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Synthetic peptides to produce antivenoms against the Cys-rich toxins of arachnids

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ABSTRACT

Scorpion and spider envenomation is treated with the appropriate antivenoms, prepared as described by Césaire Auguste Phisalix and Albert Calmette in 1894. Such treatment requires the acquisition and manipulation of arachnid venoms, both very complicated procedures.

Most of the toxins in the venoms of spiders and scorpions are extremely stable cysteine-rich peptide neurotoxins. Many strategies have been developed to obtain synthetic immunogens to facilitate the production of antivenoms against these toxins. For example, whole peptide toxins can be synthesized by solid-phase peptide synthesis (SPPS). Also, epitopes of the toxins can be identified and after the chemical synthesis of these peptide epitopes by SPPS, they can be coupled to protein carriers to develop efficient immunogens. Moreover, multiple antigenic peptides with a polylysine core can be designed and synthesized.

This review focuses on the strategies developed to obtain synthetic immunogens for the production of antivenoms against the toxic Cys-rich peptides of scorpions and spiders.

1. Introduction[1]

Scorpions and spiders (generally called arachnids) secrete venom from specialized glands in order to kill prey or enemies. Several scorpion stings and spider bites cause life-threatening envenoming in humans. The most dangerous taxa are the spiders *Phoneutria nigriventer* (Brazilian armed spider), *Loxosceles* spp (recluse spiders), *Latrodectus* spp (widow spiders), *Atrax spp*. and *Hadronyche spp.* (Australian funnel-web spiders), the scorpions of the *Buthidae* family, such as *Tityus serrulatus* (yellow scorpion) and *Centruroides sculpturatus* (bark scorpion), and scorpions of the genus *Androctonus* (Fattail scorpion), such as *A. crassicauda*, *A. australis* and *A. amoreuxi* (Hauke and Herzig, 2017; Diaz, 2004; Goyffon and Tournier, 2014).

Scorpion and spider envenomation is treated with the appropriate antivenoms, prepared as described by Césaire Auguste Phisalix and Albert Calmette in 1894 (Bochner, 2016). This method requires capturing the arachnids, a complicated process due to their small size, keeping them in captivity and using arduous electrostimulation to milk a very small amount of venom from each specimen (Meadows and Russell, 1970).

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[1] Abbreviations: APCs, antigen-presenting cells; BSA, bovine serum albumin; CSα/β, cystine-stabilized α/β scaffold; EDC.HCl, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydorchloride; ELISA, enzyme-linked immunosorbent assay; ICK, inhibitor cystine knot; KLH, keyhole limpet haemocyanin; KTxs, potassium channel specific toxins; LiD1, *Loxoceles intermedia* dermonecrotic protein isoform 1; MHC, major histocompatibility complex; NaV, voltage-gated sodium; SPPS, solid phase peptide synthesis; NHS, *N*-hydroxysulfosuccinimide ester; sulfo-SMCC, sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate; α-NTxs, alpha-neurotoxins, β-NTxs, beta-neurotoxins.

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Most of the toxins in the venoms of arachnids are extremely stable Cys-rich peptide neurotoxins (Cheek et al., 2006; Reeks et al., 2015). These neurotoxins block or modify the opening and closing mechanisms of ion channels in the cells of prey, causing an anomalous depolarization that severely impairs the neuromuscular, respiratory and cardiovascular systems (Catterall et al., 2007).

This review will focus on different strategies developed to obtained synthetic immunogens for the production of antivenoms against the toxic Cys-rich peptides of scorpions and spiders.

2. Cys-rich peptide toxins in arachnids

Cys-rich venom peptides from scorpions and spiders are neurotoxins capable of interacting specifically with potassium, sodium or calcium channels, impairing their activity and hence cell function. Many of these peptides are responsible for human envenoming, which is often treated with specific antivenoms (Cardoso and Lewis, 2019). The capacity of Cys to form disulfide bridges provides conformational rigidity to the molecule, thereby explaining why these venom peptides are extremely stable to degradation by heat or enzymes.

2.1. Cys-rich peptides in spider venom

2.1.1. Knottin peptides

Most venom peptides found in spider venom are knottins, with the so-called cystine knot structural motif, which provides exceptional stability (Postic et al., 2018). These peptides contain at least three disulfide bridges with loop regions anchored to a core of anti-parallel β strands, where two disulfide bridges form macrocycles while a third one crosses a macrocycle, thereby building a knot. These chemical, thermal, and proteolytic stable polypeptides are found in animals, plants and fungi, where they exert antimicrobial, antifungal, insecticidal and protease inhibition activity, among others. Most of these peptides are neurotoxins that interact with multiple sites on voltage-gated sodium (NaV) channels of prey. The inhibitor cystine knot (ICK), also called knottin (Fig. 1), is a subset of this family in which the disulfide bridge between the 1st and 4th Cys and the 2nd and 5th Cys form macrocycles, while the bridge between the 3rd and the 6th Cys crosses a macrocycle, forming a knot (Escoubas et al., 2000; Nicholson, 2013). In cone snails and spider venoms, ICK toxins are predominant components, and they have diverse molecular targets, including Na^+, K^+, Ca^{2+}, acid-sensing, transient receptor potential, and mechanosensitive channels. The KNOTTIN database (http://knottin.cbs.cnrs.fr/) offers a complete list of the knottins that have been described to date. Although abundant in spiders, the cystine knot structural motif is unusual in scorpion venom peptides (Quintero-Hernández et al., 2013; Rodríguez de la Vega et al., 2013).

2.1.2. Peptides with a disulfide-directed β-hairpin fold

A few peptides in spiders have a disulfide-directed β-hairpin fold, which comprises a double-stranded antiparallel β-hairpin stabilized by mandatory disulfide bridges with a current consensus sequence of $–CX_{4–19}–CX_2$[G or P]$X_2–CX_{4–19}–C–$, where X is any amino acid. The ICK motif seems to have evolved from this simpler canonical ancestral fold, which has been observed in a range of toxins with unknown targets (Nicholson, 2013).

