
Molecular Engineering of Biomolecules for Nanobio-Sciences

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Abstract: Biologically programmed molecular recognition provides the basis of all natural systems and supplies evolution optimized functional materials from self-assembly of a limited number of molecular building blocks. Biomolecules such as peptides, nucleic acids and carbohydrates represent a diverse supply of structural building blocks for the chemist to design and fabricate new functional nanostructured architectures. In this context, we review here the chemistry part of our Nanobio program, we have developed in Grenoble to manipulate such biomolecules, and organic molecules, as well as assemble combinations thereof using a template assembled approach. With this methodology, we have prepared new integrated functional systems exhibiting designed properties in the field of nanovectors, biosensors as well as controlled peptide self-assembly. Thus this molecular engineering approach allows for the rational design of systems with integrated tailor-made properties and paves the way to more elaborate applications by bottom-up design in the domain of nanobiosciences.

Keywords: template, peptide, carbohydrate, nucleic acids, molecular recognition, oxime, nanovector, glycocluster, sensor.

Biographical notes: **Pascal Dumy** (45 years) obtained his PhD thesis at the Université Montpellier-I France in 1993 in the field phosphonopeptides as transition state analog of peptide bond. From 1993, he performed postdoctoral research under the guidance of Prof M Mutter at the Institute of Organic Chemistry, Lausanne University, Switzerland, where he became an Assistant Professor in 1997. In 1998, he was appointed full professor at the Université Joseph Fourier, Grenoble, France, where he starting his research group. He was appointed as a junior member at the Institut Universitaire de France (IUF) from 2000-2005 and as Research and Higher Education Programmes adviser of the Agency for the evaluation of research and higher education (AERES). He is member of the P5 panel of ERC starting grants since 2008. His main research

interests are directed to chemical biology and nanosciences and include the chemistry of biomolecules (peptides, nucleic acids, oligosaccharides), the design of nonviral vectors for biomolecules vectorization and in vivo targeting, molecular imaging, tumor neoangiogenesis, synthetic vaccines, surface functionalization, and the use of biomolecules in nanosciences.

Olivier Renaudet studied Molecular Chemistry at the University Joseph Fourier, Grenoble, France, where he received his PhD in 2002 under the supervision of Prof. P. Dumy. Thereafter, he joined the University of Berne, Switzerland, for a postdoctoral position with Prof. J.-L. Reymond, then he moved back to the University of Grenoble, where he obtained an Assistant Professor position in 2004. His current researches are directed towards the chemistry of carbohydrates and peptides, as well as their use in Nanoscience. He is particularly interested in the synthesis of multitopic glycoclusters for biological applications such as cancer vaccines, vectors or microarrays.

Julian Garcia studied biochemistry at the University of Lyon, France. He went then to the University of Grenoble, France, where he obtained his PhD in pharmaceutical sciences 1988. In 1989 he joined the laboratory of Professor J. Lhomme in Grenoble where he assumed a position as Assistant Professor for organic chemistry. After a postdoctoral fellowship with Prof. G. Esposito, at the University of Udine, Italy, in 2002, he moved back to the University of Grenoble where he was appointed as a full professor in the laboratory of Professor P. Dumy. His research interests comprise structure elucidation using NMR and molecular modelling of oligonucleotides, peptides and proteins. Special focus is placed on the role of complementary interactions and cooperativity in peptide and protein folding as well as peptide and protein modification for the development of novel molecular tools for biochemical research.

Didier Boturyn studied chemistry and biochemistry at the University Joseph Fourier, Grenoble, France. He received his PhD thesis in 1996. From 1997 he got a postdoctoral position at the University of Virginia, Charlottesville, USA. He worked on the syntheses and biological studies of bleomycin derivatives in Professor Sidney Hecht laboratory. Then, Didier got a position at the Centre National de la Recherche Scientifique in 1999. He is currently working with Professor Pascal Dumy at the Département de Chimie Moléculaire (UMR 5250). There he started to work in the field of peptides notably biomolecular assemblies by means of chemoselective ligations to construct drug-delivery systems.

