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Review

RAFT Nano-constructs: surfing to biological applications‡

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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 we have developed to conjugate peptides with nucleic acids, carbohydrates, and organic molecules, as well as 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. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cyclopeptide; template; oxime; protein mimic; molecular recognition; targeting; synthetic vaccine; sensor

INTRODUCTION

Molecular recognition is a key feature used by nature for the design and the fabrication of biomaterials with tailor-made properties and that exhibit highly developed nanostructures. Biological assemblies are highly interesting for nanosciences and nanotechnologies since they raise a number of problems to be addressed by nm-scaled probes or devices; they also provide ideas 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 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 joined together by chemoselective and regioselective processes, 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. For protein design applications, Mutter and coworkers proposed already in the 1980s a very promising approach to control the directionally of protein secondary-structure building blocks. They showed that a central and rigid peptide template can be used as a structure inducing device for the nucleation and stabilization of secondary structures [1–3]. This approach, also known as template assembled synthetic proteins (TASP), involves the folding of peptide strands into desired supersecondary structures. The TASP approach has been used to construct several four-helix bundles (parallel and antiparallel) and $\beta\beta\alpha$-motifs [4–7]. The growing interest for template-assembled nano-scaled devices has led, to date, to the elaboration of a number of template molecules as structure-inducing devices similar to the TASP approach, e.g. porphyrins [8], calixarenes [9], and carbohydrates [10]. These template molecules as for the TASP approach limit the degree of freedom of the attached peptides and thus promote self-association to achieve a desired folding topology as well as introducing directionality.

The central peptide template typically utilized by Mutter's group is shown in Figure 1. A key feature of this template, composed of ten-amino acid residues, is the formation of two faces with regioselectively

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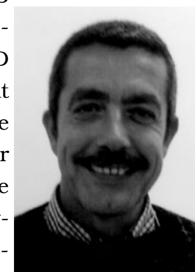
BIOGRAPHY

Didier Boturyn studied chemistry at the University Joseph Fourier, Grenoble, France. He received his PhD thesis in 1996 under the supervision of Professor Jean Lhomme. He worked on the synthesis of fluorescent probes to assess apurinic sites in DNA. From 1997 he performed postdoctoral research 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 Departement de Chimie Moléculaire (UMR 5250) in Grenoble. There he has started to work in the field of peptides notably on the syntheses and biological activities of drug-delivery systems.

Olivier Renaudet studied Biochemistry and Molecular Chemistry at the University Joseph Fourier, Grenoble, France, where he received his PhD in 2002. Under the supervision of Prof. Pascal Dumy, his PhD thesis focused on the development of chemoselective ligation strategies for the conjugation of carbohydrates and peptides. In 2002, he joined the University of Berne, Switzerland, for a postdoctoral position with Prof. Jean-Louis Reymond where he explored original iterative procedures for the synthesis of polyaromatic oligomers as protease inhibitors. Thereafter, he moved back to the University of Grenoble, France, where he obtained an Assistant Professor position in 2004, in the Département de Chimie Moléculaire. His current researches are directed towards the chemistry of carbohydrates and peptides, as well as their use in Nanoscience. He is particularly interested in developing molecular tools based on multitopic glycoclusters for biological applications related to the construction of cancer vaccines, vectors, inhibitors or microarrays.

BIOGRAPHY

Pierre Labbé was born in 1955 in Moutiers (France). After a master in Physical Sciences and a PhD in Molecular Chemistry obtained at the Université Scientifique et Médicale de Grenoble, he had a one-year Post-Doc training at the Laboratoire d'Electrochimie Organique et Analytique (CEN-Grenoble) where he studied the photoinduced reactivity of electrogenerated cation-radicals. Then he became assistant-professor at the Université de Savoie (Chambéry) where he developed the modification of clay-based materials by amphiphilic compounds and their applications to modified electrodes for analytical and catalytic purposes. 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 diagnostic and therapeutic applications. This activity is developed in strong synergy with the other activities in the group that bring together the synthesis of molecular recognition systems by chemical engineering of biomolecules (nucleic acids, peptides, oligosaccharides and related bioconjugates) and their applications in the field of life science and nanoscience. Current research activities concern protein-sugar interactions, recognition events between peptidic ligands and cellular receptors, cell adhesion, build-up of stimuli-responsive multilayered assemblies based on host-guest interactions between biopolymers.



functionalizable amino acid side chains. Owing to the presence of these selectively addressable side chains, these templates are also known as regioselectively addressable functionalized templates (RAFT) [11,12].

This possibility of regioselective functionalization extends the utility of these templates beyond structure-inducing devices to other fields of application, which was foreseen by Mutter and Vuilleumier [13,14]. They realized the functionalization possibilities of these templates, which are capable of orienting structural and/or functional units in well-defined spatial orientations, for the construction of nano-sized devices. Such templates displaying functional groups/ligands in defined spatial orientations for interacting with particular receptors were initially used in molecular recognition studies [7,15] and peptide mimicry studies as surface mimetics [6,15].

Research in the field of protein design and synthesis has opened up the possibilities for construction of elaborate functional devices by the controlled stepwise attachment of functional groups to the peptide template. Important research has been achieved in orthogonal protection strategies [11], solubilizing techniques [16], and chemoselective ligation methods [17–20]. Through application of the controlled functionalization, together with a highly defined structural core, the RAFT concept has proven extremely useful in synthetic protein design and established itself as a versatile tool in the field of peptide mimicry [21–23].

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BIOGRAPHY

Pascal Dumy (43 years) conducted the research for his PhD thesis at the Université Montpellier-I, France. From 1993 he performed postdoctoral research 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, and since 2003 he is Head of the research department, Grenoble, France. 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). His main research interests are directed to chemical biology and nanosciences and include the chemistry of biomolecules (peptides, nucleic acids, oligosaccharides), nonviral vectors for biomolecules vectorization and in vivo targeting, molecular imaging, tumor neoangiogenesis, synthetic vaccines, surface functionalization, and the use of biomolecules in nanosciences.

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 oligonucleotides synthesis and various applications such as DNA micro-arrays preparation, G-quadruplex mimetic and DNA-based nanostructures.

BIOGRAPHY

Gunnar Dolphin studied organic chemistry at Göteborg Universitet, Sweden, where he also obtained his PhD in 1997 under the supervision of Prof. Lars Baltzer. For his PhD thesis he worked on the de novo design, synthesis and structural characterization of helix-loop-helix peptides that fold into native-like proteins. Thereafter, Gunnar has continued research on structural / functional analysis of de novo designed as well as native peptides and proteins, first, at the department of Organic Chemistry at Göteborg Universitet and later from 2002 at Linköping Universitet, Sweden. In 2005 he moved to France and currently he is working together with Prof. Julian Garcia and Prof. Pascal Dumy at Université Joseph Fourier in Grenoble. There he has started research on neurodegenerative disorders such as the synthesis and structural characterization of template assembled protofibril models, associated with Alzheimer's disease. Currently he is working with high throughput screening and drug discovery projects against amyloid formation. His research interests in peptide chemistry also include structural activity relationships of antimicrobial peptides and cell penetrating peptides.

Julian Garcia received his Diploma in biochemistry from the University of Lyon, France. He went then to the University of Grenoble, France, where he earned his PhD in pharmaceutical sciences under the supervision of Professor A. Mariotte in 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.

In this Review, we summarize the research performed in laboratories based on the construction of nanosized devices with a central RAFT peptide. 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. General construction principles will be discussed as well as the underlying rules for designing multifunctional constructs.

