Protein Segments with Conformationally Restricted Amino Acids Can Control Supramolecular Organization at the Nanoscale

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1. INTRODUCTION

One of the major challenges that modern chemistry faces in the nanotechnological era is how to materialize ideas conceived in organic and inorganic chemistry laboratories to obtain *stable* nanoscale three-dimensional structures. In many cases, such ideas involve the combination of molecular components, i.e. building blocks,[1] that form complex supramolecular structures upon association. To effectively build a nanostructure, several steps need to be undertaken, such as preparing the building blocks, promoting their assembly, and processing the final product. One of the important unsolved questions is how to limit the size of the nanocomponents during the synthesis[2] (especially if they are based on polymeric compounds) and how to accurately drive the components into the desired three-dimensional assembly.[3] An illustrative case of the first problem can be found in carbon nanotubes, the most common of the nanostructures fabricated nowadays. While it is possible to produce significant amounts, controlling either their molecular size or their layer multiplicity is still a major hurdle. Their preparation requires a subsequent separation process, which depends on their molecular size and molecular shape.[4]

Another important related problem involves imposing the 'proper' shape on each building block to favor their desired assembly. Organic molecules with open-chain aliphatic segments present conformational freedom, which often characterizes the unassociated blocks. Acquiring the 'proper' molecular shape for the assembly implies promoting a preferred conformation. Since the assembly is a spontaneous process that requires a particular fold, the exploration of the conformational space presents a serious difficulty hampering the construction of nanostructures. Currently, the control over the ordered association of the molecular components is still limited. In spite of this, a significant number of successful cases of driven assembly have been reported, such as the use of heavy metal particles as nucleation points for growing the nanostructure.[5] Hence, both the conformation of the individual building blocks and their assembly process are intimately related, focusing substantial attention in nanotechnology.

The convergence of nanotechnology and life sciences has led to *nanobiology*. Various literature definitions have attempted to describe this discipline. We have recently described it as the combination of tools, ideas, and materials from more traditional fields such as physics, chemistry, mathematics, and computer science with emerging new concepts and problems in nanoscience and biology.[6] When a construct is designed for biomedical applications, the physicochemical conditions controlling the conformational ensemble and the assembly need to be mild. At the same time, such *in vivo* conditions lead to a drastic reduction in the palette of potential chemical blocks. Under these circumstances, the core components of biological macromolecules become strong candidates.

Among the natural building blocks, protein segments are the most suitable candidates given their intrinsic conformational features that allow formation of complex three-dimensional structures.[7] Several strategies were tested to bias the conformational space of peptide building blocks, to guide their proper assembly into three-dimensional nanostructures.

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The most common strategy reduces the conformational freedom by introducing topological 'obstacles' in the peptides, mainly manifested as covalent links. It is very common to bind the N- and C-termini giving rise to cyclic molecular systems. Such a strategy has been successfully used to construct nanotubes and nanochannels of different shapes, from narrow rods to hollow tubes.[8] However, cyclization is a restriction that induces the formation of rodlike structures. If narrow rods are not wished, the reduction the conformacional space can also be achieved by setting a network of disulfide bridges that affixes the desired molecular shape. Again, this approach, though effective, limits the dynamical applications of the final construct, while the amount of natural peptides that form determined conformational patterns based on covalent links is relatively small.[9] To obtain different shapes, a reasonable alternative is to bias the conformational space of specific parts of the building blocks instead of imposing global topological constraints such as through cyclization. In this case, identification of the key sequence positions that most influence the building block structure is required. The amino acids occupying these positions are then replaced by non-natural analogs exhibiting intrinsically reduced conformational freedom. These rigidify the building block in the desired conformation. This strategy has already proved successful when applied to small peptide sequences to promote the formation of distinct helix conformations or even regular nanostructures,[10] based on the ordered disposition of the targeted conformational motif.

This manuscript outlines our recent advances in the use of conformationally restricted amino acids as chemical tools to limit the conformational preferences of protein segments. As stated above, such conformational restrictions become a necessary step to promote the correct 3D organization of the isolated building block, which is an essential feature to guarantee their correct assembly without imposing severe physicochemical conditions. Overall, our strategy is based on the rational that rather than engineer a building block with a preferred conformation *ab initio*, it is preferable to choose a naturally occurring building block presenting such a conformation and rigidify it via targeted replacements.