Nicolas Spinelli studied Bioorganic Chemistry at the University Montpellier II, France, where he received his PhD in 2001 under the supervision of Dr. J.-J. Vasseur. Then, he joined the Ecole Normale Supérieure of Lyon, France, for a postdoctoral position with Dr. B. Mandrand. Thus he joined the University of Grenoble, where he obtained an Assistant Professor position in 2006. His current researches are directed towards the chemistry of oligonucleotides and their use in Nanoscience. He is particularly interested in the synthesis of oligonucleotide conjugates for improving their pharmaceutical properties and for preparation of microarrays.

Eric Defrancq received his PhD degree in organic chemistry in 1989 from the University of Grenoble (France). After a postdoctoral stay for 2 years at the Institute of Chemistry at Neuchâtel (Switzerland), he became Assistant Professor in 1992 at the University of Grenoble, where he is currently full Professor. His research interests lie in the field of modified oligonucleotides synthesis and various applications such as DNA micro-arrays preparation, G-

quadruplex mimetic and DNA-based nanostructures.

Pierre Labbé obtained in 1982 his PhD in Molecular Chemistry at the Université Scientifique et Médicale de Grenoble. After a one year postdoctoral position at the Laboratoire d'Electrochimie Organique et Analytique (CEN-Grenoble) he became assistant-professor at the Université de Savoie (Chambéry). In 1992, he got a position of professor at the Université Joseph Fourier in Grenoble, where he initiated new activities in the field of bioelectrochemistry and physical chemistry of interfaces in the Laboratoire d'Electrochimie Organique et de Photochimie Redox. In 2007, Pierre Labbé and his team have joined the group of Prof. P. Dumy (Ingénierie et Interactions biomoléculaires). The research interest of Pierre Labbé concerns the conception of new functional surfaces and interfaces for studying biomolecular interactions with the objective of applications in the field of life science and nanoscience.

1 Introduction

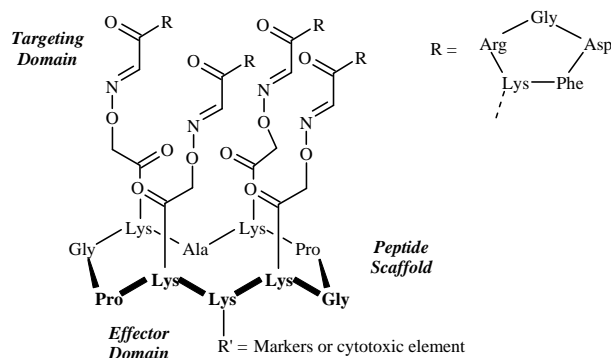
Molecular recognition is a key feature used by nature for the design and the manufacture of biomaterials that exhibit highly developed nanostructures with tailor-made properties. Biological assemblies are highly interesting for nanosciences and nanotechnologies since they raise a number of questions to be addressed by nm-scaled probes or devices; they also provide source of inspiration for the design of nanodevices, nanostructures with new types of functions, and perhaps even components for new types of devices. Biomolecules such as peptides, nucleic acids and carbohydrates exhibit intrinsically structural and functional encoded recognition properties in living systems, which represent a tremendous source of inspiration for the creation of nano-sized molecular systems. Nano-constructs formed from combinations of such molecular building blocks opens up a wide and diverse field of research, from the construction of smart drugs that can selectively target diseased tissue to the understanding of nature's fascinating principles. Further, nano-sized biomolecular devices composed of protein or nucleic acid building blocks have the advantages of a broad spectrum of functionalities and binding interactions. Combining these properties with modern synthetic strategies allows for the design and preparation of elaborate nanomaterial with multiple embedded functions. Here, nano-constructs are referred to as artificially made structures possessing sizes between one to tens of nanometers. A key factor in the preparation of such elaborate devices is the precise control by which the different building blocks are conjugated together both chemically and directionally.