CHEMICAL APPROACHES FOR THE DESIGN OF RAFT-BASED STRUCTURES

During the past decade, our research activity has focused on the use of the RAFT and the chemical attachment of a number of functional building blocks including peptides, carbohydrates, oligonucleotides, and fluorescent and electrochemical probes to obtain new integrated functional systems as paradigms of nonviral vectors for drug targeting and molecular imaging, of nanomaterials for surface functionalization

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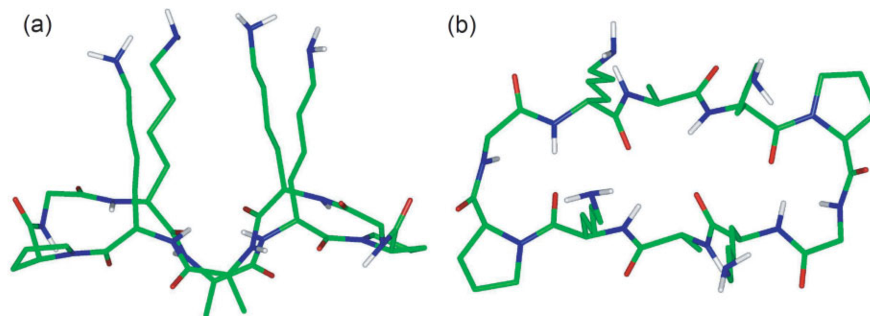
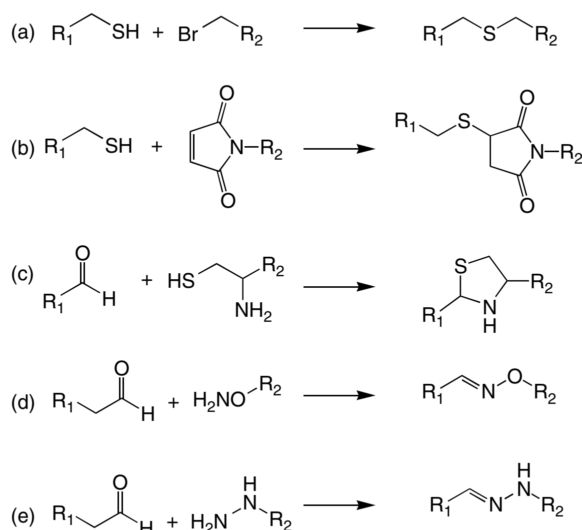


Figure 1 Molecular model showing the side (A) and top views (B) of a RAFT with four-unmodified lysine units. The characteristic structural feature of this template is the presence of two-prolylglycine sequences, as β–type II turn inducers, that constrain the backbone conformation into an antiparallel β sheet.

and transduction of protein folding processes and of synthetic vaccines. The fabrication of these systems requires facile and flexible conjugation protocols for the controlled attachment of functional building blocks to the template. Since many chemical functions are present in biomolecules that often hamper or prevent a conjugation step, highly chemoselective and regioselective mild reaction conditions are preferred for conjugation. Several chemoselective linkages have been employed for RAFT assembly (Scheme 1). For instance, thioether linkages can be formed by the reaction of a thiol group with a haloalkyl group or a maleimide group [24,25]. The thiazolidine linkage, formed by the reaction of an aldehyde group with an amino thiol group, is also used [26]. Template assembly by hydrazone linkage, formed by the reaction of an aldehyde group with a hydrazine derivative, has also been recently illustrated [27].

In our laboratory, we have focused on the use of oxime ligation for the anchoring of different functional building blocks on the RAFT. Oxime-ligation reaction has been extensively studied for the conjugation of biomolecules. It has been shown that the chemoselective oxime linkage can be successfully employed to prepare peptide-oligonucleotide conjugates bearing peptides at either the 3′- or 5′-terminus of the oligonucleotide [28,29]. The methodology has been further explored by our group for the labeling of oligonucleotides and RNA [30] and for the anchoring of oligonucleotides on glass support [31]. The earlier results from our group and others have shown that the oxime bond does have certain advantages over other types of linkages. For instance, oxime-bond formation is highly efficient, does not require the use of any activation or stabilization step and does not suffer from the lack of regiospecificity as is the case with thio- or amine-based ligations. Since the oxime ligation is carried out at slightly acidic pH, at which the free amino groups in peptides are protonated, it also helps to solubilize the peptide in water either alone or with a cosolvent.



Scheme 1 Various chemoselective ligation techniques used for the attachment of functional building blocks on the templates: (a) thioether, (b) maleimide, (c) thiazolidine, (d) oxime and (e) hydrazone.

The two faces of the RAFT can be selectively functionalized by using orthogonally protected side-chain groups. These templates are prepared by a combination of solid- and solution-phase-based methods (Scheme 2). The linear peptide sequence is first assembled on an acid-labile linker by using Fmoc-based protocols. During the chain assembly, the requisite numbers of lysine moieties with orthogonally protected side chains are incorporated into the peptide sequence. The peptide chain is then cleaved from the support and a head-to-tail cyclization is performed in solution by using PyBOP in DMF at high dilution. The orthogonal protections on the side chains (PG1 and PG2) are then selectively removed to generate free amino groups that can be used for the introduction of an aminooxy or aldehyde precursor. Alternatively, the conjugation reaction can be carried out while the peptide is still on the support.

Different strategies can be employed for the preparation of the conjugates depending on the nature of the functional groups to be anchored on the RAFT

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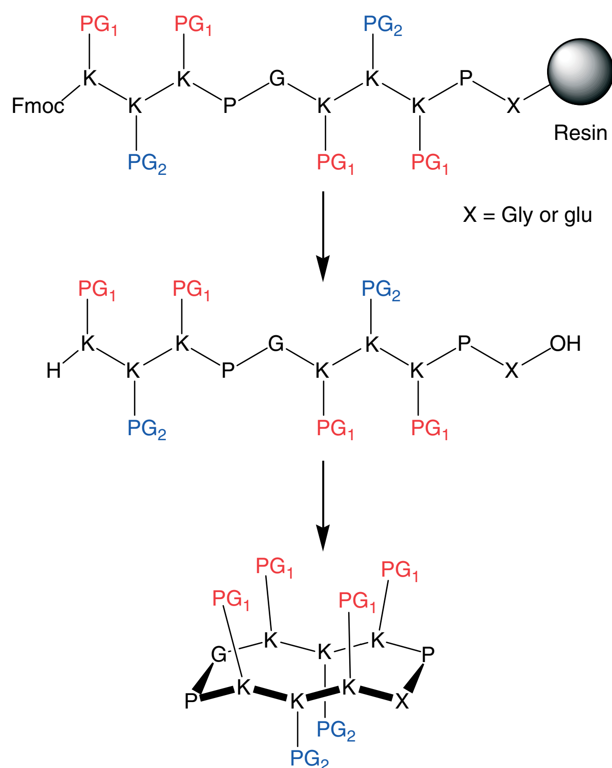

Scheme 2 Strategy for the preparation of the RAFT exhibiting orthogonal protecting groups. This scheme is available in colour online at www.interscience.wiley.com/journal/jpepsci.

(Scheme 3): the upper face can be functionalized by the aldehyde group and the lower face by the aminooxy (i) or vice-versa (ii). In both the cases, the synthetic scheme thus involves chemoselectively addressable template (CAT) intermediate in which the conjugation reaction can be performed first on the lower face followed by a second conjugation on the upper face (downside-up strategy) or vice-versa (upside-down strategy). These different strategies are illustrated with the following examples.

Bis-conjugation by a Downside-up Strategy

In this case, the first conjugation reaction is performed on the lower face of the template. This was performed by oxime-bond formation by reaction of aldehyde-containing reporters with aminooxy-functionalized RAFT. The aminooxy function on the lower face was introduced using the succinimide ester of *N*-Boc-*O*-(carboxymethyl)-hydroxylamine and the aldehyde groups on the upper face were incorporated by oxidative cleavage of serine residues attached to the lysine side chains. The CAT intermediate **1** was prepared from the suitably protected RAFT by standard protocols. Alternatively, the intermediate **2** was prepared using an entirely solid-phase supported process (Scheme 4).

As an example of this strategy, the CAT intermediate **1** was used to prepare a conjugate bearing a cluster of RGD peptides on the upper face and the antimicrobial peptide $(KLAKLAK)_2$ on the other face (Scheme 5) [32]. The ‘KLA’ peptide was first attached and the four RGD peptides were subsequently assembled on the upper face after liberation of the aldehyde function by oxidative cleavage of the serine residues [33].

Starting from **3**, a complementary alternative strategy involving only one chemoselective ligation has also been described [34]. In this strategy, the formation of an amide or thiourea bond on the lower face and the subsequent incorporation of carbohydrate recognition motifs on the upper face is realized, following an oxime-based strategy from aminooxylated modified sugar derivatives [35]. The carbonyl group was introduced on the upper face by using levulinic acid **14,** to prevent side reactions during the oxidative cleavage, commonly required to generate aldehyde functions from serine (Scheme 6).

