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Sequential oligopeptide carriers, SOC_n, as scaffolds for the reconstitution of antigenic proteins: applications in solid phase immunoassays

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Abstract

A new class of helicoid type sequential oligopeptide carriers (SOC), for anchoring antigenic epitopes, has been modeled from the repetitive Lys-Aib-Gly (SOC_n-I) and Aib-Lys-Aib-Gly (SOC_n-II) units aiming to the development of scaffolds with predetermined 3D structures. Conformational analysis showed that the SOC_n carriers adopt 3_{10}-helical structures, while the SOC_n-conjugates retain their original active conformations and they interact neither to the carriers nor to each other. It is concluded that the helicoid structure of SOC_n helps the reconstitution and/or mimicking of the native forms of the epitopes so that potent antigens are generated for developing specific, sensitive and reproducible immunoassays.

Keywords: Oligopeptide carriers; Antigenic proteins; Solid phase immunoassays

1. Introduction

Over the last decades, the rapid development of peptide technology has allowed the replacement of crude extracts with highly pure synthetic antigens. Short synthetic peptides can mimic linear and, to a certain extent, conformational epitopes and they can be used in solid–phase assays instead of complex antigens that are often difficult to purify and prepare in large quantities. Application of peptide epitopes as reliable substrates in antibody detection tests are not only a confirmation of the diagnosis, but may also point to a subset of the disease with a particular prognosis [1,2].

The conventional approach to obtain potent antigens or immunogens is to conjugate B and T cell epitopes to a protein carrier, as for example, bovine serum albumin, ovalbumin and keyhole limpet hemocyanin. However, several drawbacks as ambiguous composition and structure, of the protein–epitope conjugates, alteration of the biologically "active" conformation of the coupled epitopes and generation of non-specific immune responses originated from the protein carrier, frequently result from such conjugations [3–5]. To avoid the mentioned shortcomings, artificial carriers appeared in the literature the last decades as: the lysine core matrix (multiple antigenic peptide system) [6,7], the Pam_3-Cys-Ser (a palmitoyloxy lipopeptide) [8], the template assembled synthetic protein (TASP) [9,10] and dendrimers [11,12].

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With the aim to optimize epitope presentation and help in the reconstitution of an antigenic or immunogenic protein, a new class of carriers named sequential oligopeptide carriers (SOC), and SOC_n-I, have been modeled and successfully applied [13,14]. The SOC_n-I scaffold, formed by the repetitive motif Lys-Aib-Gly, incorporates lysine for anchoring of the antigen/immunogen, the α-aminoisobutyric residue for inducing a helicoid structure of the peptide backbone and glycine for its small stereochemical volume. One of the major guidelines in designing this new class of carriers was to build up constructions with predetermined 3D structure so that the attached antigenic/immunogenic peptide epitopes would obtain a defined spatial orientation.

Alternatively, a SOC_n-II formed by the Aib-Lys-Aib-Gly repeating tetrapeptide unit was constructed in order to study (i) the significance of the induction of the second Aib moiety in the overall conformation of the carrier and (ii) the influence of the position of the Lys-$N^{\varepsilon}H_2$ grafted antigens along the carrier's backbone in the antibody recognition.

Selected examples of SOC_n conjugates and their applications as antigenic substrates are presented in the following.

2. Synthetic aspects and conformational characterization

All the SOC_n-conjugate syntheses have been carried out according to the step-by-step solid–phase peptide synthesis principles [15–17]. Taking advantage of the big variety of protecting groups and by applying orthogonal protection is feasible to shape alternate ABAB motifs were A and B represent different epitopes (e.g. two types of B cell epitopes, B and T cell epitopes, CD4+ and CD8+ T-cell epitopes). Two N-protecting groups Boc- and Fmoc- are required for the attachment of identical copies of epitopes to the Lys-$N^{\varepsilon}H_2$ groups of the carrier on the Pam resin [18,19]. Orthogonal systems with three N-protecting groups as Boc-, Fmoc-, Alloc- (Pam resin) or Fmoc-, Alloc-, Mtt- (Wang resin) are needed for coupling two different copies of epitopes [20] (Figs. 1–3).