2.2. Cys-rich peptides in scorpion venom

2.2.1. Peptides with the Cys-stabilized α/β (CS-α/β) motif

These peptides are the most predominant components of scorpion venom. They contain three or four disulfide bonds. Two of them are strictly conserved and they link an α-helix and one strand of a β-sheet, conforming the so-called Cys-stabilized α/β scaffold (CSα/β). These peptides are potassium channel-specific toxins (KTxs). They are classified into the families α-, β- and γ- KTxs on the basis of their amino acid sequences and Cys pairing. They all show high capacity to block K^+ channels. α-KTxs are short-chain peptides of 23–43 amino acids with three or four disulfide bridges, β-KTxs are long-chain peptide toxins of 50–75 amino acids with three disulfide bridges and γ-KTxs are peptides of 36–47 amino acids with three or four disulfide bridges (Quintero-Hernández et al., 2013, Rodríguez de la Vega et al., 2013).

2.2.2. Peptides with the cystine-stabilized helix-loop-helix (CSα/α) motif

These peptides form the κ-KTx family, which are minor components found in very few scorpion species. They are poor blockers of K^+ channels. They have a purely helical structure stabilized by two disulfide bridges and they fold into an α-hairpin fold known as CSα/α (cystine-stabilized helix-loop-helix) (Quintero-Hernández et al., 2013).

2.2.3. Peptides with an α-helix and three- or four-stranded anti-parallel β-sheets

Some scorpion toxins are polypeptides comprising 61–76 amino acids, tightly bound by four disulfide bridges. They adopt a highly conserved three-dimensional structure comprising an α-helix and three- or four-stranded anti-parallel β-sheets with high chemical and thermal stability. They are Na^+ channel-specific toxins. Although being minor components in scorpion venom, these peptides are the main toxins responsible for the symptoms caused by stings in humans (scorpionism). They modify the opening and closing kinetic mechanisms of NaV channels. Given their interaction site on NaV channels, they are classified into alpha-neurotoxins (α-NTxs), also known as old world scorpion toxins and beta-neurotoxins (β-NTxs), also called new world scorpion toxins (Quintero-Hernández et al., 2013).

3. Classical arachnid antivenom production

The classical antivenom (venom antiserum) production described by Césaire Auguste Phisalix and Albert Calmette in 1894 consists of the following: a) capturing spiders or scorpions and keeping them in captivity; b) collecting their venom by means of electrostimulation; c) immunizing large mammals repeatedly with the venom; and d) purifying the immunoglobulin G antibodies from the hyperimmune plasma (Bochner, 2016).

As previously stated, acquiring and manipulating venom from

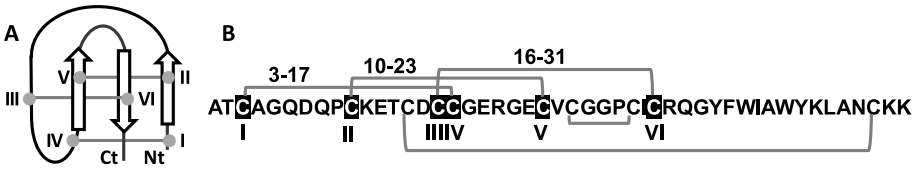

Fig. 1. A) Schematic representation of the inhibitor cystine knot or knottin: macrocycles are formed by CysI-CysIV and CysII-CysV disulfide bridges. The bridge between CysIII and CysVI crosses through a macrocycle, forming a knot-like structure. Loop regions are anchored to a core of anti-parallel β strands (arrows). **B)** Delta-ctenitoxin-Pn2a from *Phoneutria nigriventer* venom. Macrocycles are formed by Cys3-Cys17 and Cys10-Cys23 disulfide bridges. The bridge between Cys16 and Cys31 crosses through a macrocycle, forming a knot-like structure. This peptide also contains two more disulfide bridges between Cys14-Cys46 and Cys25-Cys29, which add further stability to the molecule.

spiders and scorpions is extremely complicated because these small animals are very difficult to capture and keep in captivity, and only a very small amount of venom can be milked from each specimen by arduous electrostimulation (Meadows and Russell, 1970).

4. Synthetic Cys-rich peptide toxin

Instead of using crude venom, Cys-rich peptide neurotoxins can be obtained by recombinant DNA technology expression or by chemical synthesis. These synthetic neurotoxins can be used to immunize animals against arachnid venom. The antivenom obtained with these pure immunogens has higher titers of the specific antibodies against the toxins responsible for the main symptoms of envenoming, thereby avoiding the production of antibodies against other non-toxic components of the venom (Bermúdez-Méndez et al., 2018).

Although Cys-rich peptides can be obtained by recombinant expression in a heterologous species such as bacteria, yeasts, animal cells and baculovirus expression systems, the yield of recombinant active Cys-rich toxins obtained is usually low. Furthermore, the recovery and purification of these toxin peptides from the culture broth (downstream processing) is usually a complex process involving many steps. Purification is sometimes aided by fusion tags, but the introduction of a chemical or enzymatic cleavage step is needed in this case (Clement et al., 2015 Jiménez-Vargas et al., 2017). Expression in *E. coli* is simpler and less expensive in comparison to yeast or eukaryotic systems. However, the cytoplasmic reducing environment of *E. coli* impairs correct disulfide bond formation and peptide folding, causing peptide aggregation in inclusion bodies. Therefore, after cell lysis, laborious steps including reduction, denaturalization and refolding are needed, which further reduce the yield. Fusion tags to enhance peptide solubility, low temperature culture incubation with slow peptide expression rates to achieve better folding, and periplasmatic expression to enhance disulfide formation have been assayed to improve yields. Nevertheless, the physicochemical similarity of many of the disulfide-bond isomers obtained calls for fractionation by orthogonal HPLC steps (Saez et al., 2017).