Bis-conjugation by an Upside-down Strategy

In this case, the reporters are first assembled on the upper face of the template and the other reporter is subsequently attached on the lower face. This strategy is more convenient for the incorporation of high molecular-weight elements (nucleic acids, proteins) as the effector on the lower face. The reporters to be introduced on the upper face can contain the aldehyde or the aminooxy group for the coupling reaction with aminooxy and aldehyde functionalized CAT, respectively.

By using two successive oxime-bond formations, conjugation of oligonucleotides as effectors was achieved on the lower face of the template by using 5′-aminooxy-containing oligonucleotides (Scheme **7**(A)) [33] or 5′-aldehyde-containing oligonucleotides (Scheme **7**(B)) [36]. ‘RGD’ peptides and carbohydrates have been used as targeting entities. It is important to notice that no *trans*-oximation reaction occurred [33,36].

In another method, oxime ligation was performed to attach RGD on the upper face. Thereafter, fluorescent probes were introduced on the lower face via amide-bond formation by using commercially available thiocyanate-activated cyanine 5(Cy5) probe (Scheme **8**).

APPLICATIONS

Design of Nanovectors

The development of molecular devices endowed with tumor-targeting functions should enable the specific delivery of toxic molecules or imaging probes or a combination thereof to malignant tissues, thus increasing their local efficacy. Such vectors can pave the way for the development of new classes of therapeutics and diagnosis, which could in the future be called tailor-made medicine. In line with this concept, among

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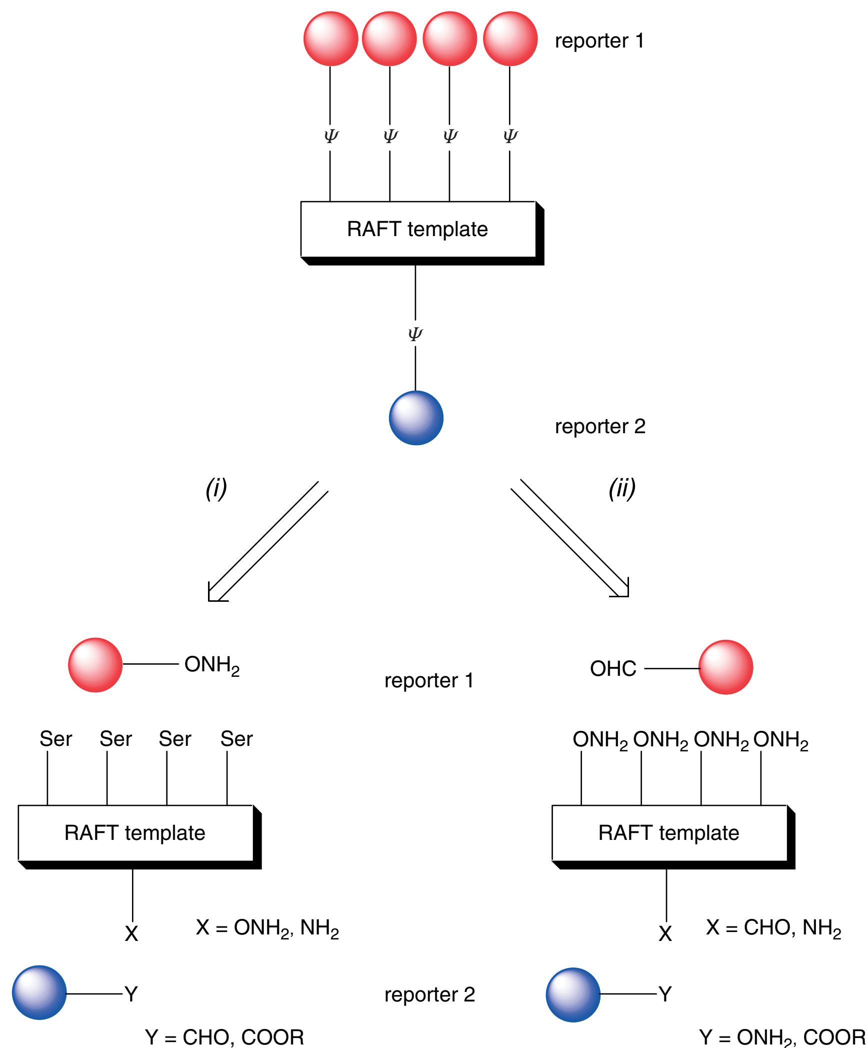
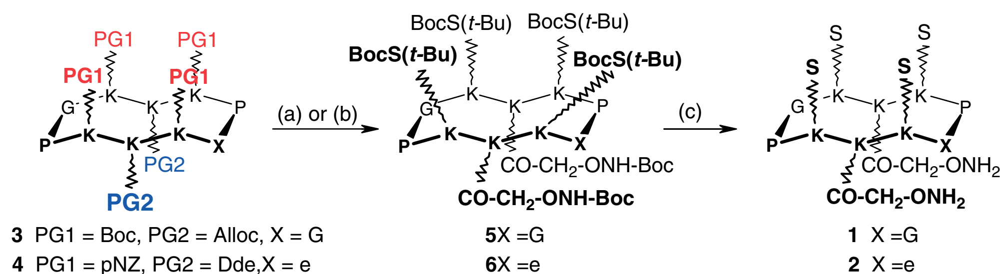


Scheme 3 Strategies for the attachment of reporters using CAT. We defined the 'upper face' and the 'lower face' containing 4 and 1–2 attachment sites respectively.



Scheme 4 Chemoselectively addressable templates **1** and **2**. Reagents and conditions: From **3** (a) 50% TFA, CH_2Cl_2, 15 min; BocSer(*t*-Bu)OH, PyBOP, DIPEA, DMF, 30 min; $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, 1 h; $BocNHOCH_2CO$-OSu, DIPEA, DMF, 30 min. From **4** (b) $SnCl_2$, PhOH, AcOH, DMF, 1 h; BocSer(*t*-Bu)OH, PyBOP, DIPEA, DMF, 30 min; 2% hydrazine, DMF, 30 min; $BocNHOCH_2CO$-OSu, DMF, 30 min; (c) 95% TFA, TIS, H_2O. e denotes D-Glu. This scheme is available in colour online at www.interscience.wiley.com/journal/jpepsci.

targeting elements, peptide ligands containing the RGD triad, which display a strong affinity and selectivity to the $\alpha_V\beta_3$ integrin, have been widely developed to target the tumor-associated cells expressing the corresponding receptors [37,38]. In this context, we have designed systems based on the RAFT, in which the upper face assembling array of four RGD motifs is used to promote programmed recognition.

Studies of such systems have shown that the tetrameric RGD-containing RAFT conjugates represent