In this Review, we summarize the research performed in our laboratory based on the construction of nanosized devices with a central template peptide named Regioselectively Addressable Functionalized Template (RAFT). Here, the biomolecule-based templates are currently intensively investigated as structure-directing building blocks to generate well-defined protein mimics, potent anticancer vaccines, enzyme active site mimics, redox-active materials, and nanovectors (smart drugs) with targeting, imaging and delivery of therapeutics. This work also belongs to the Nanobio pole in Grenoble which promote, thanks to local communities supports, research in the field of nanobiosciences.

2 Design of nanovectors

The design of molecular assemblies endowed with tumour-targeting functions should enable the specific delivery of drugs or imaging probes or a combination thereof to cancer cells. Among targeting elements, peptide ligands containing the Arginine-Glycine-Aspartate (RGD) triad sequence, which display a strong affinity and selectivity to the $\alpha_v\beta_3$ integrin, have been widely used to target the tumour-associated cells expressing the corresponding receptors.^{1,2} In this context, we have designed nanomolecules based on the cyclodecapeptide scaffold. The latter presents in a spatially controlled manner two independent functional domains: a clustered ligand domain for integrin recognition triggering cell targeting and a domain devoted to a supplementary function (cancer monitoring and/or drug delivery).³ Such molecules are synthesized via molecular assemblies through chemoselective ligations.^{4,5} Studies with scaffolds containing one to 16 RGD peptide ligands have highlighted the utility of clustered ligands that represent a new class of vectors for targeted-drug delivery as well as for molecular imaging of tumours.⁶

Figure 1 Structure of RGD-containing peptide



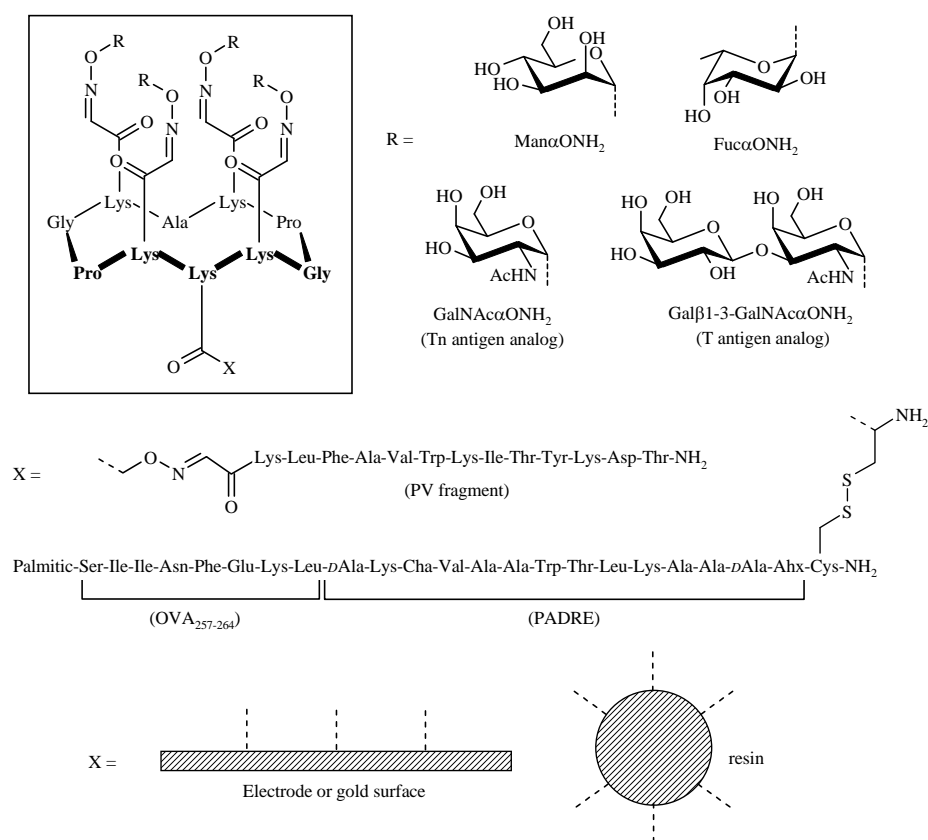
We demonstrated the propensity of such molecules for specific *in vitro* and *in vivo* targeting (Figure 1).⁷ Several markers were used such as biotin,³ fluorescein,³ ^{99m}Tc ,⁸ ^{111}In ,⁹ and very recently NIR (near-infrared) dyes such as cyanine 5 (Cy5).^{7,10} For example, *in vitro* and *in vivo* studies show that tetrameric Cy5-RAFT(c[-RGDfK-])₄ targets more specifically subcutaneous tumours as well as abdominal metastases than the cognate monovalent ligand.^{11,12,13} Altogether, this makes our molecular scaffold a very promising nano-sized vector for future clinical imaging applications: we are now validating a fluorescent compound as a surgeon helper molecule to assist in surgical removal of tumours. We recently designed our scaffold for drug delivery and analysed the capacity of our compounds to improve the destruction of targeted cells.^{14,15} We showed that one compound displays significant biological effect in $\alpha_v\beta_3$ integrin-containing tumour cells. Such targeting and killing integrated functional systems would exhibit better effects than conventional chemotherapeutic drugs for the systems since it would benefit from the selectivity of the targeting ligand and the specificity resulting from the neoangiogenesis process in cancer.