The structural characterization of SOC_n, was carried out by ^{1}H NMR experiments and molecular modeling [13,14]. Intense NOE connectivities, appearing between successive amide protons, are compatible either with a helical conformation or with a random structure. The latter has to be excluded due to the following findings: (i) the low absolute temperature

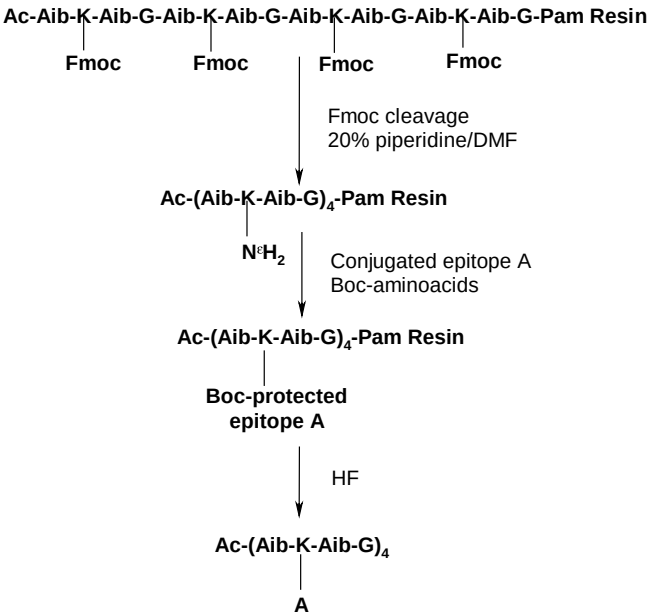

Fig. 1. Steps in the synthesis of SOC_n-II conjugates with four epitope identical A copies on Pam resin.

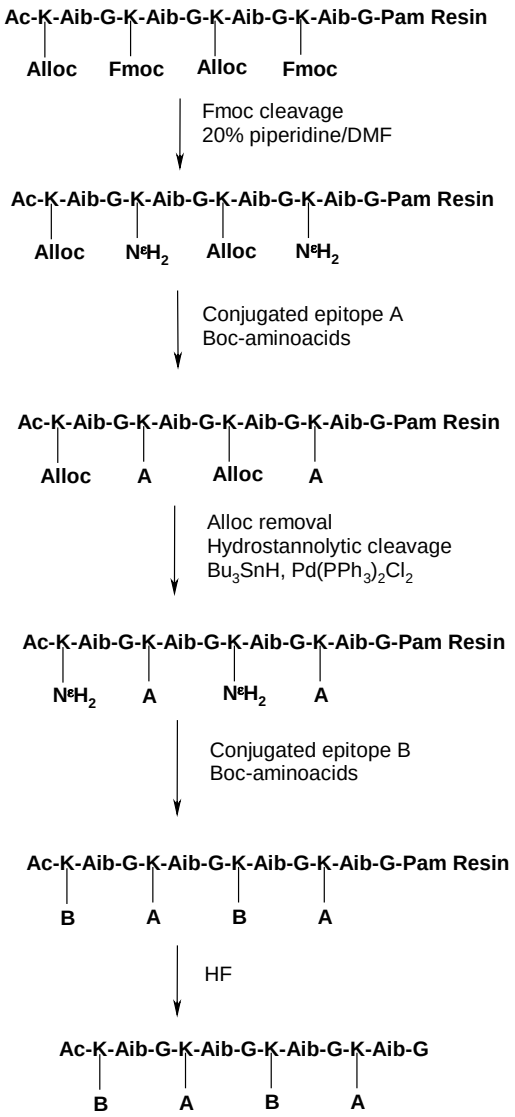

Fig. 2. Steps in the synthesis of SOC_n-I conjugates with two different epitope copies (A and B) on Pam resin.