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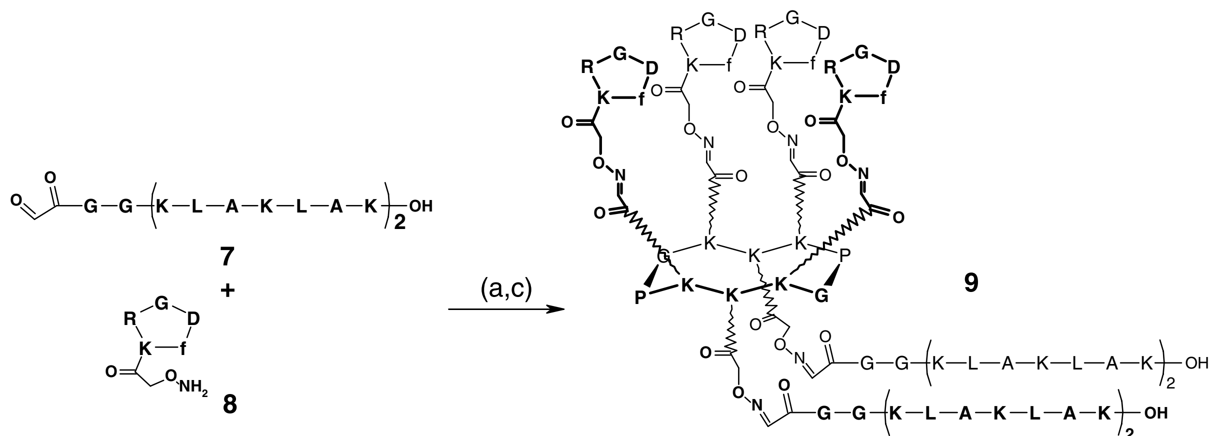
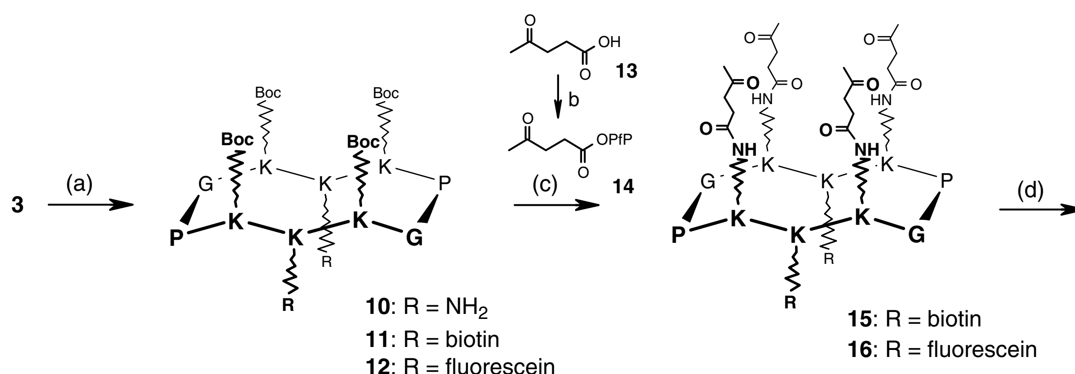


Scheme 5 Chemoselective ligation of 'KLA' peptide. Reagents and conditions: (a) **7**, 0.1 M AcONa buffer, pH 4.6; (b) $NaIO_4$, H_2O; (c) **8**, 0.1 M AcONa buffer, pH 4.6,CH_3CN (1 : 1).



17: $(Biotin)_2RAFT(sugar)_4$

18: $(Fluorescein)_2RAFT(sugar)_4$

R =

biotin =

fluorescein =

Scheme 6 Synthesis of a glyco-cluster functionalized RAFT. Reagents and conditions: (a) (i) $PhSiH_3$, $Pd(Ph_3P)_4$, DMF; (ii) biotin, PyBOP, DIEA, DMF or FITC, DIEA, DMF; (b) pentafluorophenol, DCC, CH_2Cl_2; (c) (i) TFA/CH_2Cl_2 (1/1); (ii) **14**, DIEA, DMF; (d) Aminooxy sugar, 0.1 M AcONa buffer, pH 4.0.

a new class of vectors for targeted-drug delivery as well as for molecular imaging of tumors [39,40]. Indeed, we demonstrated the capacity of such RGD-functionalized RAFT for specific *in vivo* targeting. Furthermore, these conjugates are internalized through integrin-dependent endocytosis, and thus can be used to carry additional functions for specific purposes into cells such as cytotoxicity (Figure 2) [39,41].

Design and evaluation of imaging agents for cancer monitoring. Molecular imaging of tumor cells is becoming a major field of investigation in clinical oncology, especially for the detection of cancer at

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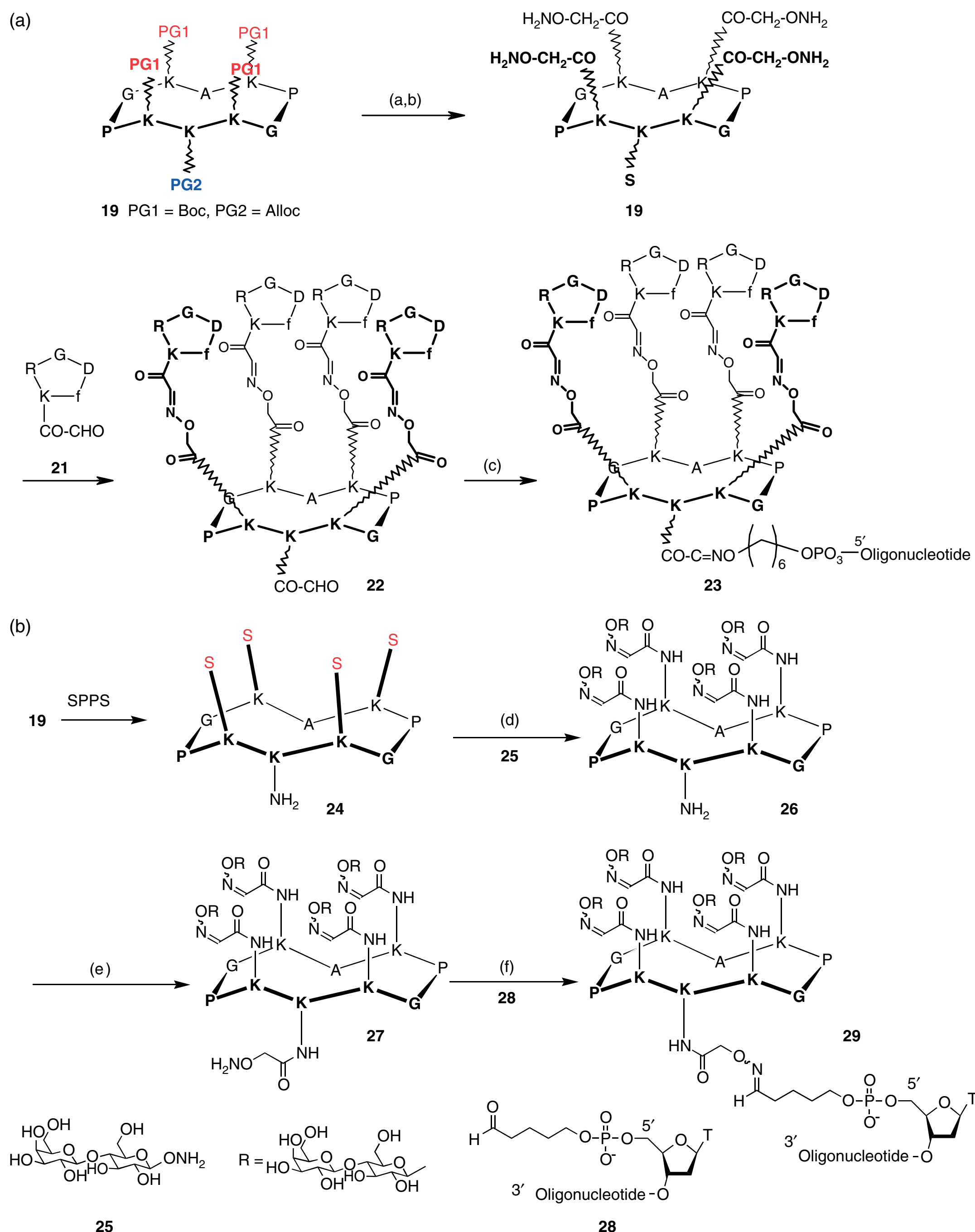

Scheme 7 Synthesis of RAFT-oligonucleotide conjugates. Reagents and conditions: (a) 0.1 M AcONa pH 4.6/CH_3CN (1 : 1); (b) $NaIO_4$, H_2O; (c) 80% AcOH, d($^{5'}$XTGGCGTCTTCCATTT$^{3'}$) [X designs the 5′-trityl-protected aminooxy linker]; (d) (i) $NaIO_4$, H_2O; (ii) **25**, 10% AcOH; (e) (i) BocAoa-OSu, DIEA, DMF; (ii) 50% TFA; (f) 80% AcOH, **28**. This scheme is available in colour online at www.interscience.wiley.com/journal/jpepsci.

its earliest stages. To prove the utility of RAFT scaffolds for tumor imaging, a broad range of labeled compounds were prepared using a convergent and regioselective assembly together with the upside-down strategy. Several markers were used such as biotin, fluorescein [39], Tc^{99m} [42], and very recently NIR (near-infrared) dyes such as Cy5 (Scheme **8**) [41,43]. For the noninvasive imaging of tumors, optical imaging using NIR probes appears as a new complementary modality to the conventional nuclear or MRI (magnetic resonance imaging) techniques because of its low cost and fewer constraints.