In contrast, solid-phase peptide synthesis (SPPS) allows the straightforward preparation of peptides in laboratories not strictly oriented to organic chemistry synthesis and it is the ideal technique to obtain Cys-rich peptides (Jaradat, 2018). Fig. 2 shows a scheme of the SPPS method, which consists of coupling α-amino and side chain protected amino acids on a solid support one by one from the C- to the N-terminus. The classical tert-butyloxycarbonyl (Boc) chemistry SPPS was first described by Merrifield (1963) and soon afterwards improved by Carpino and Han (1970), who introduced the 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry approach. The C-terminus protected amino acid is first covalently bound to the solid phase. After washing, the amino protected group (Boc or Fmoc, depending on the chemistry used) is removed and reacted with the carboxylic acid of the next protected amino acid. The cycle of washing, deprotection and coupling steps are repeated until all the desired amino acids are assembled. Finally, side chain protecting groups are removed, and the peptide is separated from the solid support in the global deprotection step. Commercial solid supports and protected amino acids are widely available. Also, D protected amino acids can be used to synthesize the retro-inverso analog, which has a reversed amino acid sequence with respect to the natural peptide and an inverted chiral center. Given that D amino acids cannot be recognized by common proteases, they will not be degraded easily and will consequently have a longer half-life as immunogens (Rai, 2019).

To obtain long peptides of high quality, SPPS can be combined with native chemical ligation (Yamaji et al., 2009; Tsuda et al., 2015; Blanco-Canosa et al., 2015; Kent, 2009), which consists of assembling two or more unprotected pure peptide segments previously obtained by SPPS (Fig. 3).

Afterwards, oxidative folding is usually performed in a one-step procedure in the presence of a redox pair, which is generally enough for spontaneous peptide folding. Although most natural peptides fold correctly, in some cases orthogonal chemical synthesis using Cys with different protecting groups is needed to ensure the correct formation of disulfide bridges (Fig. 4).

5. Immunogenicity of Cys-rich peptides in spider and scorpion venom

To obtain IgG antibodies against the Cys-rich peptide toxins, the full venom or the recombinant or synthetic pure peptides of spiders or scorpions are injected into large mammals such as horses. To initiate an adaptive immune response, protein and peptide antigens must be presented to T cells by specialized antigen-presenting cells (APCs), such as dendritic cells, macrophages and B-lymphocytes. These immature APCs ingest the polypeptide antigens by endocytosis and deliver them to endosomes and lysosomes. The endosomes and lysosomes contain proteases that degrade the antigens into short peptides, typically 10 to 25 amino acids long (antigen processing). Some of these peptides, called T-epitopes, bind to major histocompatibility complex (MHC) class II molecules and the complex is transported to the cell surface. The peptide-MHCII molecular complex is displayed on the mature APC surface (antigen presentation) and is recognized by naive CD4 T cells. This recognition process induces the proliferation and differentiation of these cells into CD4 effector T cells, which perform their function as soon as they encounter their specific antigen on other target cells. CD4 T cells differentiate into several subsets of effector T cells with a variety of functions. One of these functional classes, the effector helper T cells, stimulates B-lymphocytes to proliferate and differentiate into specific antibody-secreting plasma cells and memory B cells (antigen-specific humoral immune responses). Antibodies produced by plasma cells recognize the intact antigen and inhibit the effects of the toxins by binding to them (neutralization) (Murphy et al., 2012).

Despite of being highly toxic, the Cys-rich peptides from scorpion and spider venoms have low immunogenicity because they are extremely stable and resist attack by lysosomal/endosomal enzymes, a step that is critical for fragmentation and hence for the presentation of immunogens (Huang et al., 2019; de la Rosa et al., 2019;

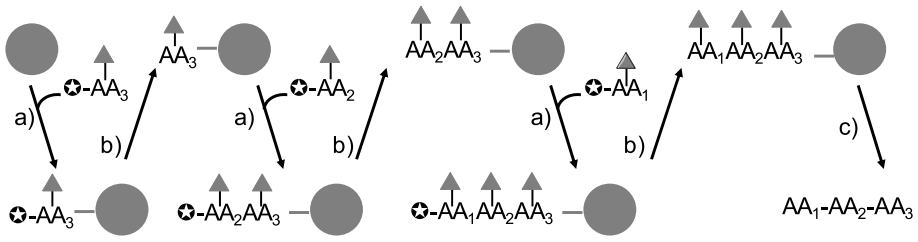

Fig. 2. Solid-phase peptide synthesis (SPPS): *N*-protected and side-chain protected amino acid (AA) is coupled to a solid phase (●) through a linker. After washing, the *N*-protected group (✪) is removed. The second *N*-protected amino acid is then coupled. Coupling (a) and deprotection (b) steps are repeated until the desired amino acid sequence has been elongated. Finally, side-chain protecting groups (▲) are removed and the peptide is cleaved from the solid support (global deprotection) (c).