3 Design of glycoclusters

Due to the importance of protein-carbohydrate multivalent interactions in biological processes, parts of our research interests are focused on the design and synthesis of cyclopeptide-based glycoclusters.¹⁶ In order to ensure an efficient and versatile assembly process, we developed an expedient synthetic strategy to introduce an aminoxy function at the anomer position of biologically relevant carbohydrates (*e.g.*, mannose,¹⁷ fucose¹⁸) or tumor-associated carbohydrate antigens (*e.g.*, Tn and Thomsen-Friedenreich antigens analogs¹⁹) with α or β anomer configuration (Figure 2). Such aminoxy carbohydrates can be efficiently conjugated in solution into cyclopeptides scaffolds containing aldehydes or ketone^{20,21} and on solid phase^{22,23} to afford glycoclusters with various composition (Figure 2). We next took advantage of the efficiency of this synthetic method to assemble molecular tools for diagnostic and therapeutic applications. With the aim of generating synthetic anticancer vaccines, we designed several multiepitopic molecular construction on the model of previous reports.^{24,25} A first generation of synthetic vaccine candidates based on the use of cyclopeptide scaffold was first developed following the sequential oxime-based strategy.²⁶

Well-defined compounds displaying clustered mucin-associated Tn antigen analogue and immunostimulant CD4⁺ helper T-cell peptide epitope from the type I-poliovirus protein were thus designed. Biological investigations have confirmed the potency of this multiepitopic construction to elicit a specific immune response towards tumours expressing the human form of Tn antigen. On the basis of this study, a more sophisticated generation of vaccine candidates was next prepared.^{27,28} This vaccine prototype contains four essential components, that are a cluster of Tn analogue, a chimeric CTL-Th peptide made of a CD8⁺ T-cell epitope from ovalbumin (OVA₂₅₇₋₂₆₄) in line with a universal CD4⁺ T helper (Th) epitope (PADRE) and a palmitic acid moiety at the N-terminal end to provide self-adjuvanting properties. After immunization of OVA-expressing mouse B16 melanoma without external adjuvant, the resulting glycolipopeptide vaccine prototype has shown a strong induction of IgG/IgM that recognized Tn-positive human tumour cell lines and an efficient stimulation of OVA₂₅₇₋₂₆₄-specific CD8⁺ T cells and PADRE-specific CD4⁺ T cells. More notably, a significant reduction of tumor size and an improved survival in mice inoculated with MO5 carcinoma cells was observed, suggesting their potential for human immunotherapy against cancers.

