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# The expression of soluble functional α7-nicotinic acetylcholine receptors in *E. coli* and its high-affinity binding to neonicotinoid pesticides

Dandan Xiang[b,1], Xiaojun Xu[b,2], Qiaoling Zhou[a,b], Ru Yan[a,b], Mengli Chen[a,b,*], Yirong Guo[b], Guonian Zhu[b]

[a] Zhejiang Provincial Key Laboratory of Biometrology and Inspection & Quarantine, College of life sciences, China Jiliang University, Hangzhou 310018, China
[b] Institute of Pesticide and Environmental Toxicology, Zhejiang University, Hangzhou 310029, China

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels mediating fast cholinergic synaptic transmission in nervous system. In insects, nAChRs are the target sites for several naturally occurring and synthetic compounds, including the neonicotinoid insecticides. So far, one of the major strategies to explore the interaction of nAChR and ligands is based on the heterologous expression of nAChR, which is tough, and needs to be explored. In this study, we expressed and purified extracellular domain of rat a7 subunit (Rα7-ECD), the binding site of the ligands in *E. coli* and determined the interactions and kinetic constants of neonicotinoid insecticides with Rα7-ECD. The recombinant Rα7-ECD is water-soluble and appears to be correctly folded. The interactions of three neonicotinoid pesticides with Rα7-ECD were assessed by surface plasmon resonance (SPR) biosensor. The results revealed a fast association and fast disassociation binding mode of Rα7-ECD/pesticides complexes with the KD value of clothianidin (6.414E-9 M) > imidacloprid (9.030E-9 M) > acetamiprid (2.874E-6 M), respectively. This study demonstrated that the nAChR expressed from *E. coli* was functional, and SPR biosensor technology would be a good alternative for characterizing members of nAChR receptor family.

## 1. Introduction

Nicotinic acetylcholine receptors (nAChRs), a family of neurotransmitter-gated ion channels, play an essential role in nerve signalling at the post-synaptic membrane of both vertebrates and invertebrates (Sattelle, 1986). The nAChRs exist as homopentamers of α subunits or heteropentamers of α subunits and non-α subunits, each subunit has four transmembrane regions. Many different subtype nAChRs are present in the central nervous system (Gotti et al., 2006). They are important drug targets for Alzheimer's disease, Parkinson's disease, and schizophrenia (Taly et al., 2009). Among all these kinds of subtype nAChRs, the neuronal α7-nAChR is one of the most abundant nAChR subtypes in the brain (Fabian-Fine et al., 2001) and it is also a good candidate for study, since it is a homopentamer and has five ligand-binding sites between its extracellular domain (ECD) formed by 6 distinct regions (loops A-F) (Fischer et al., 2001).

The currently accepted transmembrane topology of each subunit consists of a large extracellular N-terminal domain (ECD), which represents approximately half of the subunit, followed by the transmembrane and cytoplasmic regions (Le Novere et al., 1999). Many studies have shown that the ECD of human or insect nAchR displayed a typical α7-nAChR pharmacology, indicating that the ECD contains all of the structural elements contributing to the neurotransmitter binding site (Fischer et al., 2001; Lansdell and Millar, 2004; Psaridi-Linardaki et al., 2002; Tillman et al., 2016; Zouridakis et al., 2009).

High-resolution structures are essential for exploring the interaction between nAChRs and ligands which are important for rational drugs and insecticides design, but only a few are available due to difficulties in obtaining sufficient quantities of protein which is suitable for structural studies, since the nAChRs are transmembrane proteins and consist of different subuits. Currently available structures for Cys-loop receptors were obtained from proteins expressed in yeast (Zouridakis et al., 2009; Dellisanti et al., 2007) and cell lines (Hui et al., 2005; Lansdell and Millar, 2002). However, the yeast and cell lines expression

* Corresponding author at: Zhejiang Provincial Key Laboratory of Biometrology and Inspection & Quarantine, College of life sciences, China Jiliang University, Hangzhou 310018, China.
*E-mail address:* chenml88@zju.edu.cn (M. Chen).
[1] Present address: Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Key laboratory of South Subtropical Fruit Biology and Genetic Resource Utilization (MOA), Guangdong Province Key Laboratory of Tropical and Subtropical Fruit Tree Research, Guangzhou 510640, China.
[2] Present address: Zhejiang Pharmaceutical College, Ningbo 315100, China.