2. CYCLIC A-TETRASUBSTITUTED AMINO ACIDS

The conformational properties of α-alkylated α-amino acids have attracted considerable attention.[11−13] The simplest of these α-tetrasubstituted residues is α-methylalanine, also denoted α-aminoisobutyric acid (Aib), which results from the replacement of the α hydrogen of alanine (Ala) by a methyl group. This change increases the steric hindrance around the α carbon atom, promoting helical peptide arrangements.[11−14] A feasible strategy to generate α-alkylated residues with even more restricted conformational flexibility involves connecting the side chain to the α carbon (Figure 1). This cyclization process gives rise to 1-aminocycloalkanecarboxylic acids, which are cyclic α-tetrasubstituted residues known in the abbreviated form as Ac$_n$c (n refers to the size of the cycle). Among the Ac$_n$c series, the intrinsic conformational propensities of the cyclopropane (Ac$_3$c), cyclobutane (Ac$_4$c), cyclopentane (Ac$_5$c), and cyclohexane (Ac$_6$c) members (Figure 1) have been extensively investigated.[11−13,16−21] Results indicated that the preferences of these amino acids strictly parallel
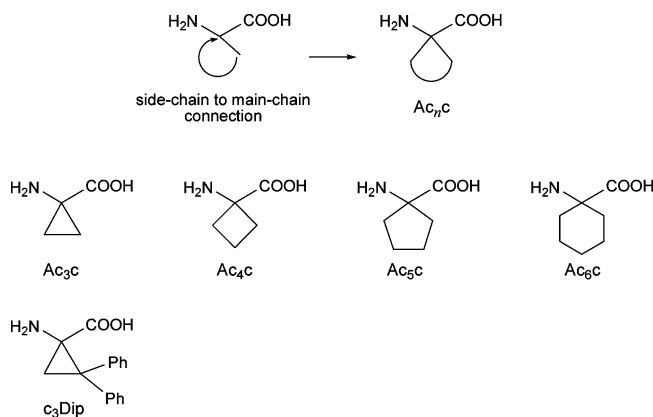

Figure 1. Cyclic α-tetrasubstituted amino acids (Ac$_n$c, $n = 3−6$) obtained by connecting the side chain to the α carbon. Structure of an Ac$_3$c derivative bearing two geminal phenyl substituents (c$_3$Dip).

those of Aib, i.e. they induce folded structures in the 3_{10}-/ α-helix region ($\varphi, \Psi \approx \pm 60°, \pm 30°$), with some distortion in the case of Ac$_3$c, which prefers the spatially close *bridge* region ($\varphi, \Psi \approx \pm 80°, \pm 0°$). In contrast, semiextended or fully extended conformations are forbidden for these residues.

The presence of additional substituents in the cycloalkane ring may modulate the conformational tendencies of the corresponding Ac$_n$c residue according to their size, orientation, and chemical nature. For example, we have carried out a structural study[22−32]—theoretical and experimental—of Ac$_n$c residues ($n = 3−6$) incorporating one or more phenyl substituents at the β position. Steric and electronic interactions between the peptide backbone and the rigidly held aromatic substituent have proved useful in stabilizing different types of β-turns or in inducing novel turn types never observed before. Recently, we have shown that the conformational preferences of an Ac$_5$c derivative bearing the arginine (Arg) side chain are dictated by electronic interactions between the amide groups in the backbone and the guanidinium substituent.[33]

3. HELICAL PROTEINS AS SCAFFOLD TEMPLATES FOR NEW NANOSTRUCTURES

The search for suitable molecular scaffolds involves a wide range of chemical disciplines and often ends up converging at protein structures. Many reasons can be found to support the use of such natural macromolecules: their biological origin makes them potential biocompatible systems, their universal abundance, their natural selection through millions of years of evolution, etc. However, the most interesting point is the fact that, in nature, they spontaneously form coherently folded structures. In many cases, they even form complex organizations of assembled macromolecules with features needed to build synthetic nanostructures. Scaffolds that best suit our needs can be picked. The easiest strategy involves the search of simple structural motifs in proteins to be used as building blocks for our nanoconstructs. Within this context, highly repetitive secondary structure motifs have already been used by several research groups to build new nanostructures.[34−37]

Taking into account these considerations, we decided to combine the idea of using repetitive secondary structure

