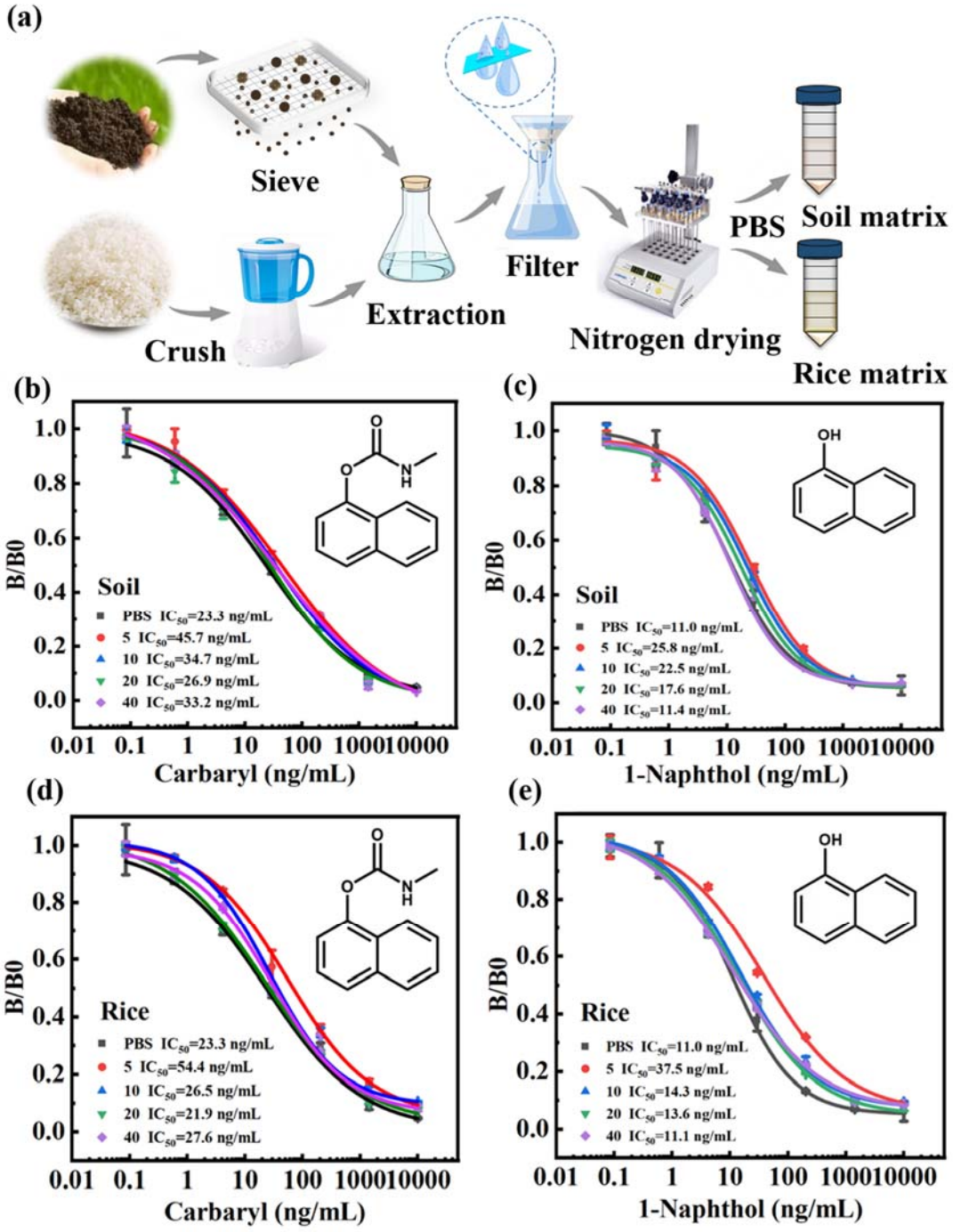

Figure S11. Calibration curves of ic-ELISA based on buffers and blank sample extract with several-fold dilution buffer solutions. (a) The procedures of samples preparation; (b-c) matrix effect with soil extraction at 1:5, 1:10, 1:20 and 1:40 dilutions for carbaryl and 1-naphthol analysis; (d-e) matrix effect with rice extraction at 1:5, 1:10, 1:20 and 1:40 dilutions for carbaryl and 1-naphthol analysis.