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systems offer the disvantanges of low yield, high cost and complicated operation. In this study, we used the *E. coli.* expression system to produce functional rat α7-nAChR-ECD (Rα7-ECD) because of its effective and inexpensive qualities. In addition, due to its limited ability for post-translational modifications, *E. coli* may produce a more homogeneous population of purified proteins.

Except the drugs for the treatment of neurological diseases and disorders, other toxins and insecticides target on nAChR, including the neonicotinoid insecticides, nereistoxin, spinosad (Millar and Denholm, 2007). The neonicotinoids are the most important new class of synthetic insecticides of the past decades, such as acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam (Tomizawa and Casida, 2005; Matsuda et al., 2005; Matsuda et al., 2001). The neonicotinoids have outstanding potency and systemic action for crop protection against piercing-sucking pests, which are highly effective for flea control on cats and dogs (Millar and Denholm, 2007). Previous studies indicated that the neonicotinoid insecticides share a common mode of action in selectively targeting insect nicotinic acetylcholine receptors (nAChRs), acting as agonists or antagonists (Tomizawa and Casida, 2005; Tan et al., 2007; Tomizawa and Casida, 2003).

SPR (Surface Plasmon Resonance) spectroscopy is a sensitive detection technology that has become a powerful tool for the analysis of biomolecular interactions such as antigen-antibody, ligand-receptor, DNA-protein, and enzyme-inhibitor (Retra et al., 2010). This technology has been recognized as a powerful tool for investigating the interactions between proteins and molecules with the advantages of permitting real-time measurement without labeling (Karlsson, 2004; Nguyen et al., 2015). Binding affinities of mammalian α7-nAChR with neonicotinoid insecticides have been determined by radioligand binding assays, but the non-labeled direct binding of α7-nAChR with these neonicotinoid insecticides has not previously been obtained from any species in a form suitable for structure determination.

Here we report that Rα7-ECD purified from *E. coli* retains signature properties of native Rat α7-nAChR, including the ability to assemble into pentameric structures, to form functional ion channels that can be activated by neonicotinoids, to bind directly with remarkably high affinities by SPR. The study demonstrates that *E. coli* is capable of producing mammalian Cys-loop receptors. It also suggests that the post-translational machinery may not be as essential as previously thought for expressing functional complex membrane proteins.

## 2. Material and methods

### 2.1. Test chemicals

All of the pesticides (clothianidin, imidacloprid, acetamiprid) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). These compounds were prepared as a stock solution in dimethylsulfoxide (DMSO, Sigma–Aldrich (St. Louis, MO, USA).) at a concentration of 100 mM and stored in the dark at 4 ˚C.

### 2.2. Construction of recombinant plasmids

The extracellular domain of rat nAChR α7 subunit (Rα7-ECD, a 630 bp fragment) was generated through polymerase chain reactions with the upstream CCCATATG CAGAGGAGGCTGTACAA and downstream CCTCGAGTGTCCTACGGCGCAT primers, which contained *Nde* I or *Xho* I restriction site (underlined) respectively. The Rα7-ECD fragment was cloned into the expression vector pET-29a in which contain genes coding for the fusion proteins (six-histidine tag). The recombinant constructs were verified by DNA sequencing.

### 2.3. Protein expression and purification

BL21(DE3) bacteria transformed with recombinant pET-29a vector were grown in LB medium containing kanamycin (50 mg/L) at 37 ˚C with 200 rpm/m shaking until reached an optical density to 0.6 at 600 nm. The culture was induced by 0.1 mM IPTG for another 12 h at 16 ˚C. The bacteria were harvested by centrifugation at 5000*g* for 10 min, washed with ice cold binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole and 1 mM phenylmethanesulfonyl fluoride, pH 7.4). The bacterial pellet from 1 L of culture medium was resuspended in 40 mL of binding buffer. The suspension was subjected to ultrasonic treatment (5 s bursts at 200 W with a 5 s cooling period between each burst) on ice for 30 mins and centrifuged at 39000*g* for 30 mins at 4 ˚C, supernatants containing soluble Rα7-ECD fusion proteins were collected.