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motifs found in crystallized proteins with our previous work on amyloid structures[38−41] to design new structures based on β-helix organization. In such investigations we explored the relative stability of different molecular assemblies using atomistic simulations based on classic Force Fields. The simulations were performed using the Molecular Dynamics (MD) technique that consists of numerically solving the Newton's equation of movement for a system represented by classical particles, i.e., without accounting for potential rearrangements in the electronic structure. The potential energy of the system is described by a summation of independent energy terms, and each of them has previously been parametrized to reproduce the organization of real molecular systems. The collection of empirical parameters and analytical expressions that account for the potential energy of a system is know as *Force Field*.[42] In our simulations we used both Charmm[43] and Amber[44] force fields, which are designed to correctly reproduce the dynamics of proteins and peptides conformation. Due to the large amount of atoms involved in realistic model systems, highly scalable codes are required in order to use as many CPU per MD simulation as possible, the NAMD[45] program being our preferred choice.

The studied nanostructures were based on the fold of β-helical proteins. This arrangement consists of a repetitive helical strand-loop motif, where each repeat contributes a strand to one or more parallel β sheet(s). The left-handed β-helical fold appears particularly useful since its tubular structure is regular and symmetrical and is often stabilized by a network of interactions between similar residues in consecutive coils.[46] We therefore decided to take advantage of the tubular nature of β-helical proteins to design nanosize building blocks to be used in the construction of fibrils without the need to perform many constitution manipulations. Our strategy was based on the massive use of molecular simulations of *de novo* built nanoassemblies,[1,47] each constructed as *n*-replicas of the selected motif (Figure 2). After studying the sequence and conformation of numerous β-helix protein crystals, small protein segments characterized by a highly regular secondary structure were selected as structural motifs (below referred to as *building blocks*). Each building block was replicated into a regular assembly of motifs, and its theoretical stability under physiological conditions was assessed using long atomistic molecular dynamics simulations. The results obtained using this approach allowed us to discard a large number of selected motifs and to focus only on those building blocks leading to stable simulated complexes. This set of simulations pointed, in particular, toward a promising segment, which became our reference structural motif for further investigations.[47]

The segment excised from *E. coli* galactoside acetyltransferase [Protein Databank (PDB) code *1krr*][48] comprising residues 131 to 165 (hereafter named *1krr*$_{131−165}$) showed an inherent propensity to retain its native fold (Figure 3). This was attributed to a complex network of inter-residue interactions involving not only amino acids that are relatively close in the sequence but also residues located in different regions of the motif.[49] The *1krr* segment and its nanoconstruct derivatives were submitted to extensive *in silico* testing under many different environments and conditions, and these demonstrated its ability to stabilize nanotube assemblies in all cases studied. In further studies, we also investigated in
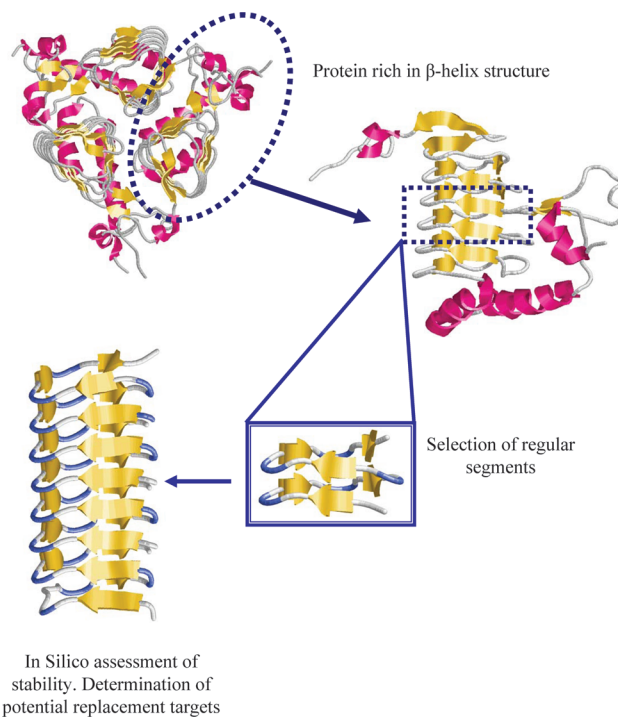