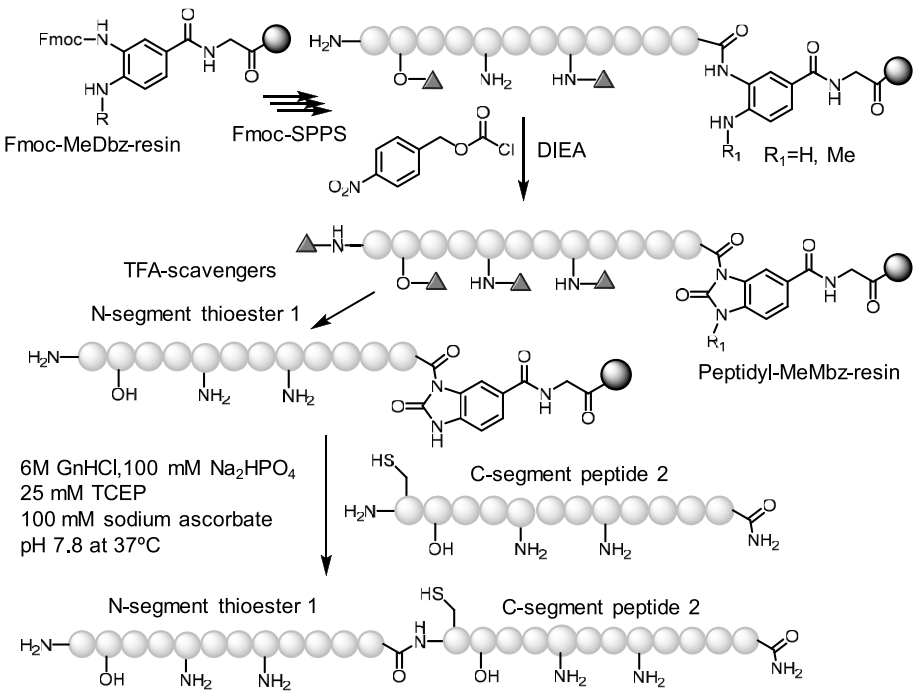

Fig. 3. Scheme of native chemical ligation of two peptides obtained by solid-phase peptide synthesis. ▲: Side-chain protecting group; ● :solid phase, ●:amino acid residue; Fmoc: 9-Fluorenylmethyloxycarbonyl; Fmoc-MeDbz-resin: 3-[(Fmoc)amino]-4-amino-benzoil resin; SPPS: solid-phase peptide synthesis; Me: methyl group; DIPEA: *N,N*-Diisopropylethylamine; TFA: Trifluoroacetic acid; GnHCl: Guanidinium chloride; and TCEP: Tris (2-carboxyethyl)phosphine hydrochloride.

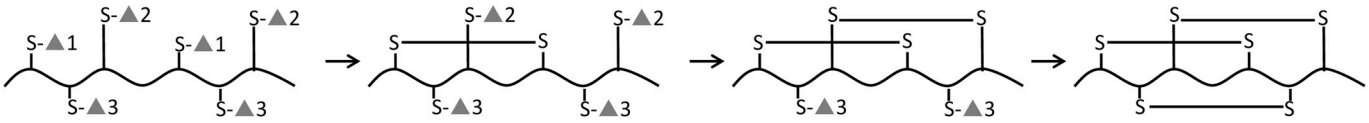

Fig. 4. Scheme of orthogonal Cys-rich peptide synthesis using different Cys protecting groups (▲).

Bermúdez-Méndez et al., 2018; Maillère et al., 1995). Intramolecular disulfide bridges need to be reduced prior to peptide digestion in endosomes. Although APC endosomes contain thiol reductases, this reduction process hinders antigen processing and leads to an unbalanced antibody content in antivenoms, with a major fraction of the antibodies targeting immunogenic but non-toxic components, and a minor fraction targeting highly toxic but poorly immunogenic Cys-rich peptides.

6. Alternative synthetic antigens

As Cys-rich peptides have low immunogenicity, alternative antigens have been developed to improve the immune response. These artificial antigens contain one or more of the epitopes of the natural immunogen.

6.1. Toxin epitope identification

An epitope is the part of an antigen recognized by the antibody, B cell receptor or T cell receptor. It can be continuous, consisting of short fragments of a protein, or discontinuous, in which different amino acids in distant positions are brought together by protein folding, thereby creating a more complex structure (Chen et al., 2009). These linear or continuous epitopes can be mirrored by short peptide sequences. Generally, an antigen has several epitopes that can be identified by different methods and then be synthesized easily by SPPS for later preparation of immunogens to immunize animals against arachnid toxins (Nilvebrant and Rockberg, 2018).

6.1.1. Synthesis and mapping

The short peptides (approximately 15-mer) covering the full sequence of the target peptide protein can be chemically synthesized and then screened to determine which ones bind to the antibody. To maximize this identification, peptides are synthesized in such a way that they overlap the previous and posterior sequence. Thus, a protein of 213 amino acids can be mapped by the synthesis and posterior screening of 208 possible overlapping hexapeptides. Although SPPS offers a great advantage over in solution chemistry in terms of efficiency, high-throughput techniques are needed to speed up the whole process. The most useful of these is probably the simultaneous synthesis of 96 (12 × 8) of these peptides on an array, so that the sequence in each position is spatially addressed.

6.1.1.1. Simultaneous synthesis and mapping using the multipin/PEPSCAN procedure. The multipin procedure (Geysen et al., 1984) entails SPPS of a large number of overlapping peptides on individual polyethylene sticks. These so-called “pins” are mounted on a block in an arrangement of 12 columns and 8 rows that fit into the wells of enzyme-linked immunosorbent assay (ELISA) plates, thereby facilitating subsequent screening by ELISA (PEPSCAN) to identify the overlapping linear peptides that bind to an antibody (Tribbick, 2002). The introduction of Chemical Linkage of Peptides onto Scaffolds (CLIPS) has allowed the use of PEPSCAN to identify conformational epitopes (Timmerman et al., 2007).

6.1.1.2. Simultaneous synthesis and mapping using the SPOT procedure. Overlapping short peptides (approximately 15-mer) covering the amino acid sequence of the target polypeptide toxin can be synthesized by the "Spot-Synthesis Method" designed by Frank (1992). Briefly, peptides are prepared by SPPS on a planar cellulose support to facilitate their screening *in situ*. Droplets of solution with the reagents are applied onto the cellulose sheet and spread over a restricted circular area (spot). Antibody binding to peptides bound on the cellulose membrane can be directly analyzed on the sheet using secondary peroxidase-conjugated anti-IgG antibody in a similar manner to dot-blot analysis (Frank, 2002). Recently, high-throughput microarray synthesis has improved the SPOT approach (McBride et al., 2016).

An important advantage of multipin/PEPSCAN and SPOT is that if a cleavable linker is inserted between the peptide and the solid support (polyethylene stick or cellulose), the unprotected peptides can be released from the support for posterior analysis and/or characterization. Finally, both methods are affordable because they are based on a miniaturization strategy, allowing the simultaneous synthesis of only the required amount of peptide and in short period.

6.1.1.3. Simultaneous synthesis using tea-bags. First developed by Houghten (1985), this method consists of synthesizing each peptide independently in a "tea bag" reactor, where the common steps (washing and removal of the amino protecting group) are carried out in a polyethylene bottle using the same solution. In contrast, the coupling of each amino acid is performed simultaneously in a separate polyethylene bottle (one for each amino acid). Final global deprotection and cleavage is carried out independently for each peptide. This method allows the preparation of larger amounts of peptide compared to the techniques previously described, but screening should be carried out in a posterior step.

