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Short Communication

Immobilization of marine toxins on carboxylic acid modified surfaces

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ABSTRACT. Saxitoxin and gonyautoxin 2 and 3 are among the most toxic components of the Paralytic Shellfish Poison from red tides. Being small molecules, they often require to be immobilized in order to be handled experimentally. Here is presented a methodology for covalently binding the toxins to carboxylate-modified surfaces. Both toxins were successfully bound to magnetic beads and saxitoxin was additionally bound to a modified golden surface in order to perform a surface plasmon resonance analysis. Success of binding to magnetic beads was evaluated through a standard immune-based toxin assay. Despite the different methods used for each toxin, the maximum binding yield for both toxins occurred when using concentration of 120 μ M.

Keywords: PSP, ELISA, magnetic beads, plasmon, resonance, saxitoxin, gonyautoxin.

Inmovilización de toxinas marinas en superficies modificadas con ácido carboxílico

RESUMEN. Saxitoxina y gonyaulatoxina 2 y 3 están entre los componentes más tóxicos del Veneno Paralizante de Mariscos de las mareas rojas. Al ser moléculas pequeñas, a menudo requieren ser inmovilizadas para manipularlas experimentalmente. Se presenta una metodología para la unión covalente de las toxinas a superficies modificadas con carboxilatos. Ambas toxinas fueron exitosamente unidas a perlas magnéticas y la saxitoxina fue unida además a una superficie de oro modificada para realizar un análisis de resonancia de plasmones de superficie. El éxito de la unión a perlas magnéticas se evaluó mediante un inmunoensayo estándar contra las toxinas. A pesar que cada toxina requirió métodos diferentes, el rendimiento máximo de ligamiento para ambas ocurrió cuando se utilizaron concentraciones de 120 μ M.

Palabras clave: VPM, ELISA, perlas magnéticas, resonancia, plasmones, saxitoxina, gonyaulatoxina.

Paralytic Shellfish Poison (PSP) is a mixture of toxins that are synthesized by certain dinoflagellates mainly from the genus *Alexandrium* (Landsberg *et al.*, 2006). During a PSP outbreak, these dinoflagellates are consumed and concentrated by bivalve mollusks and the toxins can reach concentrations that can be fatal upon human consumption (Shumway, 1990; James *et al.*, 2010). PSP is composed mainly by saxitoxin derivatives that bind with nanomolar affinity to sodium channels, ultimately leading to muscle paralysis and death by asphyxia (Mons *et al.*, 1998; Wang, 2008).

Since saxitoxin derivatives are molecules of only about 300 Da, immobilization methodologies have been developed to arrange them into formats that allow easier handling. For example, saxitoxins have been bound

to proteins, such as Keyhole limpet hemocyanin (KLH) and/or to specific antibodies (Micheli *et al.*, 2002; Handy *et al.*, 2013).

Here we present a methodology to bind saxitoxins directly to carboxylate-modified surfaces, which are available in a number of brands and formats. Binding was achieved for two of the major saxitoxin components of PSP, saxitoxin (STX) and gonyautoxin 2/3 (GTX), via two different binding protocols. For the first time, the toxins were successfully bound to carboxylate-modified magnetic beads (MB) and STX in particular was also immobilized directly to a carboxylate-modified gold chip for surface plasmon resonance analysis (SPR).

Saxitoxin derivatives are regularly dissolved in chloridric or acetic acid for better stability, so before the fixation procedure, the toxins were lyophilized and resuspended in 25 mM MES buffer (Sigma-Aldrich, MO).

MB (Dynabeads® M-270 Carboxylic Acid, Invitrogen) were washed three times in an equal volume of MES buffer 25 mM by collecting them on a Bilatest Magnetic separator (Sigma-Aldrich, MO). For STX binding, MB were resuspended in a fresh solution of 25 mg mL⁻¹ 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 25 mg mL⁻¹ of N-hydroxysuccinimide (NHS). For GTX binding, MB were washed with distilled water and then resuspended in 100 µL EDC at 70 mg mL⁻¹. After one hour incubation MB were washed with MES buffer 50 mM. Then MB were incubated during one hour with 100 µL of STX or GTX solutions. Finally, the beads were collected and incubated one hour with 100 µL of TBS pH 7.4. After that the MB were washed four times with MES 25 mM and resuspended in TBS pH 7.4.

A similar procedure to MB binding was used to bind STX to a four channel CM5 golden chip for SPR. All the fixation reagents were passed through the channels at 5 µL min⁻¹. Solutions of EDC and NHS (25 mg mL⁻¹) were pumped together during 10 min and then STX was pumped for 20 min at 4 µg mL⁻¹. SPR was performed on a Biacore 3000, reporting a curve of resonance units (RU) in time, compared to the control channel.