For instance, our *in vitro* and *in vivo* studies show that the tetrameric Cy5-RAFT(c[-RGDfK-])$_4$ **30** targets more specifically subcutaneous tumors as well as abdominal metastases than the cognate monovalent

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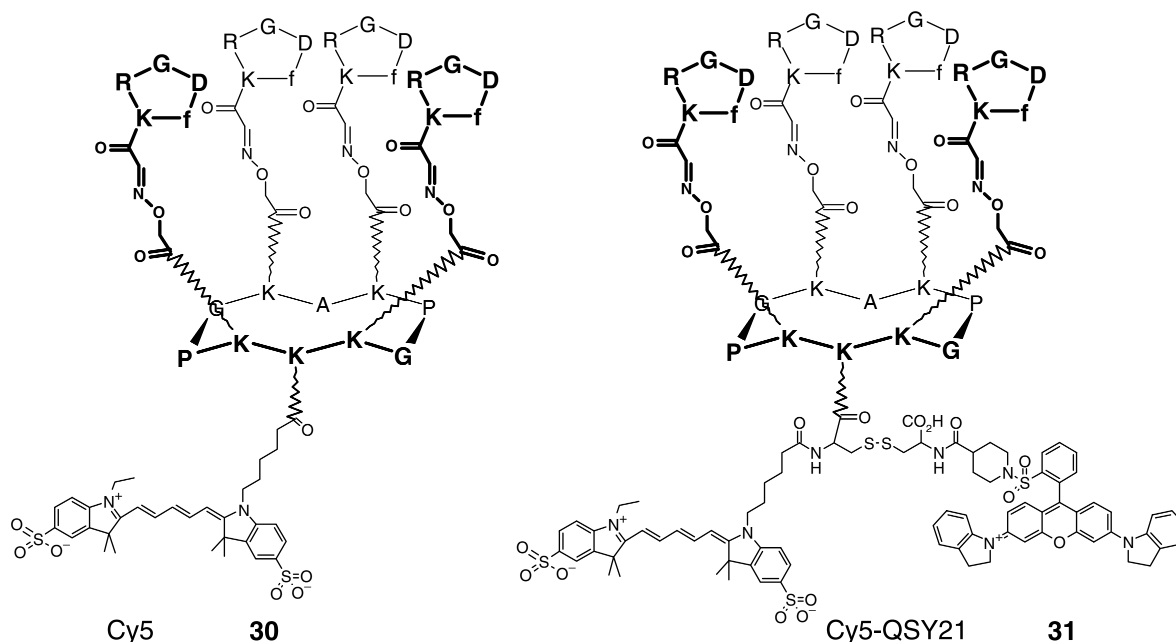

Scheme 8 RAFT bearing the RGD as recognition motif and Cy-5 **30** and Cy-5-QSY21 **31** as fluorescent probes.

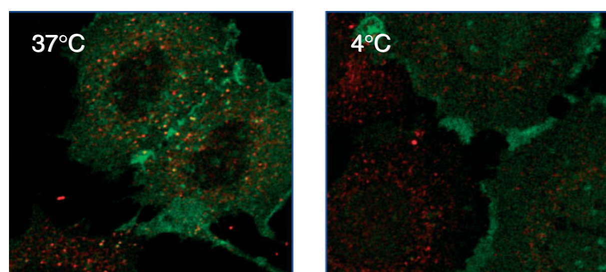

Figure 2 Internalization of the Fluorescein-RAFT (c[-RGDfK-])$_4$ system. Adherent HEK293(β3) cells were incubated for 30 min at 37 °C or 4 °C with 2 μM peptide. Early endosomes were detected by an anti-EEA1 antibody and revealed by a secondary antibody conjugated to cyanine 3 dye (red). Colocalization of endosomes stained in red and the peptide in green was demonstrated by confocal laser microscopy and results in an yellow signal.

ligand [44]. An additional study was aimed to improve RAFT(c[-RGDfK-])$_4$ performance by introducing an activatable linker (disulfide bridge) and applying the concept of 'smart-probes', initially described by Weissleder and colleagues [45]. Examples of such agents include quenched NIR fluorochromes that can be activated by tumor-associated enzymes [46,47]. We then developed a self-quenched Cy5-RAFT(c[-RGDfK-])$_4$ by linking a fluorescence quencher (QSY21) to Cy5 via a disulfide bond (Scheme **8**) [43]. Whole-body optical imaging of IGROV1 tumor-bearing mice injected with QSY21-Cy5-RAFT(c[-RGDfK-])$_4$ **31** confirmed that the quenching effect was very efficient *in vivo* since very low levels of fluorescence were measured in the skin and in the kidney during the first hours using **31**, as compared with the Cy5-RAFT(c[-RGDfK-])$_4$ **30** analog (Figure 3) [48]. Altogether this makes our molecular scaffold a very promising vector for future clinical imaging applications, currently we are investigating these new probes as helper molecules to assist in surgical removal of tumors.

Design and evaluation of therapeutic agents for cancer treatment. We analyzed the capacity of RAFT(c[-RGDfK-])$_4$ to inhibit subcutaneous tumor growth in nude mice. As shown in Figure 4, RAFT(c[-RGDfK-])$_4$ significantly slows down tumor growth whereas neither c[-RGDfK-] nor the negative control peptide RAFT(c[-RβADfK-])$_4$ exhibited any activity [41].

To improve the destruction of targeted tumoral endothelial cells, we are currently decorating RAFTs with biomolecules such as peptide or protein toxins, SiRNA, and PNA that are cytotoxic after membrane breaching. Such targeting and killing integrated functional systems would exhibit better effects than conventional chemotherapeutic drugs for the patient.

Design and immunological evaluation of synthetic anticancer vaccines. Taking advantages of the synthetic versatility of the RAFT platform, we reported recently new synthetic molecules displaying clusters

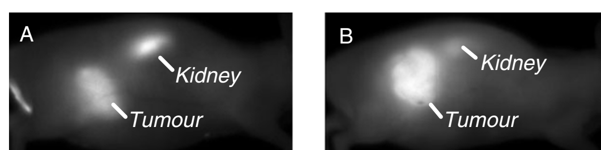

Figure 3 Representative fluorescence images after 3 h of Swiss nude mice bearing IGROV1 subcutaneous tumors after intravenous injection of 10 nmol (A) Cy5-RAFT(c[-RGDfK-])$_4$ **30** or (B) QSY21-Cy5-RAFT(c[-RGDfK-])$_4$ **31**.

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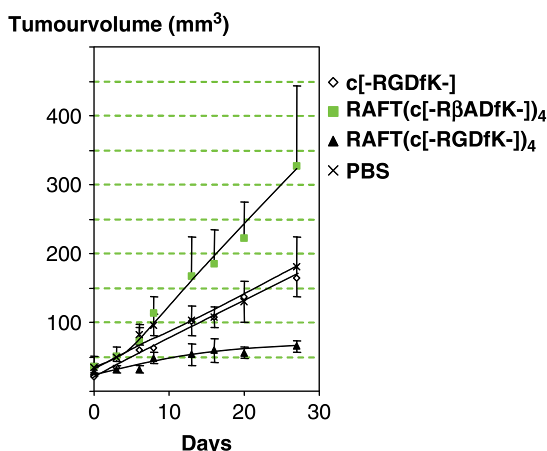

Figure 4 Nude mice inoculated subcutaneously with A549 lung carcinoma cells were treated intratumorally twice a week with 100 μl of a 20 μM solution of RGD peptides. Means value of the final tumor volumes of $n = 5$ tumors/group are presented. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

of cancer-related Tn or TF antigen analogs as vaccine candidates [49,50]. Particularly, we designed and synthesized well-defined multiepitopic RAFT scaffolds composed of Tn antigen as B-cell epitope and the $CD4^+$ helper T-cell peptide following the upside-down strategy (Figure 5) [50]. The saccharidic part at the upper face of the template mimics the clustering of the mucin-associated Tn antigen, which characterizes the surface of epithelial tumors [51]. On the other domain, we assembled the peptide fragment from poliovirus to ensure the stimulation of $CD4^+$ T-cells and activate the production of specific antibodies for the Tn antigen by B-cells. We chose the peptide fragment KLFAVWKITYKDT from the type I-poliovirus (PV) protein since it was previously proved to elicit a T-cell dependent antibody response in BALB/c mice [52].