6.1.2. Epitope mapping using phage display peptide libraries

High-throughput epitope mapping can be performed using phage display libraries, first described by Smith (1985). This method consists of a peptide library expressed as a genetic fusion of a bacteriophage coat protein. Each peptide is displayed on the surface of a virion, while the DNA encoding the peptide is inside the virion. The combinatorial library is then amplified by propagation in *E. coli*. Subsequently, the phage particles are purified and the screening is performed, incubating the phages with the immobilized target protein. Non-interacting phage particles are washed. The adsorbed particles that contain peptides with affinity for the target protein are eluted and amplified by infection in *E. coli*. Amplification and screening are repeated numerous times with buffers of increasing astringency in order to obtain high-affinity ligands. The displayed peptide of the selected phages is identified by sequencing the corresponding coding region of the viral DNA after PCR amplification (Böttger and Böttger, 2009).

6.1.3. X-ray diffraction method

X-Ray diffraction or X-Ray crystallography method involves the co-crystallization of the antigen-antibody complex and can provide information about the interface of this complex and the residues that contribute to the binding (Toride King and Brooks, 2018). Crystallographic strategies have the disadvantage that they can be time-consuming, they require large amounts of purified sample, and they are limited by the quality of the crystals. However, these approaches can still identify both continuous and discontinuous epitopes and also provide information about binding strength. Given these features, crystallographic strategies are considered to be among the most accurate ways to map structural epitopes (Potocnakova et al., 2016).

6.1.4. Nuclear magnetic resonance spectroscopy in solution

Nuclear magnetic resonance spectroscopy in solution is a technique that is sensitive to the chemical environment involved in the antigen-antibody interaction (King and Mobli, 2010; Mobli et al., 2011). The residues at the interface can be identified by changes in residue-specific signals that participate in the formation of the complex. This technique can also give information about the structure, dynamics and binding energy of the complex antigen-antibody (Rosen and Anglister, 2009). A previous structural determination and a resonance assignment of the free antigen is required. However, this technique is limited to small and isotopically labeled proteins and is therefore not commonly used for epitope mapping (Nilvebrant and Rockberg, 2018).

6.1.5. Site-directed mutagenesis

Site-directed mutagenesis is a simple technique used to identify the key residues for the antigen-antibody interaction. It involves the mutation of a single amino acid and subsequent analysis to identify any loss of binding (Benjamin and Perdue, 1996). This is a laborious technique as only one amino acid is mutated at a time and mutants must be purified and evaluated for structural integrity. Alanine scanning mutagenesis technology is a variation, where non-alanine residues are sequentially substituted by one Ala at a time. This amino acid is used because of its inert methyl group side-chain. Subsequently, the corresponding change of the peptide-antibody binding activity is measured. When a key amino acid in the antigen is replaced by Ala, a loss or change in the binding capacity to the specific antibody is detected (Gershoni et al., 2007).

6.1.6. In silico B cell epitope prediction

In silico B cell epitope prediction and other computational tools consider the correlation between the localization of the epitope and physicochemical properties such as solvent accessibility, flexibility, polarity, antigenicity, hydrophilicity, surface exposure and turns (He and Zhu, 2015.). However, these methods lack precision as they can interpret only general immunogenic regions. In this regard, further improvements of these approaches are required (Nilvebrant and Rockberg, 2018).

6.1.7. Mass spectrometry (MS) epitope mapping

This method is based on the resistance of the antigenic region to proteolytic cleavage when it is bound to the antibody (Jemmerson and Paterson, 1986). The antibody is first covalently immobilized on a chromatographic solid support to obtain an immunoaffinity chromatographic matrix. Then MS samples are obtained by two approaches, namely epitope excision and epitope extraction. For the former, the antigen is adsorbed on the immunoaffinity matrix and then the antigen-antibody complex is digested with proteolytic enzymes. After removal of the unbound digestion products, the bound antigen fragments are desorbed and analyzed by matrix-assisted laser desorption/ionization MS or electrospray ionization MS. For epitope extraction, the unbound antigen is digested with the same proteolytic enzymes and the proteolytic digest is loaded on the immunoaffinity chromatographic matrix. After removal of the unbound peptides, the epitope fractions are recovered by elution for subsequent MS analysis (Opuni et al., 2018; Suckau et al., 1990).

6.1.8. Electron microscopy and cryogenic-electron microscopy

Electron microscopy is another technique that can be used for localizing the epitopes of larger antigens. This is a low-resolution method as it is incapable of detecting contact residues. Cryogenic-electron microscopy is an alternative approach that avoids the need for staining and fixing as it allows observations of rapidly frozen antigen-antibody complexes in physiological buffers (Nilvebrant and Rockberg, 2018).

6.2. Immunogen conjugates

As discussed earlier, the small regions of the toxin that interact with the antibody can be easily synthesized by SPPS. Peptides of 10–20 amino acids are optimal as antigens. Larger peptides may adopt their own

specific conformation which is often immunodominant over any primary structural determinants, which may not be reflected in the conformation of the sequence within the intact protein (Hancock and O'Reilly, 2005). Although peptides of 10–20 amino acids are satisfactory antigens and can bind to antibodies or B cell receptors, they are poor immunogens. To increase their immunogenicity, peptides can be coupled to an immunogenic protein called carrier. The conjugated immunogen usually consists of a large carrier protein and many peptide molecules attached to it through covalent conjugation strategies. The conjugates can stimulate an immunological response when administered to an animal, causing the production of antibodies against the venom toxin. Protein carriers commonly used include bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin, thyroglobulin, tetanus toxoid, diphtheria toxoid, and tuberculin purified protein derivative (Murphy et al., 2012).