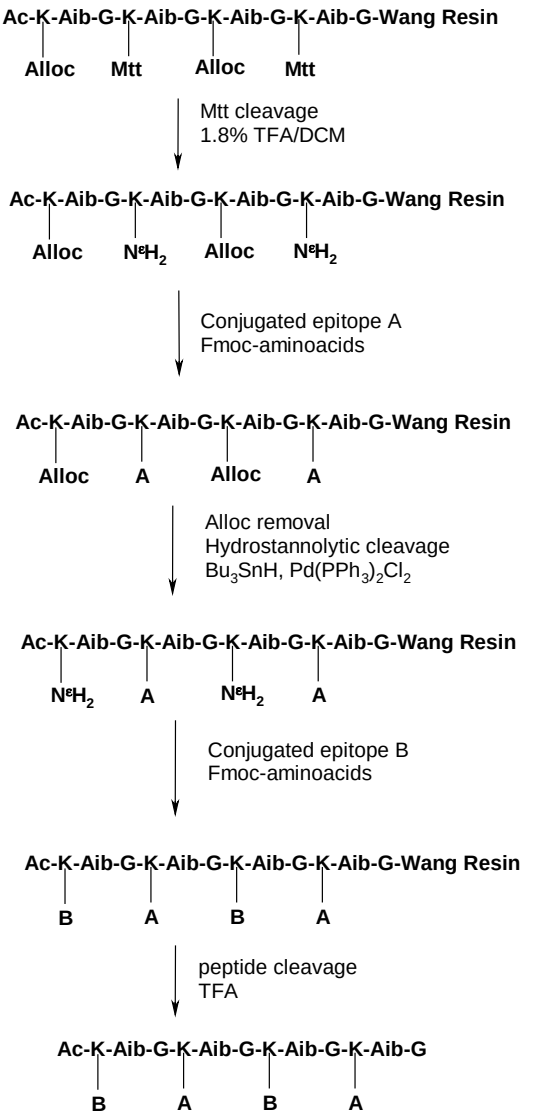

Fig. 3. Steps in the synthesis of SOC_n-I conjugates with two different epitope copies (A and B) on Wang resin.

coefficient values of all the Lys-NHs indicate that they are involved in intramolecular interactions, (ii) the variation of the $J_{N\alpha}$ and $J_{N\alpha'}$ coupling constants for the glycines, except the *C*-terminal one, versus the torsional angle Gly-Φ indicates that the Gly-Φ assumes a defined value about $\pm 70^{\circ}$ and (iii) a remarkable similarity between the amide proton chemical shifts of the same repeating residue, when increasing the carrier length, indicates that the Lys-Aib-Gly segments share a common repetitive conformation initiated from the carboxy end of SOC_n.

From the preceding NMR data it is concluded that the carrier adopts a rigid conformation with some regularity. The NMR data were introduced in MD calculations in order to refine the SOC_n structure. Considering the main conformational angles of the time-averaged structure and the average interproton distances, estimated from NOE measurements,

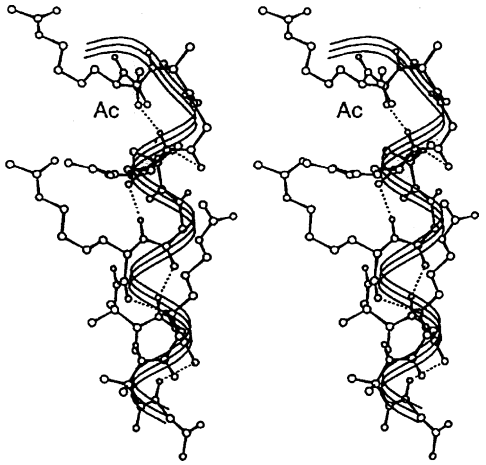

Fig. 4. Minimized average structure of the sequential oligopeptide carrier (SOC_4) from the in vacuo-restrained MD simulation during the last 20 ps. Comparison to the ribbon representing a canonical 3_{10}-helix of the SOC_4 backbone [14] (Copyright 1996, with permission from Elsevier Science.

a time-averaged structure stabilized by a network of hydrogen bonds was obtained. The most frequent ones are of the $i + 3 \rightarrow i$ type, and effectively involve the NHs having the lower absolute temperature coefficients. The time-averaged structure of SOC_n is a distorted 3_{10}-helix with somewhat curved axis and rms deviation between every atom pair of a canonical 3_{10}-helix and the SOC_n of less than 1.4 Å (Fig. 4).

Conformational studies of SOC_n-II were realized by the combined use of FTIR, CD and ^{1}H NMR. The presence of amid I band at $\sim$1660 cm^{-1} in the solid state and organic solvents provides convincing evidence in support of 3_{10}-helices, since it has been demonstrated, using model compounds incorporating Aib, that the main amide I band of fully stable 3_{10}-helices occurs at this frequency. CD measurements (Fig. 5) suggested that the helical content of SOC_n-II increases progressively as a function of the carrier's length upon addition of SDS [19].