B and T antigenicity of the vaccine candidates were investigated *in vitro*. We first studied whether the GalNAc moiety incorporated into the RAFT platform is recognized by monoclonal antibodies (mAbs), specific for the Tn antigen. ELISA assays with the two anti-Tn monoclonal antibodies IgG3 (6E11) and IgM (83D4) clearly demonstrated that the multimeric presentation in the RAFT system mimics the carbohydrate part of natural tumor-associated mucins. We next investigated, by a T-cell stimulation assay, the presentation of the PV epitope by MHC class II molecules on the antigen-presenting cell surface when incorporated into the RAFT core. By measuring the secreted interleukin-2 (IL-2) using [^{3}H]-thymidine, we observed a significant stimulating immune response by the glycosylated conjugate (PV)RAFT(αGalNAc)$_4$, indicating that the cluster of the Tn antigen improved the presentation of the T-cell determinant to the $CD4^+$ T-cells by MHC class II molecules.

We finally evaluated the immunogenicity of our RAFT constructs in BALB/c mice. The vaccine candidate was first used to immunize mice and the sera were tested by ELISA against the corresponding biotinylated conjugates. Only 0.1–1% of the Tn-specific IgG antibodies raised by the conjugate (PV)RAFT(αGalNAc)$_4$ recognized the RAFT devoid of the GalNAc moiety, confirming the suitability of the cyclic template as a nonimmunogenic carrier for vaccine constructs. To clearly determine whether the antibodies elicited by the RAFT are able to recognize the native form of Tn expressed on human tumor cells, we also tested, by flow cytometry, their binding to the Jurkat tumor cell line (Figure 6).

Thus, we confirmed that sera from mice immunized with conjugate (PV)RAFT(αGalNAc)$_4$ displaying one copy of PV fragment were able to recognize the Jurkat cells. These results show the ability of the RAFT constructs bearing both Tn- and PV-epitopes to elicit a specific immune response directed against the human form of Tn antigen. In addition to the efficiency of the oxime ligation strategy, this study suggests that the RAFT scaffold provides a promising and suitable tool for engineering potent synthetic anticancer vaccines. Research related to the design and the immunological evaluation of the three-component self-adjuvanting vaccines are currently investigated in our laboratory.

The RAFT-based multiepitopic design was exploited recently by Wang's group using a click chemistry procedure [53]. Similar constructions displaying tetravalent clusters of Man$_9$GlcNAc have been reported as mimics of the epitope of the HIV-neutralizing antibody 2G12.

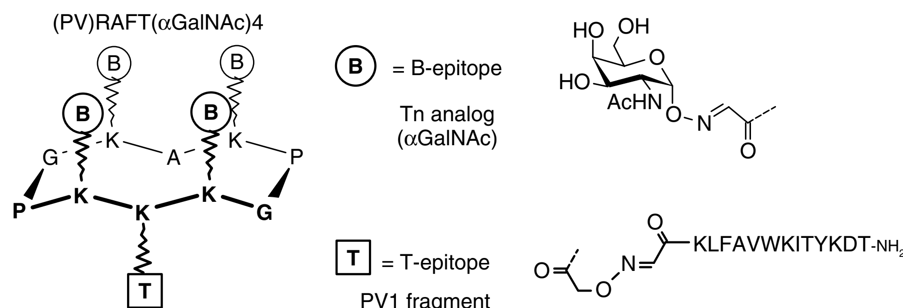

Figure 5 Multiepitopic vaccine candidate (PV)RAFT(αGalNAc)$_4$.

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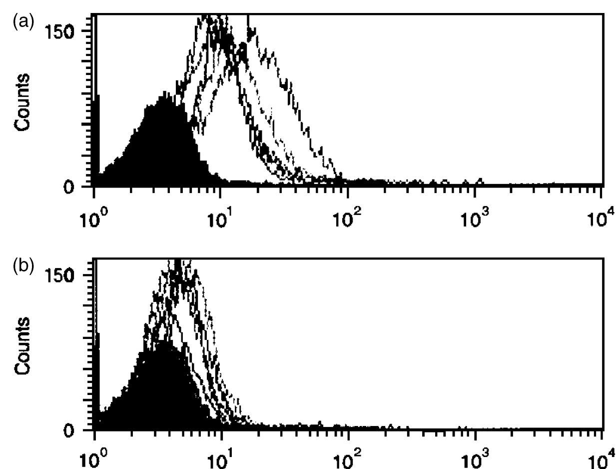

Figure 6 Antibodies induced, following immunization of mice with (PV)RAFT(αGalNAc)$_4$ (A) and RAFT(4Ser,1PV) (B) recognize the native form of Tn on human Jurkat cells. Sera obtained from mice were analyzed by flow cytometry. The binding of antibodies to Jurkat cells was revealed with phycoerythrin-conjugated anti-mouse IgG antibodies. Staining of Jurkat cells with secondary reagent alone (plain histogram), and with sera from the different groups (empty histogram) are shown.

Using SPR studies, the authors have found that such multivalent constructions ensure the expected cluster effect in 2G12 recognition. They assumed that incorporation of T-helper antigen consisting of a sequence of tetanus toxoid toxin may provide a new type of immunogen against HIV.

Proteins Mimics

The key feature of the RAFT concept is that it offers an appropriate area for the spatial arrangement of protein secondary structures that are in close vicinity upon folding in the native protein. This construct has emerged as an elegant approach for the *de novo* design of peptides and proteins. By reducing the complexity and the size of native proteins, the minimal requirement to attain a specific protein function can be studied.

Application to four-helix bundles. Of particular interest are artificial proteins that can incorporate coenzymes involved in the active site. This class of proteins is important since it covers a very large range of biological functions from electron transfer to catalysis or oxygen storage. In this context, the structural motif of four-helix bundles for the design of synthetic proteins is particularly well-suited and has been extensively investigated.

When four α helices are linked to the RAFT, structures similar to nanotubes can be formed giving rise to cavities than can accommodate a single transition metal ion, or a more complex prosthetic group. The general structure can be represented as depicted in Figure 7.

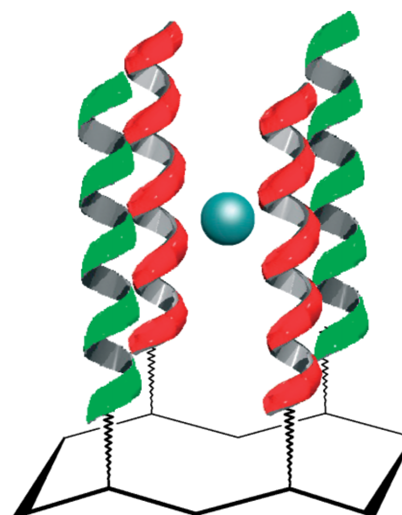

Figure 7 Schematic representation of a modular four-helix bundle protein. The ball represents a metallic ion or a prosthetic group.

Haehnel's group was one of the pioneers in this research field [54–60]. For example, they synthesized a water-soluble cytochrome *b* model protein constructed with two pairs of antiparallel helices. The heme group was attached to two-histidine residues localized in the hydrophobic interior and the four helices were selectively fixed to the template via thioether bridges [55].

Another [61] who designed a chimeric four-helix-bundle RAFT scaffold mimicking the surface of the A1 domain of the von Willebrand factor (VWF) involved in the platelet adhesion. This construct was able to inhibit the platelet aggregation induced by botrocetin and illustrated the potential of the RAFT approach for designing novel molecules with antiadhesive and antithrombotic activities.

Amyloid β-peptide protofibril models. It is widely believed that the conversion of the amyloid β-peptide Aβ into amyloid deposits is a causative event in the pathogenesis of Alzheimer's disease. Understanding the aggregation of Aβ on a molecular basis might help in the development of new approaches to eliminate or at least reduce the destructive effects of Aβ. On the pathway to amyloid, the Aβ peptides form intermediate oligomers and misfold into protofibrils. However, owing to the noncrystalline, insoluble and heterogeneous nature of fibrils, no high-resolution structure is yet available.