In addition to antibodies against the target toxins, animals immunized with peptide-carrier conjugates also produce anti-carrier and anti-conjugate antibodies. Nevertheless, these antibodies do not generally imply any problem; however, in the case that they do, they can be easily removed by affinity chromatography with the carrier immobilized on a chromatographic solid support (Hancock and O'Reilly, 2005).

Many studies have reported the administration of immunogen conjugates to animals in order to stimulate the production of specific antibodies against scorpion and spider venoms (Bermúdez-Méndez et al., 2018; Duarte et al., 2010; Comis et al., 2009; Fischer et al., 2007; Inceoglu et al., 2006; Alvarenga et al., 2002; Zenouaki et al., 1997; Bahraoui et al., 1986). These antigens have several advantages over full venom, including high specificity, a good safety profile, ease of production and storage, and stability.

The peptides are covalently conjugated to the carrier molecule using an appropriate bifunctional reagent involving the amine or sulfhydryl groups of the peptide and the carrier protein. The choice of crosslinking methodology depends on the functional groups present in the carrier protein and the peptide, as well as the orientation of the peptide desired

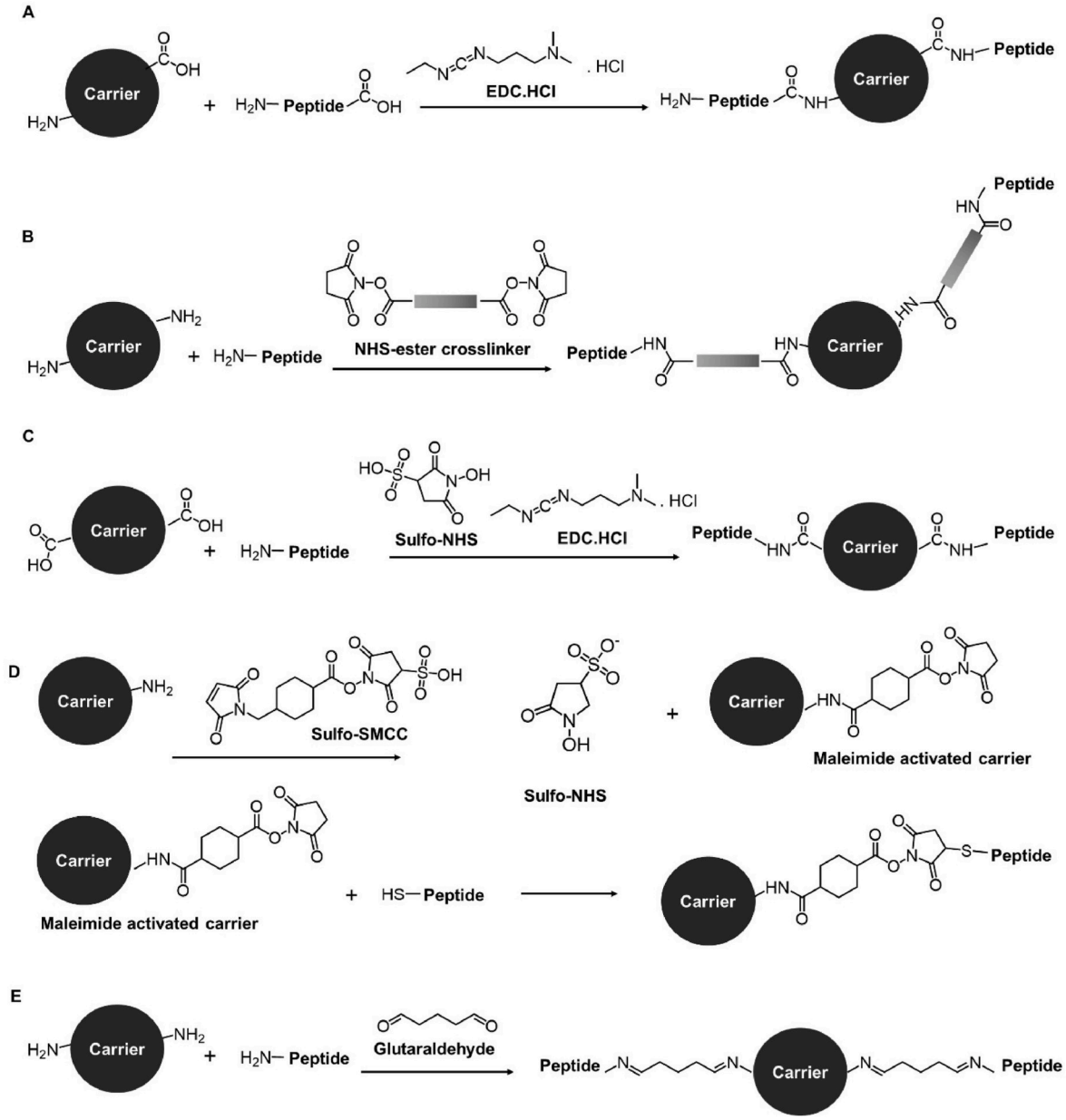

Fig. 5. Immunogen conjugate production by peptide epitope and carrier protein conjugation mediated by: A) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl); B) N-hydroxysulfosuccinimide ester (NHS) crosslinker; and C) reactive sulfo-NHS ester and EDC.HCl; and D) NHS ester-maleimide heterobifunctional crosslinker sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC); E) glutaraldehyde.

for appropriate presentation to the immune system. Here is a brief description of each of the techniques used, which are explained in detail in Hermanson (2013).

6.2.1. Epitope-carrier conjugation mediated by water-soluble carbodiimide

Water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl) reacts with the carboxylic groups present on either the carrier or the epitope to form a reactive O-acylisourea intermediate. The activated carboxylic group then reacts with a primary amine to form an amide bond, with the release of the EDC mediator as a soluble urea derivative. With this method, there is no bridging molecule between the epitope and the carrier (zero-length crosslinking procedure), thereby avoiding the generation of antibodies against the crosslinker bridge (Hermanson, 2013) (Fig. 5A).