^{1}H NMR analysis of SOC_n-II using COSY, HOHAHA and NOESY experiments revealed the occurrence of intense NOE connectivities between successive amide protons and medium-range dNN (i, $i + 2$), dαN(i, $i + 2$) and dαN (i, $i + 3$) cross peaks. These findings argue in favor of a helical structure, whereas the appearance of dαN (i, $i + 2$) are diagnostic for a 3_{10}-helix [19,21,22].

The SOC_n-conjugates were also studied by ^{1}H NMR spectroscopy. It was found that the SOC_n carriers retain their helical structure even after anchoring, from the Lys-N$^{\varepsilon}H_2$ groups, of the peptide epitopes. Moreover, the coupled peptides do not interact either with each other or with the carrier and they also preserve their initial structural elements and bioactivity [23,24]. This was further confirmed by comparing the ^{1}H NMR data of SOC_5-I-$(PPGMRPP)_5$ and SOC_4-II-$(PPGMRPP)_4$ conjugates, which both have approximately the same backbone length of carrier. As reference compound used was the PPGMRPP-NH_2 epitope, it was found that the PPGMRPP sequence anchored either to SOCn-I or to SOC_n-II and adopts similar to the free PPGMRPP-NH_2 conformation [19].

3. SOC_n-conjugates as antigenic substrates in immunoassays

3.1. *[Ala76]MIR-$SOC_{4,5}$-I*

Myasthenia gravis (MG) is an extensively studied autoimmune disease, and mainly a well characterized autoantigen involved in the disease, the skeletal AChR of the neuromuscular junction, and a pentameric membrane glycoprotein. MG is characterized by weakness and fatigability of the skeletal muscles. The detection and measurement of anti-AChR antibodies in the sera of suspected MG patients constitutes the most objective and quantitative diagnosis of the diseases [25]. In particular, the main immunogenic region (MIR) of the α subunit of the AChR, spanning the sequence 67–76, WNPADYGGIK, is the principal target of autoantibodies in myasthenic patients resulting in loss and blockage of the AChR function and failure of neuromuscular transmission [26,27]. Alanine substitution experiments showed that the [Ala^{76}]MIR analogue is better recognized by anti-MIR mAbs, compared to MIR. Soluble [Ala^{76}]MIR-SOC_n–I conjugates were found at least as efficient as the [Ala^{76}]MIR monomer in inhibiting, after preincubation, two anti-MIR mAbs suggesting a specific antibody recognition. It was also found that the SOC_n-I carrier bearing four or five copies of [Ala^{76}]MIR experiences up to a 10-fold increase in MIR binding capacity (Fig. 6). These findings indicate a clear advantage in using