In this context, our laboratory is currently engaged in studying amyloid structures built onto a cyclic decapeptide RAFT scaffold [62,63]. This construct is termed $(A\beta_{16-37}Y_{20}K_{22}K_{24})_4$, and forms a module of the Aβ protofibril, containing four Aβ_{16-37}-peptide fragments linked to the RAFT (Figure 8). From molecular modeling studies, the peptide fragments were mutated to

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increase solubility and to create charge repulsions between protofibrils, thus reducing uncontrolled amyloid formation. Our results with these building blocks showed that the kinetics of protofibril formation was very sensitive to the concentration of HPO_4^{2-} and fibrils were formed without a lag-phase. The molecule is highly soluble and the formation of protofibrils with cross-β structure has been demonstrated by transmission electron microscopy and specific binding of the dyes thioflavin-T and Congo Red. Since Aβ fibrils are known to be polymorphic, our locked conformation suggests only C2y symmetry for the formed protofibrils. This assembly is expected to provide useful insights about the structure, as well as the mechanisms of fibril formation, as the kinetics of protofibril formation is highly controllable with the HPO_4^{2-} concentration.

Zinc finger protein mimics. Zinc finger proteins are transcription factors that interact with DNA controlling gene expression. They are usually constructed of two β–strands and a helical unit that form a binding site for the complexation of a Zn(II) ion. For example, zinc finger mimic was presented by Tuchscherer and coll [4,64]. According to a molecular kit approach, peptide fragments were assembled as building blocks on a RAFT template to mimic structural and functional features of zinc finger motifs (Figure 9). The helix, which represents the functional part of the protein, was covalently attached to the RAFT as a structure-inducing device. It was demonstrated that addition of Zn resulted in an increase in helicity upon complexation of Zn(II) ions (Figure 9), whereas the unattached helical block displayed a predominantly random coil conformation. This concept represents a chemical way to overcome the protein-folding problem in the design of artificial proteins.

Metallochaperone mimics. A RAFT scaffold was also used in the design of a model-binding loop from the copper metallochaperone Atx1 [65]. Metallochaperones

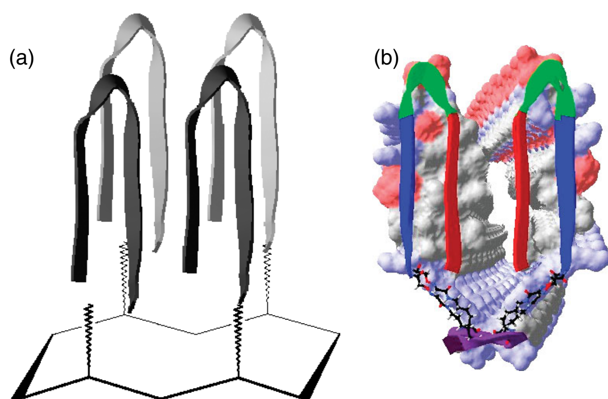

Figure 8 (A) Schematic representation of a protofibril model constructed of four β–strands linked to a scaffold. (B) Representation of the quaternary structure for the $(A\beta_{16-37}Y_{20}K_{22}K_{24})_4$ protofibril.

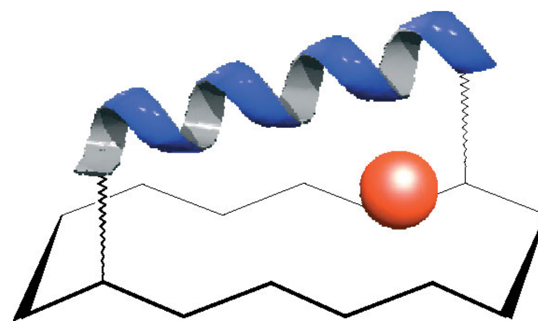

Figure 9 General features of a zinc finger motif. The template is designated to mimic two β–strands bearing an α helix. The ball represents a Zn ion.

are a class of metal-binding proteins implicated in the transport of metal ions (Cu^+, Hg^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+}) and their mimics should be of great importance for complexation of heavy metals, since at high concentrations they can lead to poisoning. A relevant task is thus the selectivity of the peptide motif that results from the binding loop of the metalloprotein for these different metal ions. We have used the Atx1 loop that binds Cu^+ by means of a MXCXXC motif. The 10-mer cyclopeptide c(-MTCSGCSRPG-) was chosen to mimic Atx1 (Figure 10) [65,66]. It provides (i) the binding sequence -MTCSGCS- of the copper-chaperone, (ii) a charged amino acid (R) to mimic a proximal lysine next to the metal binding site of Atx1 and to increase the solubility in water, (iii) the XPGX motif is able to form the β-turn found in RAFT structures. The coordination properties of the peptide were investigated by the use of complementary analytical and spectroscopic methods. Among a wide variety of metal ions, we found a strong selectivity for Hg^{2+} and Cu^+ [66].

Galactose oxidase mimics. In other studies, we have developed a model compound to mimic the active site of radical enzymes, such as the copper(II)-active site of galactose oxidase (GOase) [67]. Such models should widely contribute to the understanding of spectral properties, structural attributes and even reactivity of GOase. The potential of the RAFT cyclodecapeptide

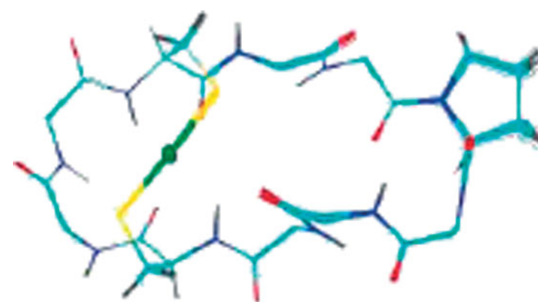

Figure 10 NMR (solution) structures calculated using *X-PLOR*. Superposition of the 20 lowest-energy structures of the Hg^{2+} complex.

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to bear phenoxyl radicals has thus been evaluated (Figure 11). The electron-donating methoxy substituent allows the phenol to be more easily oxidized. The radical species were electrochemically generated and characterized by electron paramagnetic resonance (EPR) spectroscopy. GOase mimic ($R = OMe$) differs from its analog ($R = t$-Bu) owing to its electron density, which is significantly delocalized over the methoxy substituent. These peptides are among the first radical-peptide species that have been generated and are persistent enough in solution to be characterized. Since these results concern peptides containing chelating residues (histidines, amidates, and phenolates), this work could open new possibilities in the design of model complexes for the copper(II) radical active site of GOase.

Sensors

The recent emergence of glycomics is the source of increasing efforts on probing the functional and structural features of complex multivalent carbohydrate–protein interactions which regulate many biological processes [68]. Particularly, a large number of analytical tools have been developed either in solution or by anchoring carbohydrate probes on diverse surfaces for diagnostic applications. For this purpose, we demonstrated that the immobilization of the RAFT scaffold displaying clustered carbohydrates on different supports might constitute a promising tool for the detection of carbohydrate-binding proteins with weak binding affinity and for the analysis of protein glycopatterns.

Carbohydrate microarrays. We reported recently a synthetic approach providing labeled neoglycopeptides displaying four copies of carbohydrates on one face of the RAFT core while a biotin was incorporated on the other face [34]. This structural feature first permits the direct immobilization of the glycocluster on a surface through biotin/avidin bridges. Thus, we designed biotinylated RAFT molecules displaying a cluster of lactosyl moieties as a simple ligand for the detection of peanut (*Arachis hypogaea*) agglutinin by an electrochemical approach [69]. Biotin-lactosyl conjugates were synthesized and subsequently immobilized on a polypyrrole-coated electrode for the electrochemical detection of lectin without a labeling step. A conducting copolymer film, poly(pyrrole-biotin) and poly(pyrrole-ammonium tetrafluoroborate), was first electrogenerated on glassy carbon rotating-disk electrodes and successively coated with an avidin monolayer, then with the RAFT-biotin conjugate (Figure 12). The electrode-coated assembly was incubated with the unlabeled *A. hypogaea* lectin and the binding evaluated by diverse electrochemical measurements.