Figure 2. Schematic illustration of the strategy followed for the selection of building blocks from proteins. A crystal structure is chosen when presenting domains with high contents of secondary structure. Then, highly repeating motifs are excised from the protein to build motifs featuring high structural regularity (framed). A nanostructure is constructed by stacking (self-assembling) several copies of a given building block. The stability of the nanoconstruct is checked using Molecular Dynamics simulations.
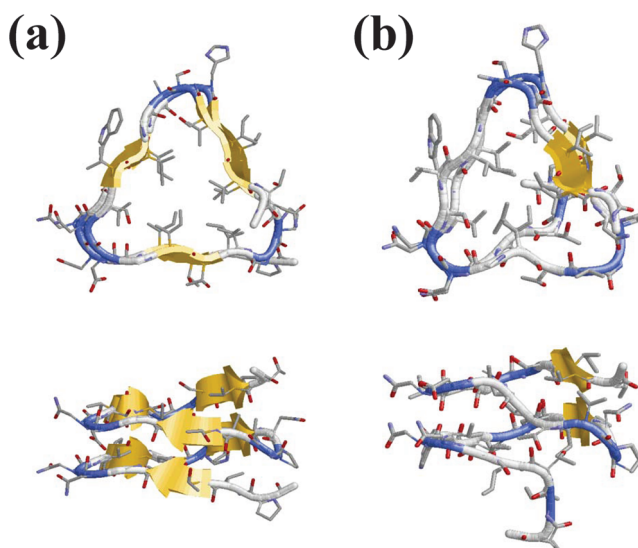
Protein rich in β-helix structure

Selection of regular segments

In Silico assessment of stability. Determination of potential replacement targets

Figure 3. β-Helical building block excised from *E. coli* galactoside acetyltransferase (*1krr*$_{131−165}$). Comparison of the crystal structure (a) with a snapshot (b) recorded after 30 ns of a MD trajectory in aggressive conditions (360 K and high ionic strength) that reflects the stability of the building block.

silico the relative stability of these assemblies based on *1krr*$_{131−165}$ versus the polymerization of the same segment via covalent links.[50] Additionally, the results obtained by simulating both the excised segment and its nanoconstructs provided us with the necessary information about the conformational dynamics of each residue in the selected sequence for subsequent targeting of the positions most susceptible to replacements by non-natural amino acids.

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4. ENHANCING THE STABILITY OF B-HELIX MOTIFS WITH NON-NATURAL AMINO ACIDS

4.1. The Flexibility of Protein Segments Arranged As a β-Helix in Their Native Fold. Atomistic MD simulations of the segments, as individual units and assembled into nanostructures, assist in describing their intrinsic folding tendencies. The dynamical behavior is essential in identifying the most flexible positions in the sequence which can promote unfolding. This information can be used for enhancing the desired fold in the segment.

The conformational space of polypeptides includes a vast number of energy minima. Among these, the observed state is the most populated under a given set of conditions. Most peptides excised from folded proteins do not populate the native fold under the same conditions as in the original protein.[51] However, if capped, the conformation that they originally presented as a part of a protein is among the more populated minima. Thus, the problem of how to induce a particular secondary structure in a peptide chain can be reduced to the question of lowering the conformational flexibility of the selected segment, i.e. biasing the population of low energy conformations to increase the probability of the desired arrangement.

Our approach combines the information obtained from atomistic MD simulations of wild-type protein segments with the accumulated knowledge on the conformational preferences of the non-natural cyclic amino acids presented above. Our results of the designed nanoassemblies showed that the most flexible sites of the β-helix motifs are located mainly at the hinges connecting different β-sheet strands. Such flexible segments exhibit folded turnlike conformations. Given the known propensity of members of the Ac_nc series to induce turn motifs,[11–13,16–21] replacement of specific amino acids in these mobile regions by Ac_nc residues should decrease the local conformational flexibility stabilizing the β-helix in both the isolated building block and the nanoconstruct.

4.2. Stabilizing Single Turns in the *1krr*$_{131–165}$ Protein Segment. Initially, we used Ac_3c-based residues as stabilizing tools for the hinges connecting two consecutive β-strands in the *1krr*$_{131–165}$ segment.[52,53] In particular, we focused on the region presenting the highest flexibility, namely the turn comprised between residues 159 and 161 (Gly-Ala-Gly sequence). The cyclopropane amino acid Ac_3c and its substituted derivatives are suitable candidates for this purpose since the conformational restrictions imposed by the three-membered ring favor folded arrangements, with a remarkably high preference to occupy the $i+2$ corner position of β-turns.[11–13,16–18,22,24]