The supernatants were passed through 0.22 μm filter prior to the purification. The expressed histidine-tagged recombinant proteins were purified by the commercially available $Ni^+$ columns (HisGraviTrap™ HP column, GE Healthcare Life Sciences; Pittsburgh, PA) following the manufacturer's instructions. In general, 10 mL binding buffer was used to equilibrate the column and the supernatants were transferred into it. Once the cell extract has completely entered the column, wash the column 5 times with 5 mL binding Buffer. Step-gradient of Ni-nitrilotriacetic acid elution buffer (20 mM sodium phosphate, 500 mM NaCl, and 100, 200, 300, 400, 500 mM imidazole separately) was used to elute the protein. The elutes were analyzed by 12% SDS-PAGE followed by Coomassie brilliant blue staining and Western blot analysis using anti-His mAb antibody (Abcam, Cambridge, UK). The 300 mM imidazole elute was used for further binding assay.

### 2.4. Measurement of insecticides binding with Rα7-ECD

Direct binding of neonicotinoid insecticides with Rα7-ECD was measured by surface Plasmon resonance (SPR) technique on a Biacore T200 analytical system (Biacore, Uppsala, Sweden) at 25 ˚C. The expressed recombinant Rα7-ECD was immobilized on the CM5 sensor chip (GE Healthcare Life Sciences; Pittsburgh, PA) surface using the standard amine-coupling procedure and HBS-N buffer (0.01 M HEPES, 0.15 M NaCl, pH 7.4) containing 0.05% surfactant P20 was used as the running buffer. Briefly, with this coupling method, the carboxymethylated dextran layer on the CM5 sensor chip surface was activated with a 10 min injection of an EDC/NHS (1-Ethyl-3-(3′-Dimethylaminopropyl) Carbodiimide)/N-Hydroxysuccinimi de) mixture. The Rα7-ECD (50 μg/mL in 10 mM sodium acetate (pH 4.5)) was injected onto the activated sensor surface for 10 min at a rate of 10 μl/min. After immobilization of the protein, the remaining activated group on the surface was blocked by a 10 min injection of 1 M ethanolamine hydrochloride (pH 8.5) at a flow rate of 10 μL/min. The stability of the sensor chip surface was demonstrated by the flat baseline achieved at the beginning (0–100 s) of each sensorgram.

Assay solutions of pesticides were prepared by dissolving in HBS-N (0.01 M HEPES, 0.15 M NaCl, pH 7.4) containing 5% DMSO and 0.05% surfactant P20 from 156.2 nM to 2500 nM (156.2, 312.5, 625, 1250, 2500 nM) for pesticides. The buffer samples containing 4.5–5.8% DMSO were injected to construct a DMSO calibration plot to correct for bulk refractive index shifts. The assay solutions were injected at a flow rate of 30 μl/min using HBS-N containing 5% DMSO and 0.05% surfactant P20. For each test chemical, the association phase of the SPR measurement was 120 s and on completion of injection, the buffer flow was continued to allow a dissociation time of 600 s. The kinetic constants were obtained by fitting the experimental data to a reversible 1:1 bimolecular interaction model included in the Biacore T200 evaluation software 3.0.

### 2.5. Data analysis

All the data were presented as mean ± SEM (standard error). The responses from the reference surface were subtracted from the binding responses collected over the reaction surfaces to correct for bulk