6.2.2. Epitope-carrier conjugation mediated by NHS ester

6.2.2.1. Using a homobifunctional reagent containing NHS ester groups. This procedure consists of using a homobifunctional reagent containing NHS ester groups on both its ends. This approach allows conjugation of amine-containing peptide epitopes to amine-containing protein carriers in a single step and leads to the formation of stable amide bonds. To ensure multipoint attachment to the carrier, the peptide is added at a molar excess of 20–30 times that of the carrier (Hermanson, 2013) (Fig. 5B).

6.2.2.2. Creating a reactive sulfo-NHS ester. This approach consists of activating the carboxylate groups on the carrier protein with a carbodiimide such as EDC. HCl in the presence of sulfo-NHS, which is more compatible with the aqueous media than the NHS derivative. The resulting amine-reactive sulfo-NHS ester then reacts with amine-containing peptide epitopes, yielding an amine bond between the peptide and the carrier protein (Hermanson, 2013) (Fig. 5C).

6.2.3. Epitope-carrier conjugation mediated by NHS ester-maleimide heterobifunctional crosslinker

This method involves the use of a heterobifunctional crosslinker containing an NHS ester and a maleimide group such as sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC). Initially, the active sulfo-NHS end of sulfo-SMCC reacts with available primary amine groups on the carrier protein, forming an amide bond between the protein and the crosslinker, with the release of sulfo-NHS as a byproduct and leaving the carrier protein with a reactive maleimide group. The carrier protein is purified by gel filtration to remove excess reagents. Finally, the maleimide groups on the carrier protein react with sulfhydryl groups on the peptide epitope such as Cys residues, resulting in a stable thioether bond (Hermanson, 2013) (Fig. 5D).

6.2.4. Epitope-carrier conjugation mediated by glutaraldehyde

Glutaraldehyde is an effective homobifunctional crosslinker. Although this compound is frequently used to conjugate peptides to carrier proteins, the conjugates formed are usually of high molecular weight and can partially precipitate. In aqueous solution, glutaraldehyde undergoes many cyclization and polymerization reactions to form hemiacetal and aldol products, which can react with primary amine groups on carrier proteins and the peptide epitope. Glutaraldehyde can react by several routes to form covalent crosslinks with amine-containing molecules (Hermanson, 2013) (Fig. 5E).

6.3. Multiple antigenic peptide (MAP)

Another strategy to increase epitope immunogenicity is the use of a synthetic polylysine core typically containing four to eight branched amino acids to which peptide epitopes can be attached at each pendent side-chain amine group. This technique makes use of the epsilon amino group of Lys residues to obtain a branched core matrix, which can be used as a scaffold for subsequent peptide synthesis (Tam, 1988). This design is called the "multiple antigenic peptide" (MAP) system, and the final conjugate has many copies of the peptide antigens attached to each core structure (Fig. 6) (Joshi et al., 2013). The presence of a Cys residue allows straightforward dimerization to achieve the desired copies of the peptide.

7. Examples of applications of synthetic immunogens

7.1. Synthetic immunogens against spider venoms

7.1.1. Peptide immunogens against Loxosceles intermedia

Loxoscelism is the term used for envenoming by the South America *Loxosceles* genus spiders. The venoms of these arachnids are composed of a complex mixture of proteins.

Phospholipases D (PLDs), also called dermonecrotic toxins, sphingomyelinases D or SMases, are the primary agents responsible for loxoscelism. They comprise a family of toxin isoforms that may be involved in the adaptation of spiders and the effectiveness of the venom (Fukuda et al., 2017). Felicori et al. (2009) studied one isoform of PLD, the *Loxosceles intermedia* dermonecrotic protein isoform 1 (LiD1). Six different antigenic regions of LiD1 were found using the SPOT method and these were chemically synthesized. Animals immunized with these peptides or with the recombinant protein (rLiD1) were protected from dermonecrotic, hemorrhagic and edema-forming activities, thereby showing that the use of synthetic peptides for immunization is an effective approach (Felicori et al., 2009). An Ala scan of one of the epitopes indicated that four residues were conserved in the dermonecrotic family of *Loxosceles* proteins. These residues are key contributors to the antigenic recognition of peptide by antibodies (Dias-Lopes et al., 2010). Also, using the phage-display technique, together with informatic tools, other epitopes of LiD1 were identified. Peptide sequences that mimic epitopes (mimotopes) were synthesized and used to neutralize the toxicity of the spider venom, thereby showing their utility to produce therapeutic sera or for vaccination approaches (de Moura et al., 2011).

7.1.2. Peptide immunogens against Tegenaria agrestis

The bite of the North America hobo spider *Tegenaria agrestis* can cause local pain and necrosis. Using *in silico* approaches, segments of the toxins ITX-1 and ITX-3 of this spider, likely to be antigenic and useful in the design of a synthetic peptide vaccine, were found (Ingale, 2010; Sherkhane and Gomase, 2014).

7.1.3. Peptide immunogens against Atrax robustus

In Australia, a great number of human fatalities are attributed to the funnel-web spider *Atrax robustus*. Its venom knottin peptide robustoxin is a lethal neurotoxin with four disulfide bridges. It has been confirmed that the polypeptide loses its toxic properties when these bridges are broken. An open-chain derivative of robustoxin was chemically synthesized, protecting the eight Cys residues with acetamidomethyl group to prevent the formation of these bridges. Conjugation of this derivative to the carrier protein KLH via glutaraldehyde was necessary to increase its immunogenicity and for the generation of protective immunity to *A. robustus* venom in primates. Immunization with the same immunogen but in the absence of KLH had no effect against the toxin (Comis et al., 2009). Also, an open-chain derivative of robustoxin was chemically synthesized using enantiomeric D-amino acids to obtain the retro-inverso isomer, with the amino acid sequence reversed and chiral center inverted. The immunogenicity of the native sequence with natural L-amino acids was compared with the retro-inverso form. Both peptides were conjugated to carrier proteins. After mice immunization by intraperitoneally injection of KLH-peptide conjugates, good titers of anti-peptide antibodies were detected for the native and retro-inverso