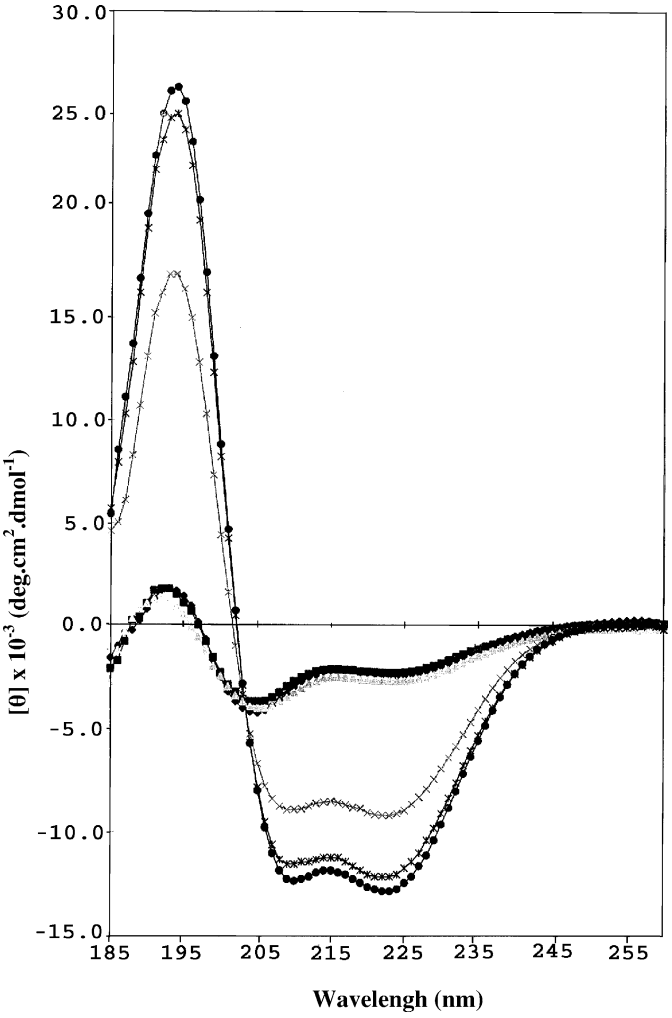

Fig. 5. CD spectra of SOC_4-II in phosphate buffer as a function of SDS concentration. Peptide concentration 10^{-4} M. SDS concentrations: (◆) 0, (■) 1.5, (▲) 3, (X) 6, (★) 15, and (●) 30 mM [19] (Copyright 2000, with permission from John Wiley & Sons).

[Ala^{76}]MIR-SOC_n rather than MIR peptides as potent antigens in diagnostic assays or in elaborating specific resins for antibody depletion [13,14].

3.2. *(La/$SSB_{349-364}$)$_4$-SOC_4-I*

The humoral autoimmune response in patients with Sjogren's syndrome (SS) and systemic lupus erythematosus (SLE) is primarily directed against the protein components of the Ro/La RNP ribonucleoprotein complex in which at least three immunologically distinct proteins participate namely, Ro/SSA (60 kDa), Ro/SSA(52 kDa) and La/SSB. Antibodies against the La/SSB autoantigen belong to a clinically important group of antinuclear antibodies that recognize ribonucleoprotein autoantigens. These autoantibodies are found in 40–87% of patients sera with Sjogren's syndrome (pSS) and in 10–15% of sera from patients with systemic lupus erythematosus [28].

Recently, four linear distinct B cell epitopes of the La/SSB autoantigen have been determined, using overlapping synthetic peptides. These epitopes TLHKAFKGSIFVVFDSIESA (145–164), ANNGNLQLRNKEVTWEVLEG (289–308), VTWEVLEGE-