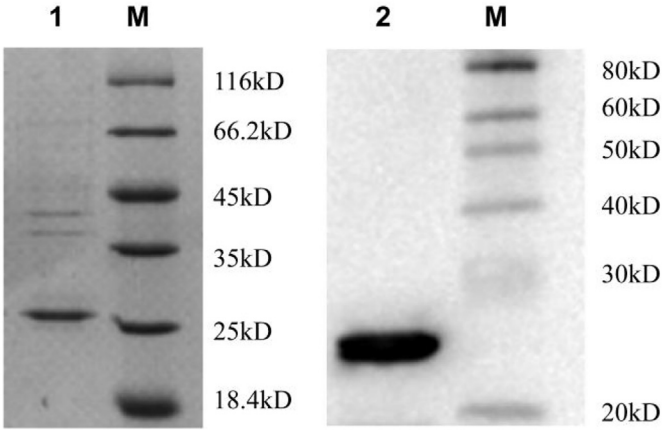


**Fig. 1.** Identification of the purified recombinant Rα7-ECD. The protein was purified and electrophoresed on an SDS-12% polyacrylamide gel. The gel was stained with Coomassie Blue (lane1). A protein band in lane 2 of 25 kDa was detected by western blot analysis of a gel prepared as in lane1 incubated with anti-His-tag antibody and then was detected by binding to goat anti- (mouse IgG) antibody.

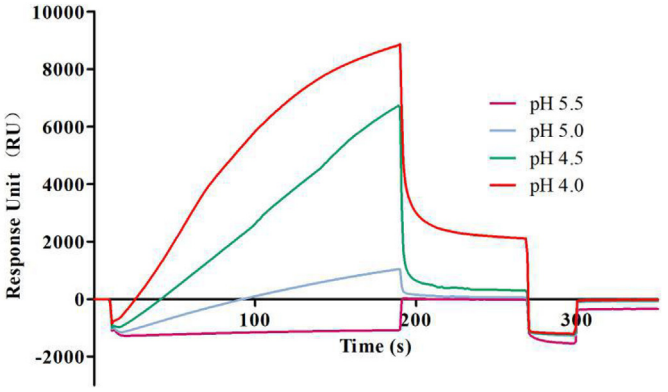


**Fig. 2.** The optimization pH of coupling buffer for Rα7-ECD coupling on CM5 chips. The purified Rα7-ECD was injected in coupling buffer for 120 s with different pH (pH 4.0, 4.5, 5.0, 5.5) with the final concentration of 20 μg/mL over a surface that has not been activated with EDC/NHS and the coupling buffer with pH 4.5 was selected for the final immobilization of Rα7-ECD.

refractive index changes. Second, the response from an average of the blank injections was subtracted to remove any systematic artifact observed between the reaction and reference flow cells.

## 3. Results

### 3.1. Obtaining of Rα7-ECD protein

A six-histidine tag was added to the C terminus to aid in purification for the expressed Rα7-ECD. After optimization, BL21(DE3) was the most suitable *E. coli* strain for Rα7-ECD expression, long induce time (12*h*) and low induce temperature (16 ˚C) after low concentration of IPTG applied were inclined to generate the functional recombinate protein, otherwize the recombinate Rα7-ECD was in low expression level and most of the protein was in the formation of inclusion bodies. The *E.coli* derived purified recombinant Rα7-ECD was confirmed by SDS/PAGE analysis and Western blotting. As shown in Fig. 1, the protein has an apparent molecular weight of 25 kDa, which is consistent with the predicted molecular weight. The concentration of the recombinant protein was collected at 300 μg/mL and used for following experiment.