Toxins bound to MB were detected with a commercial kit for saxitoxin detection in solution (Ridascreen Fast PSP SC, R-Biopharm AG, Germany) according to manufacturer instructions. The kit, is a competitive immune detection system that yields an inversed correlation between the amount of toxin and absorbance, measured in an ELISA plate reader. Toxins immobilized on MB (MB-STX and MB-GTX) resuspended in TBS, were diluted 1:1 with the buffer supplied by the kit for toxin detection. Positive controls corresponded to the purified toxins at 5 µM concentration and the negative controls corresponded to the MB subjected to the same immobilization procedure, but adding water instead of the toxins.

All assays performed with the above protocol ended with the successful fixation of the toxins to the MB. The levels of absorbance obtained were similar to those of the free toxin (positive control). However, since the MB probably influenced the efficiency of the test, the exact concentration of bound-toxin cannot be extrapolated. Performing the above fixation procedure with different toxin concentrations yielded different results, but the maximum fixation rate was achieved when using 120 µM of toxin. Higher toxin concentrations achieved similar readings in the detection kit. Up from

120 µM, MB-STX yielded an average absorbance of 0.18 and MB-GTX yielded 0.33. Negative controls yielded absorbances of 1.2 and 2.0 for MB-STX and MB-GTX, respectively. The difference in absorbance between MB-GTX and MB-STX is probably due to the fact that while the kit is designed specifically for STX detection, it is known to cross react with GTX with an approximately yield of 70%.

Stability of the MB-toxin complex was evaluated by storing it for two month at 4°C before performing the Ridascreen Fast PSP SC detection. In this condition the complex MB-toxin did not greatly diminish their activity towards the antibody, presenting similar absorbance than that of the MB-toxin that was freshly prepared.

SPR analysis confirmed binding of STX to the CM5 chip with the present methodology, presenting a stable signal of a 560 RU differential after binding. Figure 1 shows the stages of binding for the SPR procedure.

Saxitoxin derivatives are very small, most of them with molecular weights around the 300 Da. Therefore it is usually necessary to derive these toxins into bigger, easier to handle formats. For example, they have been conjugated with agarose (Watanabe *et al.*, 2006), microtiter plates (Kralovec *et al.*, 1996), horseradish peroxidase (Usleber *et al.*, 1991), biotin (Koehn *et al.*, 1981). Methods for *in vitro* and *in vivo* selection have required strategic handling as well. For example, for the development of specific antibodies, STX has been conjugated to KLH, which allows it to elicit a proper immune response in the host (Micheli *et al.*, 2002). Recently, the same conjugated STX-KLH was used to select aptamers *in vitro*, through the immobilization of the

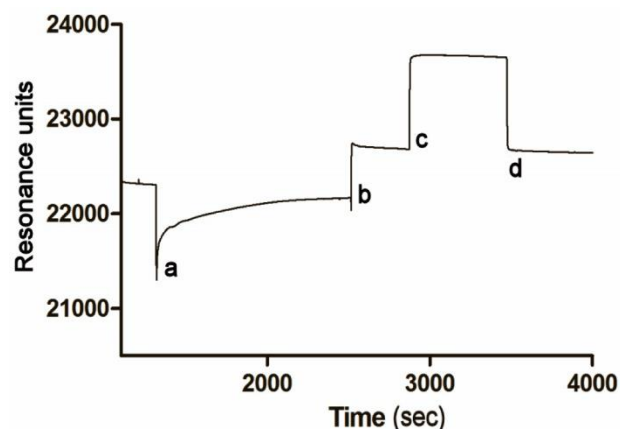


Figure 1. SPR graphic for the saxitoxin immobilization procedure. After priming the CM5 chip, NHS/EDC is added to the channel (a). Then, STX is added (b) and then washed with MES (c). Persistence of the Resonance units differential after washing (d) indicated successful STX binding.

STX-KLH conjugate with an anti-KLH antibody which was previously conjugated with MB (Handy *et al.*, 2013). While the last method might seem cumbersome, the authors needed to collect the immobilized STX though several selection steps. Here we proposed a much simpler method to achieve the immobilization and ulterior collection, by binding the toxins directly to the MB. The methodology is simple and of low cost, and it avoids hiding the molecule within a much bigger construct. In fact, the immune assays performed here, indicated that the immobilized toxin is available for antibody binding, and thus it should be accessible enough for further binding reactions. The obvious advantage of having these toxins immobilized onto MB is the versatility of its use, allowing it to be washed and exposed to a variety of reagents and then be recovered with a standard magnet.

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