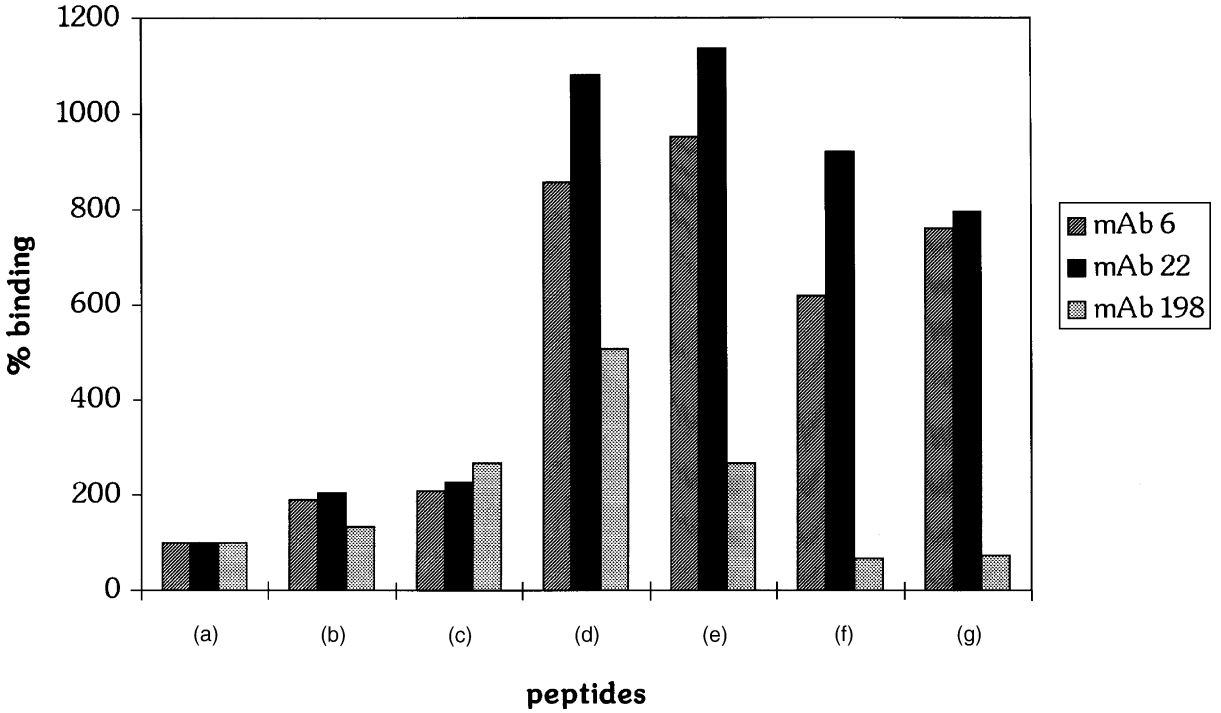

Fig. 6. Monoclonal antibody binding to [Ala^{76}] MIR (a) and (WNPADYGGIA)-SOC$_n$: (b) $n = 2$, (c) $n = 3$, (d) $n = 4$, (e) $n = 5$, (f) $n = 6$, (g) $n = 7$ [24] (Copyright 1999, with permission from Academic Press).

VEKEALKKIIE (301–320), and GSGKGKVQFQG-KKTKFASDD (349–368) were recognized by anti-La/SSB positive sera from SS and SLE patients [29].

The most sensitive and specific peptide 349–364 was covalently attached to the tetrameric carrier SOC$_4$-I, (Lys-Aib-Gly)$_4$, and used for immunoassay ELISA development [30]. Assays based on the recombinant native La/SSB protein, the La/SSB-*C* terminus (215aa) and the *N*-terminus were also developed and compared with the SOC$_4$ peptide-based assay. Eigthy-eight percent of anti-La/SSB positive sera were reactive with both the synthetic peptide (La/SSB$_{349–364}$)$_4$-SOC$_4$-I and the recombinant La/SSB, 83% of sera were reactive with the *N*-terminus and 68% of sera with the *C*-terminus. Using sera that were anti-Ro/SSA positive but anti-La/SSB negative, 37% were reactive with the recombinant protein, 26% with the La/SSB *N*-terminus, 33% with the La/SSB *C*-terminus and only 11% with the synthetic peptide epitope coupled to the SOC$_4$-I carrier (Table 1). These results suggest that (La/SSB$_{349–364}$)$_4$-SOC$_4$-I exhibits high sensitivity and specificity for the detection of anti-La/SSB antibodies and consequently it can be utilized as reliable substrate in immunoassays. Moreover, the (La/SSB$_{349–364}$)$_4$-SOC$_4$-I synthetic construct has the same sensitivity for the detection of anti-La/SSB positive sera as the recombinant protein underlining the significance of the presented construct.

3.3. *(PPGMRPP)$_5$-SOC$_5$-I*

The main target of autoimmune reactivity in SLE, the prototype immune disease in humans, are the double-stranded DNA (ds DNA), the histones and the small nuclear ribonucleoprotein particles (UsnRNPs), which are complexes of URNAs with proteins. Two main autoantibody reactivities have been recognized against UsnRNPs, the anti-Sm (U1-U6 RNA-protein complex) antibodies, which mainly recognize four polypeptides B′, B, D and N in complex with U1, U2, U4/U6 and U5 RNAs (Sm autoantigen) and the anti-U1RNP(U1RNA-protein complex) antibodies, which recognize the peptides 70 kDa, A and C in

Table 1
Comparison of the $(La/SSB_{349–364})_4$-SOC_4-I construct with recombinant La/SSB(rLa), *N*-terminus of La/SSB(La-N) and *C*-terminus of La/SSB(La-C) in anti-La/SSB(±)/Ro/SSA(+) ELISA tests

Antigen	Reactivity (%)		Specificity (%)
	anti-La/SSB(+)/anti Ro/SSA(+)	anti-La/SSB(−)/anti Ro/SSA(+)	
$(La/SSB_{349-364})_4$-SOC_4-I	88	11	89
rLa/SSB	88	37	63
La-N	83	26	74
La-C	68	33	67

complex with U1RNA (U1RNP autoantigen) [31,32]. Anti-Sm antibodies are usually considered highly specific for SLE, while anti-U1RNP antibodies are found in high titers in patients with mixed connective tissue disease (MCTD), although anti-U1RNP antibodies are also present in SLE [33].