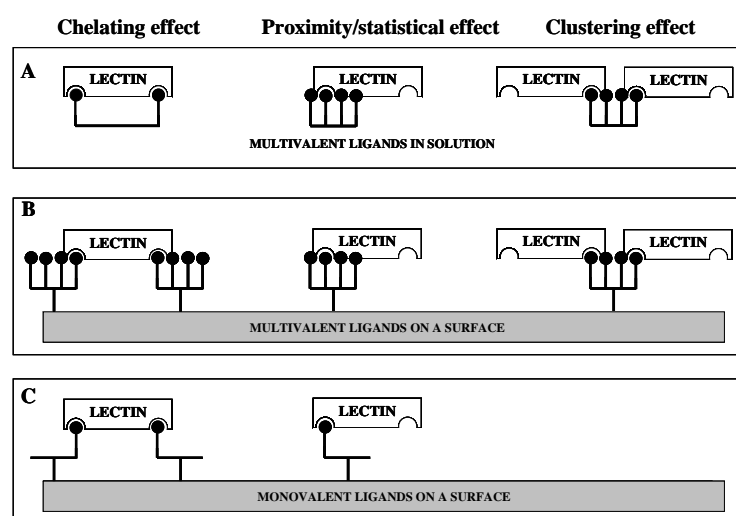
Figure 2 Structure of cyclopeptide-based glycoclusters



The recent emergence of glycomics researches²⁹ prompted us to study carbohydrate-protein interactions either after generation or immobilization of a glycocluster on different solid surface. We first investigated an on-bead procedure for the synthesis of glycoclusters and binding assay with peroxidase-labelled lectins.²² In this study, we observed that a clustered presentation of carbohydrates favours a selective and improved recognition with carbohydrate binding proteins, suggesting that the effective interaction is due to cooperative multivalent effect. A similar effect was confirmed after immobilization of a glycocluster on streptavidin-coated electrodes³⁰ and by oxime ligation onto the inner wall of fused-silica capillary tubes.³¹ These complex binding mechanisms with lectins were next investigated by Quartz Crystal Microbalance with energy Dissipation monitoring (QCM-D) and SPR on gold chips.³² Lectins, carbohydrate binding proteins, contain two or more specific sugar-combining sites and comprise a large family of recognition molecules, especially in the immune system. While the affinity between lectin and monosaccharides is weak, K_D in the 0.1 - 1mM range, sugar-protein interactions are very efficient and specific due to multivalent events commonly known as the “glycoside cluster effect”. This effect has previously been defined as an “affinity enhancement achieved by multivalent ligands over monovalent ones that is greater than would be expected from a simple effect of concentration increase”. Multivalent carbohydrate derivatives that can simultaneously interact with several binding sites of a multivalent lectin (chelating effect) are relevant for medicinal interest.

In this context, some small multivalent ligands, in which the distances between their carbohydrate moieties are too low to enable their binding to multiple sites of the same lectin, have proved to be also more efficient than monovalent ones. This improvement could be attributed either to local ligand concentration effects, also defined as proximity/statistical effects, or, as rarely demonstrated, to a favorable clustering of lectins (interaction of one multivalent ligand with several lectins). These various possibilities related to the “glycoside cluster effect” are shown pictorially in Figure 3.

Figure 3 Schematic representation of the three main effects at the origin of the “glycoside cluster effect”, illustrated by the interaction of a bivalent lectin with small multivalent ligands (A) in solution, (B) immobilized on a surface, and (C) comparison with the interaction of a bivalent lectin with monovalent ligands immobilized on a surface.



In particular, the binding affinity was measured as a function of the grafting ratio of the glycocluster on the surface. Interestingly, the binding between a model lectin and multivalent or monovalent ligands immobilized at low surface density, a higher affinity was measured for the tetravalent ligands. Moreover, at comparable immobilization ratio, we observed that tetravalent ligands can bind higher amounts of lectin due to their ability to form clusters of lectins. We presume that this clustering process could be at the origin of the higher affinity exhibited by those ligands, although it could be postulated that the presence of a high local concentration also participate through a proximity/statistical effect. While this study using QCM-D and SPR with the sugar immobilized on a surface allows evidencing the clustering and the proximity/statistical effects of our low molecular weight (LMW) multivalent carbohydrate ligands in the enhanced affinity, it was not possible to quantify both effects independently. Work is in progress for optimizing our model system in order to separate both effects. SPR was chosen as this technique allows easy assessment of the kinetics and thermodynamics parameters of sugar-protein interaction, even for LMW ligands in solution whereas the lectin is immobilized. In addition, the lectin immobilisation provides a simplification of the processes involved in the recognition. Indeed, a monovalent ligand could only recognized immobilized lectin