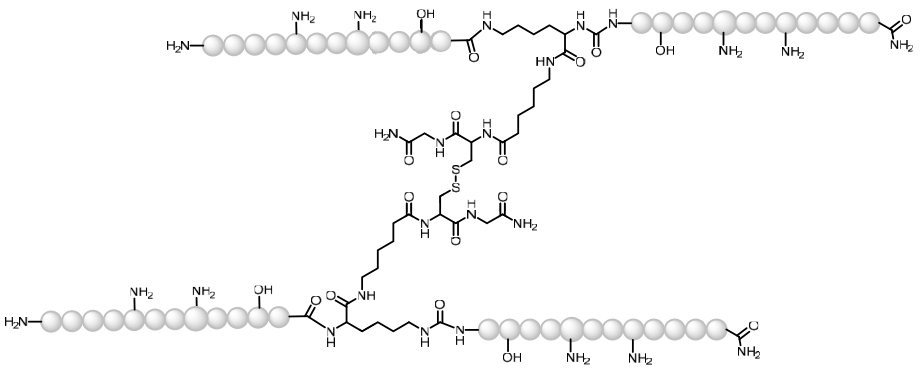

Fig. 6. Example of a Lys multiple antigenic peptide (MAP) with four copies of the antigenic peptide obtained by a dimerization through a Cys residue. ●: Amino acid residue.

forms of the peptides. The cross-reactivities of anti-sera with peptide-BSA conjugates were strong. Immunization by oral administration with natural peptide did not elicit anti-peptide antibodies whereas immunization with the retro-inverso showed high titers of anti-retro-inverso antibodies. These retro-inverso peptide antibodies had full cross-reactivity with the natural peptide conjugate. Most of the immunized mice survived after being challenged with *A. robustus* crude venom. This observation therefore demonstrates the usefulness of oral synthetic vaccines based on retro-inverso peptides (Fischer et al., 2007).

7.2. Synthetic immunogens against scorpion venoms

7.2.1. Peptide immunogens against Androctonus australis hector

A synthetic peptide mimicking toxin II of the North African scorpion *Androctonus australis hector* was designed and produced by SPPS. This peptide contains the entire sequence of toxin II (64 amino acid residues), with each half-cystine being replaced by the isosteric residue α-amino-butyric acid, thereby eliminating the disulfide bridges. This construct was non-toxic in mice. The synthetic peptide, either as a monomer or polymerized by means of glutaraldehyde, induced the production of antitoxin-neutralizing antibodies in immunized mice and rabbits. The immunized mice were protected against several lethal doses of the corresponding native toxin or whole scorpion venom (Zenouaki et al., 1997). Similar results were obtained by binding the synthetic peptide (sequence 50–59) of *A. australis hector* toxin II to BSA. Polyclonal antibodies raised against the immunogen conjugate neutralized the effects of toxin II in vivo (Bahraoui et al., 1986). Also, Devaux et al. (1993, 1997) identified epitopes in neurotoxin AaH II from the venom of this scorpion using the PESCAN method.

7.2.2. Peptide immunogens against Tityus serrulatus

Machado de Avila et al. (2004) used the SPOT method to characterize epitopes of the *Tityus serrulatus* toxins, such as the β-type toxins TsII and TsVII and the α-type toxin TsIV, the latter being the major lethal component of *T. serrulatus* venom. Linear epitopes of TsIV were discovered by this method. Also, linear and discontinuous epitopes of *T. serrulatus* TsNTxP, a non-toxic and immunogenic protein, were also identified. Anti-peptide antibodies were generated against the peptide epitopes covalently coupled to KLH and were found to neutralize the toxic effects of the venom (Alvarenga et al., 2002; Duarte et al., 2010).

7.2.3. Peptide immunogens against Centruroides noxious

Calderon-Aranda et al. (1995, 1999) studied seven synthetic peptides corresponding to the amino acid sequence of the Na^+-channel-affecting toxin II from the scorpion *Centruroides noxious*, the major component of the venom of this Mexican scorpion and one of its most toxic elements. All the peptides were immunogenic and some of them produced neutralizing antibodies, as verified by injecting the antisera with toxin into naive animals. On the other hand, Gazarian et al. (2000) used phage display libraries to map the epitope on the K^+ channel-blocking toxin, noxiustoxin, a short-chain toxin from the venom of *C. noxious hoffmann.* The most reactive mimotopes were injected into mice and showed the ability to induce antibodies reacting with noxiustoxin.

7.2.4. Peptide immunogens against Parabuthus transvaalicus

Inceoglu et al. (2006) reported on the ability of a polyclonal antibody raised against a single conserved synthetic peptide crosslinked to KLH to protect against the crude venom of *P. transvaalicus* in mice. The presence of many peptides with minor differences in their sequences explains the protective effect of an antibody generated against a common epitope of these peptides.

8. Conclusions

Although scorpion and spider venoms are still used for antivenom production through mammalian immunization, the great difficulty in capturing specimens and collecting their venom increasingly encourages the use of synthetic peptides as immunogens. Peptides can be synthesized in a straightforward manner by SPPS and immobilized on carrier proteins to increase immunogenicity. Significant advances in epitope design, as well as improvements in SPPS, assures the development of effective immunogens for the production of antivenoms with higher titers of antibodies against the most toxic components of these venoms.

Ethical statement

This review work does not involve the use of human subjects and animals.

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Silvia A. Camperi: Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Visualization, Resources, Funding acquisition. **Gerardo Acosta:** Investigation, Writing - original draft, Writing - review & editing, Visualization. **Gabriela R. Barredo:** Investigation, Writing - original draft, Writing - review & editing, Visualization. **Lucía C. Iglesias-García:** Investigation, Writing - original draft, Writing - review & editing, Visualization. **Cleópatra Alves da Silva Caldeira:** Investigation, Writing - original draft, Writing - review & editing, Visualization. **María C. Martínez-Ceron:** Writing - review & editing. **Silvana L. Giudicessi:** Writing - review & editing. **Osvaldo Cascone:** Writing - review & editing. **Fernando Albericio:** Investigation, Writing - original draft, Writing - review & editing, Visualization, Resources, Funding acquisition.

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