As expected, we observed a lower permeability value with the RAFT-lactosyl conjugate than with monovalent lactosyl control, suggesting that the higher amount of lectin bound to the RAFT-lactosyl might be due to a stronger interaction through a cooperative multivalent effect of the four-lactosyl units. Similar results were obtained by electrochemical impedance spectroscopy. Additionally, the lectin binding was quantitatively evaluated with a peroxidase-labeled PNA by amperometric detection. We noticed a 15-fold enhanced affinity per lactosyl unit of the RAFT-cluster for the PNA lectin. A comparable effect was previously observed with a RAFT displaying a mannosyl cluster and concanavalin A (ConA) [70]. This work demonstrates for the first time the use of a full electrochemical sensor for the direct detection of carbohydrate/protein interactions without an additional labeling step. It is expected that the combination of such electrochemical approaches with the RAFT clustered presentation, may open a new way for the design of carbohydrate microarrays.

On-bead synthesis and screening. A complementary study has been performed on resin beads [71]. We developed recently a fully supported protocol involving the chemoselective assembly of RAFT-based glycoclusters and binding assays with lectins. Using the solid-phase strategy described previously [33], we first prepared a protected cyclodecapaptide core

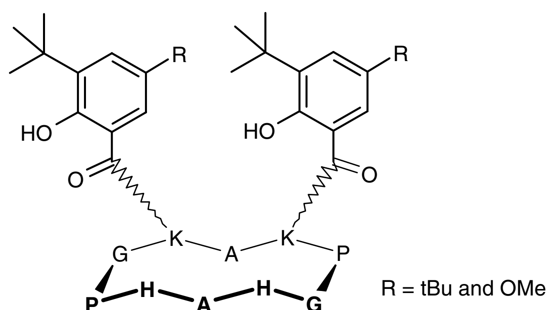

Figure 11 Formula of GOase mimics.

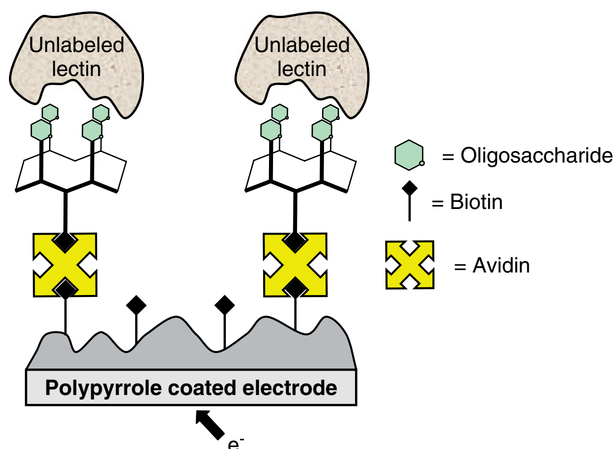

Figure 12 Strategy for immobilization of glycoclusters on a polypyrrole-coated electrode and electrochemical detection of lectin. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

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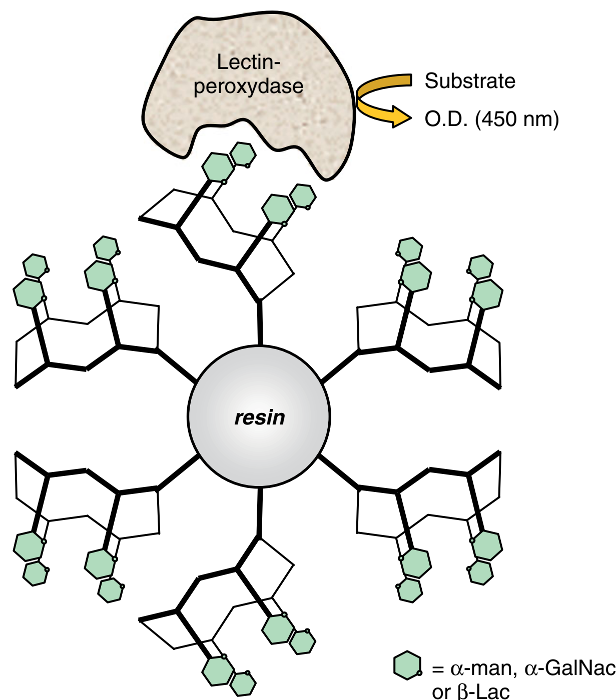

Figure 13 Supported strategy for assembly of glycoclusters and binding assays with lectins. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

on NovaSyn Tentagel resin (Figure 13). Successive cycles of deprotection and incorporation of serine residues, followed by treatment with sodium periodate, provided the cluster of aldehyde functions required for oxime ligation. After the final chemoselective assembly with aminooxy β-Lac, α-GalNAc, and α-Man, the resin beads were incubated with the corresponding labeled lectins from *A.rachis hypogaea* (PNA), *Helix pomatia agglutinin* (HPA) and aminooxylated ConA from *Canavalia ensiformis*. First, we demonstrated that the peptides displaying a cluster of sugars ensured selective recognition with the corresponding lectins, confirming thus that neither the solid support nor the RAFT core interfered with the binding. In addition, by comparing with beads, derivatized with the monovalent glycopeptide control, we noticed an enhanced affinity with multivalent ligands. This interesting result suggests that not only the multivalent presentation of the sugar ligands is important to improve the interaction but also that the control of its local density is crucial. This on-bead methodology is currently used in our laboratory as a high-throughput screening tool for the discovery of new selective ligands from combinatorial libraries and for the analysis of protein glycopatterns.

Nanoscale Redox Active Material

The RAFT scaffold has also been used for the design of a nanometer scale redox active biomolecular architecture (Figure 14) by using ferrocenyl units [72]. 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 (Figure 14). 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 nano-structured materials led to enhanced electrochemical recognition properties as proved by the amperometric type of sensing monitored in acetonitrile upon

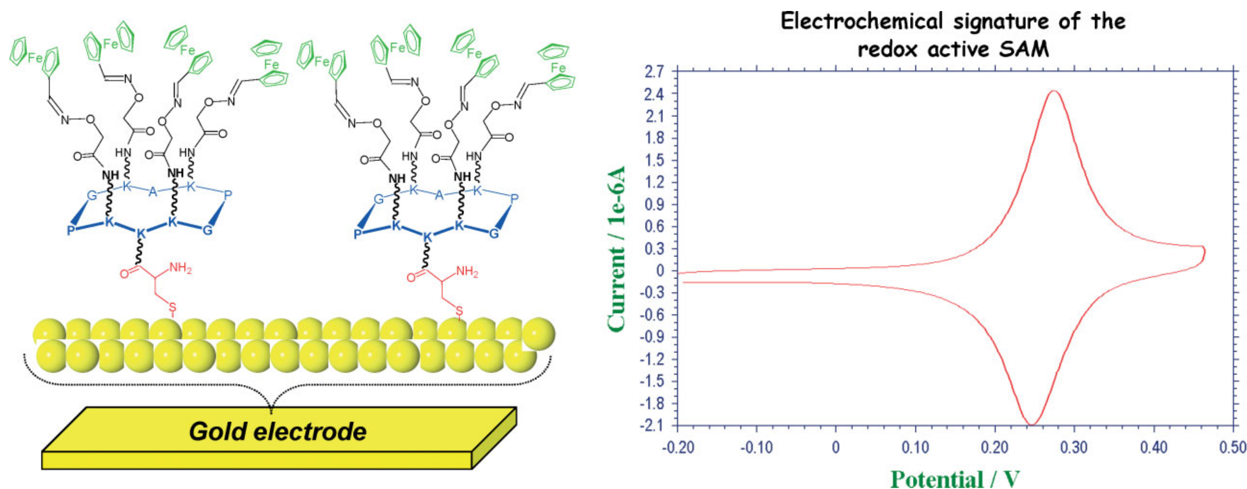

Figure 14 Preparation self-assembled monolayer on gold surface with Fc-containing peptides and cyclic voltamperometry response.

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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.

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 feasible. 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 or labels for *in vivo* imaging or drugs for their targeted delivery. Thus, these templates can be used to design and develop complex molecular nano-sized architectures and pave the way to more elaborate applications by a bottom-up design in the domain of nanobiosciences.

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