via a 1:1 interaction. In contrast, it is worth mentioning that the interaction with the same monovalent sugar immobilized on the solid surface yields to at least two more effects: the multivalency induced by the solid surface presentation of the carbohydrate could allow both chelating effect as well as proximity/statistical effect (Figure 3). The recognition between our multivalent LMW ligand in solution and the immobilized lectin is expected to arise from two effects: a clustering effect and a proximity/statistical effects. The control of the lectin surface density could thus appear as an efficient mean to separate both effects. At low surface density, the distance between immobilized lectins should avoid any clustering possibility from the multivalent ligands. Clustering effect of multivalent ligands can be observed at high surface lectin densities whereas its proximity/statistical effect could be explored only at low lectin surface coverage. Work is in progress to verify these hypothesis.

In another approach, we reported recently an original strategy to generate randomized mixture-based combinatorial libraries of glycoclusters which were expected to reflect the heterogenic composition of the cell surface glycocalix by presenting various carbohydrates and/or amino acids residues.³³ This new version of glycoclusters has revealed an attractive potential to assess how secondary binding interactions affect the recognition processes with proteins.

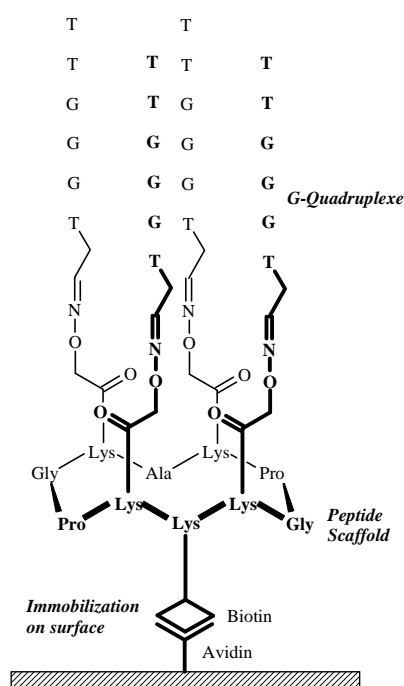
4 Design of models of G-quadruplexes

The cyclopeptidic scaffold has also been used for developing new models for studying DNA (or RNA) secondary structures. In this context, the design of G-quadruplexe mimics is of great interest. Indeed, the formation of such structures has been involved in different biological processes such as telomere stabilization,³⁴ oncogene activation³⁵ and regulation of immunoglobulin switch region.³⁶ The G-quadruplexe structures consist in the association of four G-rich DNA sequences based itself on the association of planar G-quartet of four guanine residues held together by Hoogsteen-type hydrogen bonds. Those structures are stabilized by monovalent cations such as K^+ or Na^+ .³⁷ Due to the biological importance of these DNA structures, the design of molecules that can bind to them has thus received great attention and a large number of putative ligands have been evaluated for their binding with this kind of motif.^{38, 39}

However, the G-quadruplexe motif can adopt different topologies depending on the experimental conditions that can confuse the study of recognition phenomena. For this purpose, we recently described the use of the cyclopeptidic scaffold as a topological template that can direct the intramolecular assembly of covalently attached oligonucleotides into a parallel G-quadruplexe.⁴⁰ This conformationally constrained G-quadruplex mimic was prepared by functionalization of the four lysine on the upper face of the cyclodecapeptide with four G-quadruplex forming oligonucleotides ($d(5'TTAGGG^3')$) *via* oxime chemistry (Figure 4). This oxime strategy has been proved efficient for the conjugation of oligonucleotides with various reporters as well as for the attachment on glass surfaces.^{41,42,43} Preorganisation afforded by the scaffold confers a greater stability of the model compared to “natural” G-quadruplex forming nucleic acids. In order to exemplify this stability, it has been observed by CD that the mimic is organised in quadruplex structure even in the absence of cations (Na^+ or K^+) residue allowing immobilization on a streptavidin coated surface. We showed that this molecular

system immobilized on a surface can be used for studying by surface plasmon resonance (SPR) the interactions of G-quadruplexe with small organic molecules. Thus, this novel SPR-based approach using the peptide scaffold is of interest for the screening of various G-quadruplexe ligands, providing key information on their affinity, selectivity and binding mode. Preparation of models of others nucleic acids secondary structures such as RNA based G-quadruplexes or i-motif, are currently in progress.