Table S1. Forward (F)/ Reverse (R) primer sequences of (G_4S)n-C-N-VHH and $(G_4S)_3$-N-C-VHH.

BsNbs	**Primer**	**Base Sequence**
C-N-VHH	C-N-F1	CCGTGGCCCAGGCGGCCCAGTTGCAGCTCGTGGAG
	C-N-R1	CCCAGACTGCACCAGCTGCACCTCGCCTTGTGGTTTTGGTGT
	C-N-F2	CCCAAGACACCAAAACCACAAGGCGAGGTGCAGCTGGTGCAG
	G_4S-C-N-R1	AGAGCCGCCGCCACCGCCTTGTGGTTTTGGTGTCTT
	G_4S-C-N-F2	GGTGGCGGCGGCTCTGAGGTGCAGCTGGTGCAGTCTGGGGGAGGCTCGGTG
	$(G_4S)_2$-C-N-R1	GCTTCCGCCTCCTCCAGAGCCACCACCACCGCCTTGTGGTTTTGG
	$(G_4S)_2$-C-N-F2	GGAGGAGGCGGAAGCGAGGTGCAGCTGGTGCAGTCTGGGGGAGGCTCGGTGCAG
	$(G_4S)_3$-C-N-R1	GCTTCCGCCTCCTCCAGAGCCACCACCACCGCCTTGTGGTTTTGGTGT
	$(G_4S)_3$-C-N-F2	GGAGGAGGCGGAAGCGGTGGCGGCGGCTCTGAGGTGCAGCTGGTGCAG
	$(G_4S)_4$-C-N-R1	AGAGCCGCCGCCACCGCTTCCGCCTCCTCCAGAGCCACCACCACCGCCTTGTGGTTTTGG
	$(G_4S)_4$-C-N-F2	GGTGGCGGCGGCTCTGGTGGCGGCGGCTCTGAGGTGCAGCTGGTGCAGTCTGGGGGAGGC TCGGTGCAG
	C-N-R2	CTGGCCGGCCTGGCCTGAGGAGACGGTGACCAG
N-C-VHH	$(G_4S)_3$-N-C-F1	CCGTGGCCCAGGCGGCCGAGGTGCAGCTGGTGCAG
	$(G_4S)_3$-N-C-R1	AGAGCCGCCGCCACCTGAGGAGACGGTGACCAG
	$(G_4S)_3$-N-C-F2	GGTGGCGGCGGCTCTCAGTTGCAGCTCGTGGAG
	$(G_4S)_3$-N-C-R2	CTGGCCGGCCTGGCCGCCTTGTGGTTTTGGTG

Linker：G_4S—GGTGGCGGCGGCTCT;

$(G_4S)_2$—GGTGGTGGTGGCTCTGGAGGAGGCGGAAGC;

$(G_4S)_3$—GGTGGTGGTGGCTCTGGAGGAGGCGGAAGCGGTGGCGGCGGCTCT;

148 $(G_4S)_4$—GGTGGTGGTGGCTCTGGAGGAGGCGGAAGCGGTGGCGGCGGCTCTGGTGGCGGCGGCTCT.

149 **Table S2.** Identification of BsNb and optimization results of parameters.