The conformational propensities of this three-residue sequence when the central Ala$_{160}$ is replaced by an Ac_3c derivative bearing two geminal phenyl substituents (c_3Dip, 1-amino-2,2-diphenylcyclopropanecarboxylic acid) (Figure 1) were systematically explored by using quantum mechanical calculations.[53] This investigation revealed that not only one of the lowest energy conformations of Gly-L-c_3Dip-Gly presents the exact turn fold found in the crystallized protein but also that all the conformations exhibiting a low energy, including the global minimum, adopted turnlike folds that could fit the β-helix arrangement (Figure 4a). MD simulations of the whole *1krr*$_{131–165}$ building block showed that replace-
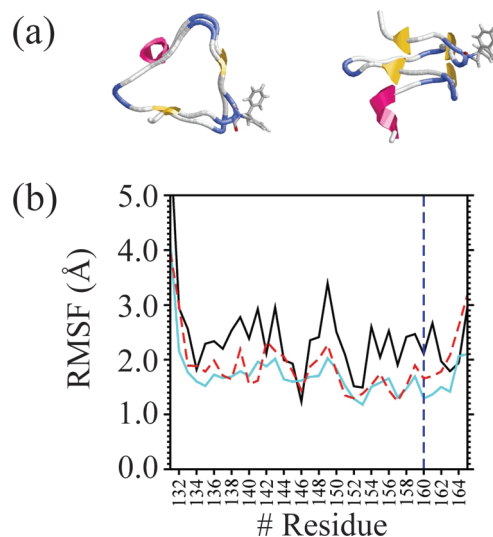

Figure 4. (a) Axial (left) and equatorial (right) views of the left-handed β-helix obtained after replacing Ala$_{160}$ in the *1krr*$_{131–165}$ building block by c_3Dip. The structure corresponds to a snapshot recorded after 10 ns of MD simulation. Explicit atoms, with the exception of those of the non-natural residue, are omitted for clarity. (b) Root mean square fluctuation (RMSF) accumulated after 10 ns of simulation of the *1krr*$_{131–165}$ building block for the wild-type sequence (black line) and the mutants obtained by replacing Ala$_{160}$ by either Ac_3c (dashed red line) or c_3Dip (solid cyan line). A vertical dashed blue line indicates the replacement site.

ment of Ala$_{160}$ by c_3Dip contributed in a major way to reduce the structural fluctuations of the loop. Moreover, the overall conformational fluctuations of the excised segment were dramatically reduced, thus demonstrating that local stabilization assists in retaining a preferred conformation in short protein segments. This is clearly reflected in Figure 4b, which compares the Root Mean Square Fluctuations (RMSF) determined for the *1krr*$_{131–165}$ segment in its wild-type sequence and after replacing Ala$_{160}$ by c_3Dip. It is noticeable that reduction of the flexibility at the replaced site induces a global stabilization of the structural motif.

Despite these promising results, our successful substitution presented a drawback when considering the assembly of the chosen building blocks into nanoconstructs. The replacement of Ala$_{160}$ by c_3Dip implies the presence of a carbon atom bearing two phenyl groups in the space previously occupied by a methyl group. Assembly of building blocks containing c_3Dip as a turn stabilizer was not favored due to the steric hindrance introduced by the bulky side chain of the non-natural residue.[52] Hence, even if the presence of the phenyl groups in the cyclopropane ring helped confining the accessible conformational space of Ac_3c, the volume of the new residue prevented the formation of stable assemblies due to steric hindrance between building block.

The targeted replacement of Ala$_{160}$ by the unsubstituted Ac_3c also led to a reduction in the conformational flexibility of both the three-residue turn motif and the whole *1krr*$_{131–165}$ building block. Although this effect was less significant than that achieved with c_3Dip (Figure 4b), in which the presence of two phenyl substituents aided rigidifying the motif, it proved strong enough to stabilize the nanoassembly, and the lack of substituents in the cyclopropane ring did not perturb the nanoconstruct organization.[52]

Stabilization of another loop connecting β-strands in *1krr*$_{131–165}$, namely that involving residues 148–150, was