Figure 4 Preparation of conformationally constrained G-quadruplex mimic.

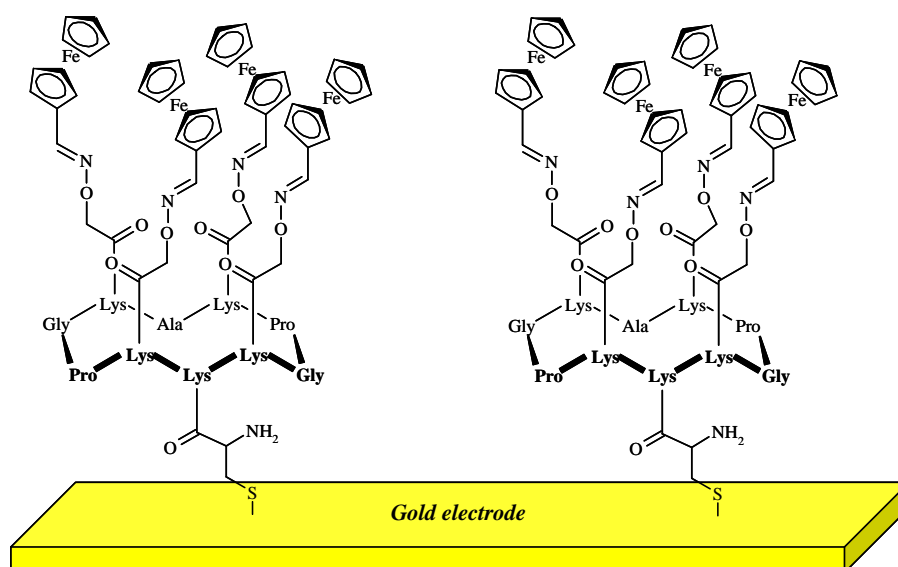


5 Nanoscale Redox Active Material

The RAFT scaffold has also been used for the design of a nanometer scale redox active biomolecular architecture (Figure 5) by using ferrocenyl units.⁴⁴ This molecular tool exhibits electronic, structural and chemical properties driven by the biomimetic recognition activity of the polypeptide skeleton, which is associated to the well-defined electrochemical activity of metallocenyl probes. Biomolecular materials on gold electrodes were obtained by the attachment of redox cyclopeptides in a self-assembled monolayer. Marcus heterogeneous kinetics, implying significant reorganization energy, consecutive to the electron transfer processes, had to be considered to account for the unusual cyclic voltametry (CV) shapes observed at high-sweeping rates. Kinetic studies strongly suggest an efficient through-bond electronic coupling of immobilized ferrocene groups via the peptidic backbone. Concentration of anion binding sites in nanostructured materials led to enhanced electrochemical recognition properties as proved by the

amperometric type of sensing monitored in acetonitrile upon adding increasing amounts of dihydrogen phosphate. We believe that gold electrodes with self-assembled monolayer of peptidic structures bearing electrophore moieties is a promising pathway towards *in situ* redox sensing of biological events. These results thus open up new exciting perspectives in the field of biomimetic electrochemical sensing and/or activation relying on the intimate association between protein-like receptors and redox active reporters/initiators.