Parameters	BsNb	
	Carbaryl	1-Naphthol
Linker peptide	G_4S	
Linker mode	C-N	
PCR annealing temperature	53-65 °C	
Coating concentration (ng/mL)	250	250
Dilution of BsNb (ng/mL)	250	250
Reaction temperature (°C)	4/25	4/25
Thermal stability (°C)	90	90
Methanol tolerance (%)	10	10
Acetonitrile tolerance (%)	20	20
Acetone tolerance (%)	10	10
Acid-base tolerance (pH)	5.4-10.4	2.4-10.4
IC_{50} (ng/g)	18.8	6.3
Limit of detection (IC_{10}, ng/g)	0.8	0.4
Linear response range ($IC_{20\text{-}80}$, ng/g)	2.1-270.9	1.1-112.0

150

151 **Table S3.** Parameters of LC-MS/MS (AB 4500).

Items	Parameters
methanol solution (A)	5 mmol/L ammonium formate and 0.1% formic acid (v/v)
water solution (B)	5 mmol/L ammonium formate and 0.1% formic acid (v/v)
Injector temperature	40 ^{o}C
Injection volume	10 μL
Thermo Accucore AQ Column	150 mm*2.1 mm, 2.6 μm
Flow rate	0.3 mL/min
Gradient elution	0~0.5 min, 90% B~90% B; 0.5 ~ 21 min, 90% B~0% B; 21~22 min, 0% B ~ 0% B; 22 ~ 22.1 min, 0% B~90% B
MS conditions	Electrospray ion source, positive ion mode
The parent ion	202.0/145.1 (carbaryl); 145.1/127.1 (1-naphthol)
The daughter ion	202.0/127.1 (carbaryl); 145.1/115.1 (1-naphthol)
The declustering voltage (V)	54 V (carbaryl); 140 V (1-naphthol)
The collision voltage (V)	15, 37V (carbaryl); 29, 35V (1-naphthol)

152

153 **Table S4.** Recovery of carbaryl and 1-naphthol from real samples by ic-ELISA and HPLC-

154 MS/MS.

Analytes	Spiked level [μg/kg]	Samples	ic-ELISA		HPLC-MS/MS	
			$\overline{X}$ ±SD [μg/kg]	Recovery ±CV (%)	$\overline{X}$ ±SD [μg/kg]	Recovery ±CV (%)
carbaryl	0	Soil/ Rice	-0.21±0.1	-	0	0
	20	Soil	19.2±2.0	96.3±10.5%	12.5±0.5	65.0±4.0%
		Rice	23.3±3.1	112.7±13.0%	16.1±1.4	80.0±8.5%
	100	Soil	81.2±2.9	81.7±3.6%	81.7±1.8	82.0±2.2%
		Rice	77.1±10.0	76.5±13.0%	84.8±2.3	85.0±2.7%
	500	Soil	439.6±16.4	84.8±3.81%	449.6±19.8	90.0±4.4%
		Rice	400.4±30.3	80.4±7.44%	414.0±10.0	82.0±2.4%
	2500	Soil	2210.4±85.9	88.6±3.89%	2373.3±52.2	94.8±2.2%
		Rice	2059.8±119.5	82.5±5.82%	2484.7±215.2	99.2±8.7%
1-naphthol	0	Soil/ Rice	-0.14±0.2	-	0	0
	20	Soil	22.4±3.4	110.8±12.5%	12.1±0.0	60.0±0.3%
		Rice	21.4±2.2	102.2±9.5%	14.7±1.5	75.0±10.2%
	100	Soil	77.4±2.6	77.4±2.6%	82.4±1.5	82.0±1.8%
		Rice	78.7±8.1	78.7±8.1%	87.1±8.4	87.0±9.6%
	500	Soil	398.2±24.7	79.6±6.3%	438.3±15.0	88.0±3.4%
		Rice	411.0±56.9	82.2±13.9%	452.0±41.6	90.0±9.2%

	2500	Soil	2043.3±87.8	81.7±4.3%	2357.3±105.1	94.4±4.5%
		Rice	2124.6±197.0	85.0±9.3%	2447.3±200.6	98.0±8.2%

155 $\overline{X}$: Average of measured value; SD: Standard Deviation; CV: Coefficient of Variation; ND:

156 No Data.