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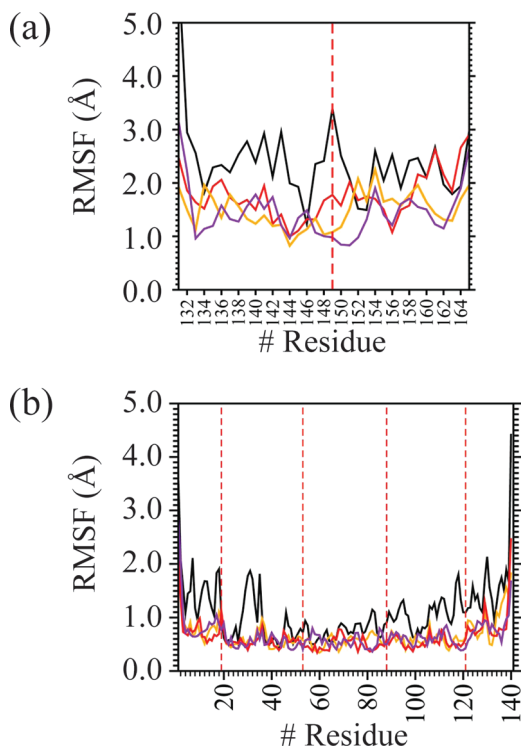
(a)

(b)

Figure 5. Root mean square fluctuation (RMSF) of the α carbon atoms accumulated after 10 ns of MD simulation for (a) the *1krr*$_{131-165}$ building block and (b) the nanoconstruct obtained by assembling four replicas of the building block. In both (a) and (b), the lines correspond to the wild type sequence (black line) and the mutants obtained by replacing Gly$_{149}$ by Ac$_3$c (red line), Ac$_5$c (orange line), or Ac$_6$c (violet line). In the nanoconstruct, the Gly$_{149}$ site of each building block has been replaced by the Ac$_n$c residue mentioned. A vertical dashed red line indicates the replacement site.

also successfully achieved following this strategy.[54] Thus, replacement of Gly$_{149}$ by either Ac$_3$c, Ac$_5$c, or Ac$_6$c led to a drastic reduction in the flexibility of the turn region, stabilizing the β-helix conformation of the fragment and the nanoassembly (Figure 5).

4.3. Nanoassemblies and Nanowires Using β-Helix Motifs. Our most recent efforts focused on combining these strategies with polymer science principles in order to design new peptide-based structures with selected conformational preferences. Given the previously observed small building block size limitations in the constructed assemblies, we explored the possibility of engineering fibril-like structures by linking consecutive *1krr*$_{131-165}$ segments through covalent bonds. As expected, the global stability of the construct was enhanced several fold.[50,55] This helped in addressing two main weaknesses: (i) the difficulties in setting up the proper conditions to drive *1krr* derivatives toward the desired fold (despite the incorporation of conformationally restricted amino acids) and (ii) the potential formation of amorphous aggregates with no structural coherency during the assembly process. The polymerization of short segments rich in secondary structure elements when embedded in the native protein is currently a standard method to build nanoconstructs based on peptide units.[34,35] Obviously, the conformational stability of this new family was greatly enhanced by the accumulative effect of bonded replicas of peptide segments that already presented a high tendency to keep a preferred folded motif (Figure 6a). However, even in this context, the
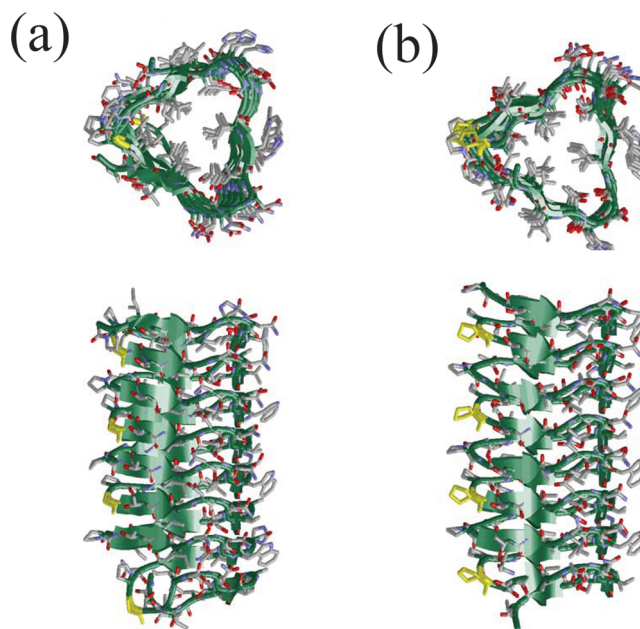