The PPGMRPP epitope exists in three copies in Sm (B, B′ and N proteins) and appears to be one of the dominant proline rich epitopes of the autoantigen [34]. This epitope was coupled in five copies in SOC_5-I and the $(PPGMRPP)_5$-SOC_5 construct tested against sera with different autoantibody specificities as anti-Sm, anti-U1RNP, anti-Ro(SSA)/La(SSB), ANA^+/ENA^- (antinuclear antibody positive, but negative for antibodies to extractable nuclear antigens) exhibited, in ELISA tests, 98% sensitivity and 68% specificity for anti-Sm antibodies and 82 and 86%, respectively for anti-snRNPs.

Taking in consideration that in ELISA experiments, using the Sm/U1RNP purified complex (snRNPs), the sensitivity in detecting anti-snRNP was 74%, we conclude that $(PPGMRPP)_5$-SOC_5 is a better alternative as an antigenic substrate in ELISA tests [35].

3.4. $[(PPGMRPP)_2(PPGIRGP)_2]$-SOC_4-I

The PPGMRPP and PPGIRGP main epitopes of Sm, which are not found in U1-A and U1-C autoantigens, were coupled in duplicate to the SOC_4 carrier to form the $[(PPGMRPP)_2, (PPGIRGP)_2]$-$SOC_4$ diepitopic construct, as a mimic of the native Sm, in order to minimize the U1-RNP cross-reactivity. In fact the diepitopic conjugate is poorly recognized by the anti-U1RNP positive sera in ELISA tests [20]. Therefore, the combined application of the diepitopic construct and the $(PPGMRPP)_5$-SOC_5 [35] could be an easy way to discriminate SLE and MCTD patients sera (Table 2).

3.5. $(PPGMRPP)_4$-SOC_4-II

The Sm epitope PPGMRPP was coupled to the SOC_4-II carrier and the $(PPGMRPP)_4$-SOC_4-II conjugate was tested [19]. Comparison of the sera reactivity with anti-Sm and anti-U1RNP specificities in anti-$(PPGMRPP)_4$-SOC_4-II and anti-$(PPGMRPP)_5$-SOC_5-I ELISA experiments, where the amount of the PPGMRPP epitopes is the same in both ELISA, is illustrated in Fig. 7. Surprisingly, the binding capacity of the tested sera to the SOC_n-II conjugate was found rather limited compared to SOC_n-I. Although the distance between consecutive lysines is longer in SOC_n-II than in SOC_n-I, $d(i, i+3) < (i, i+4)$ and

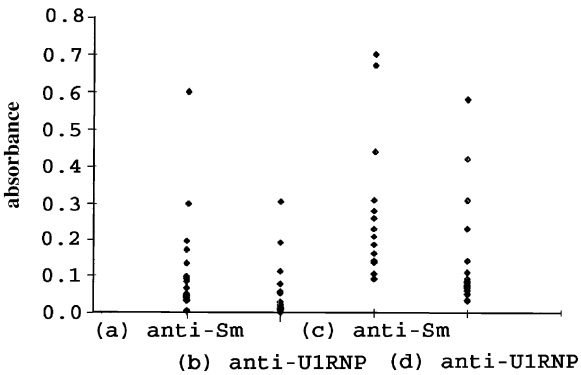

Fig. 7. Reactivity of sera with anti-Sm and anti-U1RNP specificities in anti-$(PPGMRPP)_4$-SOC_4-II (a and b) and anti-$(PPGMRPP)_5$-SOC_5-I (c and d) ELISA. The upper limit of normal sera was calculated as the mean of optical density plus 3 SD and was 0.085 absorbance units for (a and b) and 0.120 absorbance units for (c and d) [19] (Copyright 2000, with permission from John Wiley & Sons.

Table 2
Reactivity of sera with various autoantibody specificities in anti-$(PPGMRPP)_5$-SOC_5-I and anti-$[(PPGMRPP)_2,(PPGIRGP)_2]$-$SOC_4$-I ELISA tests

Antigenic substrate	Anti-Sm (%)	Anti-U1RNP (%)	Anti-Sm/anti-U1RNP (%)	Normal (%)
$(PPGMRPP)_5$-SOC_5-I	98	65	98	3
$[(PPGMRPP)_2, (PPGIRGP)_2]$-$SOC_4$-I	95	13	88	3

one should expect a more favorable antibody recognition free of steric hindrances, it seems that this is not the case.