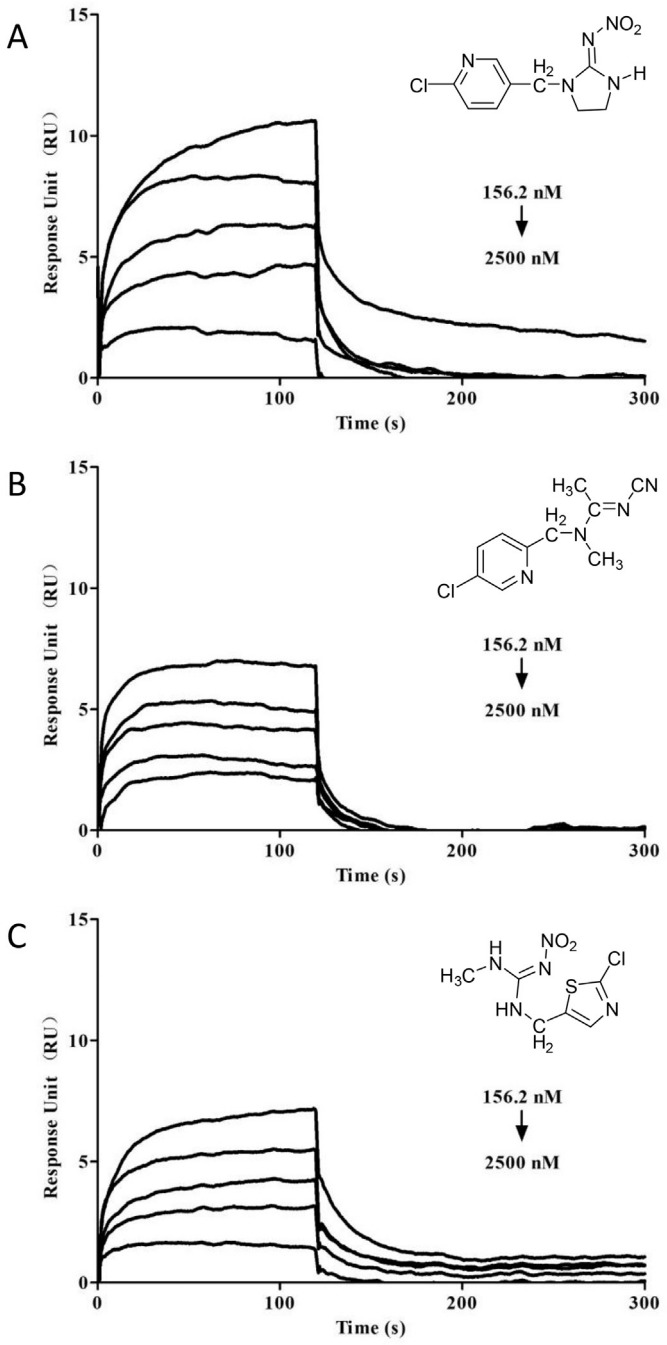


**Fig. 3.** Kinetic analyses of pesticides and α7-nAChR-ECD interactions. Each compound imidacloprid (A), acetamiprid (B), clothianidin (C) were tested in triplicate in a two-fold dilution series (0, 15.625, 31.25, 62.5, 125, 250 nM) and the responses were fit to a 1:1 bimolecular interaction model and the kinetic parameters obtained are reported in Table 1.

**Table 1**
Kinetic parameters of the binding of Ra7-nAChR-ECD and the tested compounds.

| Analyte | Ligand | ka (1/Ms) | kd (1/s) | KD (M) |
|---|---|---|---|---|
| Ra7-nAChR-ECD | imidacloprid | 1.014E+6 | 0.009155 | 9.030E-9 |
| | acetamiprid | 1.832E+4 | 0.05264 | 2.874E-6 |
| | clothianidin | 1.421E+5 | 9.113E-4 | 6.414E-9 |

### 3.2. Capturing of Rα7-ECD protein on CM5 chips

In order to identify the suitable conditions of coupling without permanently modifying the sensor chip surface, the purified Rα7-ECD was injected in coupling buffer with different pH (pH 4.0, 4.5, 5.0, 5.5) at the final concentration of 20 μg/mL for 120 s over a surface that has not been activated with EDC/NHS. The traces of Rα7-ECD was removed by injecting 50 mM NaOH before the next injection. The results of pre-concentration tests showed that, the binding level increased as the pH was reduced from 5.5 to 4.0. At pH 4.0, the binding level seemed to have reached saturation point after injection even it had a higher binding level than pH 4.5 (Fig. 2). Therefore, the coupling buffer with pH 4.5 was selected for the final immobilization of Rα7-ECD.

### 3.3. Binding kinetics of tested chemicals to Rα7-ECD protein

The immobilization of Rα7-ECD protein to the sensor chip by the standard amine coupling protocol resulted in a final immobilization level approximately 8500 resonance units (RU). The interactions of pesticides with Rα7-ECD was measured by serial 120 s injections of increasing concentrations of each compound (0, 156.25, 312.5, 625, 1250, 2500 nM) until a steady state maximum binding level was approached (association phase). The recorded sensorgrams were subjected to global non-linear regression analysis using a 1:1 interaction model. The colored experimental curves generally matched well with the black fitted curves. The concentration-dependent responses rapidly reached equilibrium and return to baseline in seconds, indicating a fast association and a fast disassociation between these pesticides and Rα7-ECD (Fig. 3). The association (ka) and dissociation (kd) rate constants and the equilibrium dissociation constants (KD) estimated by the 1:1 binding model are listed in Table 1. Concerning KD values were calculated as the ratio of kd/ka, all the three tested pesticides exhibited strong binding affinity to Rα7-ECD with following order, clothianidin (6.414E-9 M) > imidacloprid (9.030E-9 M) > acetamiprid (2.874–6 M).