Figure 6. Atomistic representation of the nanofibril obtained by polymerizing the *1krr*$_{131-165}$ building block at the end of 10 ns of simulation for (a) the wild type sequence and (b) the mutant with all Gly$_{149}$ sites replaced by Ac$_5$c. In both cases, the equatorial (top pictures) and axial (bottom pictures) projections are shown. Non-hydrogen atoms are represented by solid sticks, using the *CPK* color convention; main chain atoms are remarked with cartoons representation and dark green color. The replacement sites have been highlighted in yellow color.

effect produced by conformationally restricted amino acids was noticeable. When key positions of the building blocks were replaced by Ac$_n$c residues, the conformational stability of the resulting nanostructure became comparable to that of fibrillar proteins. Such an enhancement of the structural stability was evidenced by the regularity that the β-helical nanofibril kept even after the system was put under thermal stress (Figure 6b).

Finally, the stabilization of β-helical motifs is not significantly affected by the inner flexibility of the cyclic side chains of the Ac$_n$c residues. Two different cases were considered:[47,52] (i) Ac$_5$c, whose side chain presents a conformational equilibrium associated with the puckering of the five-membered ring, and (ii) Ac$_6$c, in which the cyclohexane ring exhibits an equilibrium between two different chair conformations separated by a boat arrangement. As the smallest member of the Ac$_n$c series (Ac$_3$c), both Ac$_5$c[47] and Ac$_6$c[52] proved to be excellent replacements for residues located in flexible turnlike regions of the β-helical motif. As can be seen in Figures 5b and 6b, the flexibility of the cycle not only did not interfere with the nanostructure organization but also helped relax global conformational tensions introduced by the rigid backbone. Moreover, the higher the ring flexibility of these non-natural backbone restricted residues, the more positive was the effect they had over the nanoconstructs.

Mesoscopic models of nanoconstructs formed by a large number of self-assembled *1krr*$_{131-165}$ building blocks were built to investigate the optimal length of the resulting nanofibers.[56] Results indicated that the nanofibril becomes an unfavored supramolecular assembly when the number of building blocks is higher than 45. This effect was attributed to the interaction between building blocks that are not direct

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neighbors, i.e. 1-m interactions with $m > 2$, which was slightly repulsive or zero when m is very large. Accordingly, the attractive interaction between consecutive building blocks (1−2 interactions) is largest when the number of self-assembled units is under 45, but the repulsive term dominates when the length of the system is above such threshold.[56] This feature represents another important drawback of fibril-like self-assembled nanostructures with respect to the polymerized ones.

5. PERSPECTIVES: ONE STEP CLOSER TO TAILORING NANOSTRUCTURES

De novo nanodesign can be an extremely costly project, requiring a tremendous amount of work. Within this framework, our efforts were centered on controlling the conformational preferences of molecular scaffolds (specifically homologous repeating sequences in β-helix proteins) to build nanostructures. To this end, we focused on the reduction of the conformational flexibility of the fragments by limiting the mobility at specific points in these sequences. Our investigations have shown that reduction of the local mobility promotes specific peptide conformations by increasing the energy difference between such arrangements and all other possible low-energy conformations.

The choice of segments is based of the conformations they exhibit when present in crystallized proteins. Sequences that are part of β-helix motifs are selected provided they present high regularity both in sequence and conformation. Therefore, such regular organization is assumed to be part of their conformational space. Massive *in silico* characterization of the conformational dynamics for each selected segment allows the identification of the structural deficiencies once the peptide is excised from the original protein. Extensive MD simulations are used to locate the positions that most contribute to destabilize the target conformation in terms of conformational mobility. Our strategy relies on the targeted replacement of the residues occupying these highly flexible positions by non-natural amino acids that present restricted conformational preferences. In particular, α-tetrasubstituted residues bearing cyclic aliphatic side chains (Ac$_n$c) have proven to be suitable chemical tools to bias the conformational space of the selected polypeptides. Due to the structural requirements imposed by their chemical constitution, these amino acids are characterized by a strong tendency to induce peptide turns. Our simulations indicated that loops (turnlike motifs) connecting β-strands are the most flexible regions of the nanostructures and are therefore responsible, to a large extent, to lowering the stability of the β-helix arrangement. Our strategy proved successful in reducing the mobility not only of the region where the target replacement was made but also of the whole protein segment. Through the incorporation of non-natural restricted amino acids, the selected conformation is stabilized and the energy difference between this arrangement and the rest of conformational minima is increased.

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