One may note that the functional groups of all lysines, which occupy the (i, $i + 4$) positions of SOC_n-II, are located all around the axis in every three residue turn of the 3_{10}-helix, while in the SOC_n-I carrier the lysine side chains (i, $i + 3$) are located on one site of the helix (Fig. 8). Consequently, it is likely that the adsorption of the $(PPGMRPP)_n$-SOC_n-II conjugate to the ELISA plate, which is realized mainly by the PPGMRPP peptides, is less strong due to their hydrophilic nature, compared to the $(PPGMRPP)_n$-SOC_n-I, which is achieved by the rather hydrophobic oligopeptide backbone of the carrier. Thus, the limited coating of the $(PPGMRPP)_n$-SOC_n-II to the ELISA plate, as well as the fact that the adsorbed PPGMRPP antigenic peptides of the carrier are not disposed to the antibodies, may explain the rather low binding level of the $(PPGMRPP)_n$-SOC_n-II to the anti-Sm/U1RNP sera, compared to $(PPGMRPP)_n$-SOC_n-I.

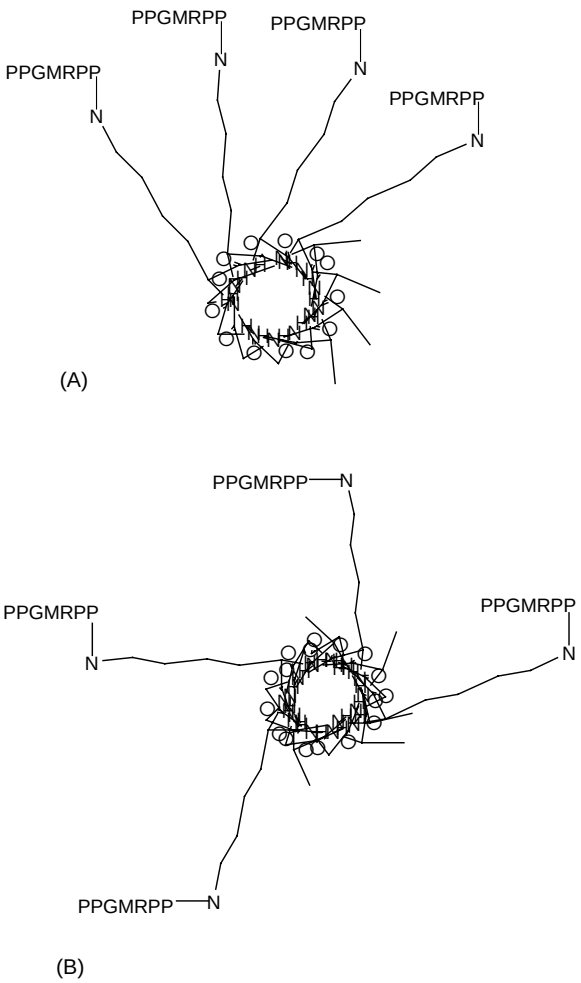

Fig. 8. Distribution of the Lys-$N^{\varepsilon}H$ groups occupying the i, $i+3$ and i, $i+4$ positions in SOC_n-I (A) and SOC_n-II (B), respectively.

4. Conclusions

The major target in the design of the presented oligopeptide carriers (SOC_n) was to construct an artificial vector with structural rigidity and regularity, so that peptide epitopes could be anchored without conformational restrictions and steric hindrances. ^1H NMR studies and molecular modeling showed that SOC_n adopt a distorded 3_{10}-helical structure, which allows a favorable orientation of the lysine side chains and therefore of the attached peptides.

Conformational analysis by ^1H NMR spectroscopy, of the SOC_n-conjugates pointed out that the peptides anchored to SOC_n retain their original "active" conformation and they do not interact neither to the carrier nor to each other confirming thus our initial design.

The significance of the position of the Lys-$N^{\varepsilon}H_2$ grafted epitopes along the SOC was highlighted by comparing SOC_n-I and SOC_n-II. It was assumed that despite the well-defined structural motif (pronounce 3_{10}-helix) of SOC_n-II, the distribution of lysines (i,

$i + 4$) all around the axis of 3_{10}-helix is less favorable, compared to the SOC_n-I lysines (i, $i + 3$) located on one site of the helix for a satisfactory antibody recognition.

The SOC_n-conjugates, when used as antigens, displayed significant biological reactivity, while the developed immunoassays were sensitive, convenient and reproducible in screening antibody specificities related to autoimmune diseases. It is very probable that the helicoid structure of SOC_n offers an optimal epitope presentation and helps the reconstruction and/or mimicking of the native antigen.

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