## 4. Discussion

It is imperative in understanding structural and functional properties of a protein in basic research and drug discovery and one of important prerequisite enabling such detailed studies is the access to large quantities of recombinant proteins. The high yield functional nAChR is widely known to be particularly difficult to express among the Cys-loop superfamily of ligand gated ion channels (Tillman et al., 2016; Aztiria et al., 2000). As we mentioned the α7-nAChR appears to be more suitable than other heteromeric neuronal or muscle-type nAChRs to conduct hetereologous expression and characterization (Tsetlin et al., 2002). It has been reported that only 62% of the total α7-nAChRs protein present in the cell is in a mature functional form even in permissive cells (Peng et al., 2005).

Previous studies reported α7-nAChRs were expressed in *E.coli* (Le Novere et al., *1999*; Tsouloufis et al., *2000)* and cells (Claudio, 1987; Beeson et al., 1996; Loutrari et al., 1997). Compared with the cell line expression system, the *E.coli* expression system could produced higher quantity of protein, however, the majority of the α7-nAChRs were inclusion bodies and renaturation was necessary (Schrattenholz et al., 1998). In this study, We optimized the expression condition, the recombinant protein expressed with high efficiency at 0.1 mM IPTG with the induction time of 12 h at 16 °C. It turned out good expression condition is a key factor to get large amount of functional recombinant proteins. $(His)_6$ tag was also introduced in the C-terminal for the production of recombinant Rα7-ECD which facilitated easy purification. The success in gaining functional human α7-nAChRs in *E. coli* indicates that specific chaperone proteins and post-translational modifications are not required for channel function, but instead are a consequence of sub-cellular trafficking in eukaryotic cells (Tillman et al., 2016).

The most commonly used strategies for detecting soluble nAChRs-binding ligands require radioisotope-labeled, which is not appropriate for real-time binding analysis (Alberts and Bartfai, 1976). SPR-based technology incorporating sensor chips directly immobilized with Rα7-ECD, the neonicotinoids, as an alternative to the traditional $^{125}$I-labeled compounds, were injected with serial dilutions across the Rα7-ECD and yielded the sensorgrams for Rα7-ECD binding detection. This radioisotope-free methodology introduces a convenient and innovative tool for the investigation of Rα7-ECD-binding ligands in real time.

In previous studies, the nicotinic receptors were prepared in membrane from insect heads, such as *Musca domestica*, *Drosophila melanogaster*, *Myzus persicae* and *Aphis craccivora*. The binding affinity of [3H] imidacloprid to nicotinic ranged from 0.5 to 7.1 nM (Kayser et al., 2016; Kagabu et al., 2002; Zhang et al., 2000). In this study, each of the binding responses of the three neonicotinoids were well-described by a 1:1 interaction model. Upon review, the binding trends of the three neonicotinoids are characterized by fast association and dissociation phases and their KD values are in the order of clothianidin > imidacloprid > acetamiprid. The sensitivity of the Biacore assay is comparable to that of the $^{125}$I-labeled-binding assay and we achieved a detection limit of approximately 5 nM pesticide using the standard binding assay.

In summary, The *E. coli* heterologous protein expression system was employed to express the whole extracellular domain of Rα7-nAChR (residues 1–210) and recombinant expressed in *E. coli* appears to be soluble and correctly folded. We have succeeded in directly immobilizing Rα7-ECD onto a general-use Biacore sensor chip, and with this modified sensor chip we measured the binding of Rα7-ECD with neonicotinoids in a concentration-dependent manner.

## Declaration of Competing Interest

The authors have declared no conflicts of interest.

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