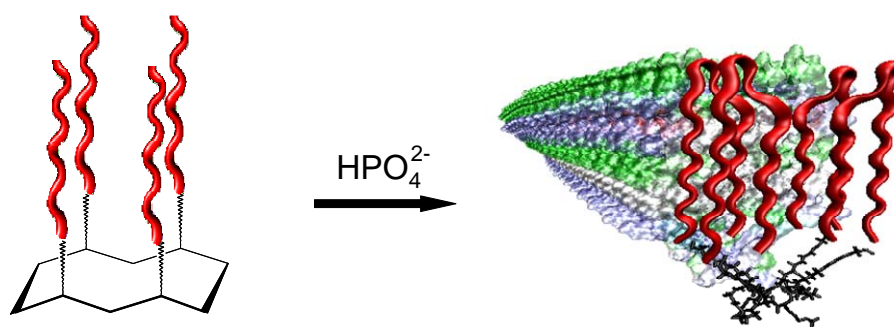
Figure 5 Preparation self-assembled monolayer on gold surface with Ferrocene-containing peptides



6 Design of amyloid fibril models

Protein misfolding is involved in many lethal diseases including Alzheimer's diseases (AD), Parkinson's disease and others non neurodegenerative diseases.^{45,46} AD is characterized by amyloid fibrillar deposits in the brain, which consists mainly of the amyloid β -peptide ($A\beta$) with 40 to 42 residues.⁴⁷ As $A\beta$ accumulation is widely believed to be the causative event of AD pathogenesis, therapeutic approaches are in development that target different sites on the pathway to production and aggregation of $A\beta$. However, research in this direction is limited by the lack of high resolution structure information available for the amyloid structure. Thus, we have designed and synthesized a water-soluble $A\beta$ fibril model for high-resolution structural determination by NMR and to aid the search of fibril inhibitors against AD.⁴⁸ We have covalently attached four $A\beta$ -fragments to the cyclic decapeptide scaffold, designed to form a building-block of the $A\beta$ fibril and represented in figure 6. The $A\beta$ fragments consist of the structural forming residues 16-37 of the full $A\beta$ length and attachment to the scaffold is designed to create a high local concentration to induced fibril formation. Further aggregation of the fibrils to insoluble amyloid was avoided by a designed large net positive charge that creates charge repulsions between fibrils and also increases the fibril solubility. From our main results, this building-block has been shown to fold and aggregate in the designed way. Formation of fibrils was determined by binding of amyloid specific dyes, CD and TEM. The current important result for this assembly is that it folds with no lag-phase and that the kinetics is highly controllable by the divalent anion HPO_4^{2-} concentration (Figure 6). As a consequence of this result, we have studied the folding of the peptide construct with previously documented amyloid inhibitors and found the construct to be an interesting tool for high-throughput screening (HTS) applications to generate novel hit compounds for $A\beta$ fibril inhibition, i.e. potential drugs for Alzheimer's diseases.⁴⁹

Figure 6 Structure of the amyloid β model.



PERSPECTIVES/CONCLUSION

Recent advances in the chemistry of coupling reagents, protecting groups and solid-phase synthesis have made the chemical synthesis of peptides with conformational control and complex structures achievable. Besides their use as structure-inducing devices, these

peptide templates can also be utilized to construct novel structures with tailor-made functions. Herein, we presented recent advances in the field of peptide template-based approaches with particular emphasis on demonstrated utility in molecular recognition along with related applications. The utility of these templates can be extended beyond their intended use as structure-inducing devices. These molecules can be used as a platform to construct multivalent ligand assemblies as these templates have constrained backbone conformation, which helps in presenting the ligand clusters in well-defined and controlled spatial orientations. In addition, the other benefit associated with the use of these molecules is that they contain two independent and chemically addressable domains that give them bifunctional characteristics. This implies that one domain is used to attach multiple ligands for binding to various receptors and the other domain can be utilized to attach self-assembling molecules for surface assembly for nanobiomaterials or labels for *in vivo* imaging or drugs for their targeted delivery. Thus, these templates can be used to design and develop complex molecular nanosized architectures and pave the way to more elaborate applications by a bottom-up design in the domain of nanobiosciences. Such orientations are currently under investigations particularly the role of loading and local density of molecule on surface to provide nanoparticle with de novo designed properties. This work was realized thanks to the support provided by local communities which makes it possible to install in Grenoble strong facilities and infrastructures for research in the field of nanobiosciences.

Acknowledgements

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