

Synthesis and properties of modified oligonucleotides



Ramon Eritja

Solid-phase synthesis protocols have become highly optimised and consequently synthetic oligonucleotides are ubiquitously found in most laboratories. These protocols allow the synthesis of a large variety of modified oligonucleotides. Our group studies the methodology used for the synthesis of DNA and RNA derivatives, in order to obtain new compounds with new and/or improved properties. The projects undertaken during 2007 address: the conjugation of small molecules (peptides, lipids, carbohydrates) to DNA and RNA for potential use in DNA/RNA therapeutics; the effect of modified bases on the structural and biological properties of oligonucleotides; the use of modified oligonucleotides in the assembly of nanomaterials and biosensors; and the preparation of new DNA-binding drugs. A review of our results in the field of oligonucleotide synthesis has been published this year (Eritja, 2007).

Synthesis of oligonucleotide-peptide conjugates

The use of synthetic oligonucleotides to control gene expression has triggered the search for new oligonucleotide derivatives with improved therapeutic potential. Oligonucleotide-peptide conjugates are chimeric molecules consisting of oligonucleotides covalently linked to peptides. As a result, synthetic oligonucleotides acquire some of the biological and/or biophysical properties of peptides.

In collaboration with the groups directed by Fernando Albericio (IRB Barcelona) and José Luis Mascareñas (University of Santiago de Compostela), we have undertaken the preparation of conjugates comprising double-stranded DNA (dsDNA) and selected fragments of biologically relevant transcription factors. Specifically, we have focused on the synthesis of conjugates between DNA and peptide regions from the leucine zipper domain of FOS. These constructs, which display a dual recognition surface provided by the presence of both the peptide and the DNA, were devised as potential specific receptors for selected FOS partners, such as the well known oncogenic transcription factor JUN. Hypothetically, recognition involves a specific interaction between the leucine-rich region of JUN and the hybrid, and an additional interaction between the oligonucleotide portion of the constructs and the basic region of JUN. The synthesis of these conjugates was complex due to the presence of long peptide sequences with a large number of tri-functional amino acids. In spite of these difficulties, the desired oligonucleotide-peptide conjugates were

prepared in good yields and showed JUN-binding properties of interest (Portela *et al*, 2007).

Synthesis of oligonucleotide conjugates carrying lipids, steroids and carbohydrates

Modified oligonucleotides are used to inhibit gene expression. To enhance the activity of oligonucleotides and their analogues, they have been covalently linked to intercalating, alkylating, photo-crosslinking and radical generating reagents. In addition to increasing the affinity for the target sequence, some of these compounds promoted the uptake of oligonucleotides by cells and improved their resistance to nucleases. Lipid moieties, such as cholesterol, linked to oligonucleotides enhance the antisense activity of these compounds as well as the silencing properties of small interfering RNA (siRNA).

During the year we have prepared several oligonucleotide (DNA and RNA) conjugates carrying lipids, steroids and carbohydrates. The properties of these new compounds are now being studied. We are collaborating with several groups in this field. The group led by José Carlos Perales (University of Barcelona) is working on the evaluation of the inhibitory properties of conjugates *in vivo*. Juan Carlos Morales' group (CSIC, Seville) is preparing carbohydrate derivatives required for solid-phase synthesis. There is a collaborative project with Syntentis-PharmaMar. Oligonucleotide-steroid conjugates are also being used for the development of bioanalytical devices for anti-doping and food control of the illegal use of steroidal anabolic hormones, in collaboration with the groups direct-



Research Group Members | Principal Investigator: Ramon Eritja
| Associate Researcher: Anna Avinyó | Postdoctoral Fellows: Clara Caminal, Alejandra Garibotti, Santiago Grijalvo, Sónia Pérez | PhD Students: Margarita Alvira, Francesc X Blasco, Sandra Ocampo | Lab Manager: Roger Ramos | Visitors: Stefania Mazzini (Italy)

ed by Pilar Marco (CSIC, Barcelona), Josep Samitier (IBEC, Barcelona), Iraida Loinaz (CIDETEC, San Sebastian) and Maria Teresa Martínez (CSIC, Zaragoza).

Synthesis of oligonucleotide clamps for triplex formation

Oligonucleotides can interact in a sequence-specific manner with homopurine-homopyrimidine sequences of duplex and single-stranded DNA and RNA to form triplexes. Nucleic acid triplexes have potential applications in diagnostics, gene analysis, and therapy. Depending on the composition and orientation of the third strand vis-à-vis the central homopurine Watson-Crick strand, triplexes are classified into two main categories: (i) parallel and (ii) antiparallel. The most well characterised parallel triplex is the one formed between a double-stranded homopurine-homopyrimidine helix (duplex DNA) and a single-stranded homopyrimidine track (triplex-forming oligonucleotide). In this type of triple helix, the triplex-forming oligonucleotide binds to the major groove (parallel to the homopurine strand of Watson-Crick double-helical DNA) via Hoogsteen hydrogen bonding, and is stabilized under acidic conditions. In the antiparallel triplexes, the third strand comprising purine bases binds in a pH-independent and antiparallel fashion to the homopurine duplex strand via reverse-Hoogsteen hydrogen bonds.

Our group, in collaboration with Modesto Orozco (IRB Barcelona) found that the introduction of an amino group at position 8 of the Watson-Crick purine produces a high stabilization of parallel triplexes. The triplex-stabilization properties of the amino group at this position results from a combined effect of the gain of one Hoogsteen purine-pyrimidine H-bond and the propensity of the amino group to be integrated into the 'spine of hydration' located in the minor-major groove of the triplex.

Sequence-specific triple-helix structures can also be formed by DNA clamps. Parallel-stranded DNA clamps consist of purine residues linked to a homopyrimidine chain of inverted polarity by 3'-3' or 5'-5' internucleotide junctions, which interact with single-stranded homopyrimidine nucleic acid targets. In this triplex, the homopurine strand of the clamp binds the homopyrimidine target through Watson-Crick bonds; and the homopyrimidine strand of the clamp forms the triplex via Hoogsteen bonding. Our group has found that the stability of triple helices is enhanced by replacing natural bases with some modified bases, such as 8-aminopurine residues (Aviño *et al*, 2007).

The classical method for the preparation of parallel-stranded clamps requires the use of reversed phosphoramidites, which are less efficient and more expensive than standard phosphoramidites. Recently, our group has reported that copper-catalysed 1,3-dipolar cycloaddition reactions between oligonucleotides carrying azido and alkyne groups can be used for the

non-templated chemical ligation of two oligonucleotides (Alvira *et al*, 2007). The cycloaddition reaction can also be performed when one of the oligonucleotides is still linked on the solid support, thereby facilitating the removal of the excess of the reagents. We have also found that solid-phase ligation of oligonucleotides using Cu⁺-catalysed cycloaddition reactions is clearly enhanced using ChemMatrix supports because of the good swelling of the support in polar (acetonitrile/water) solvents. In addition ChemMatrix supports allow both the synthesis of oligonucleotide sequences and the analysis of the binding properties of these sequences in spite of the range (anhydrous/salty aqueous) of solvent conditions used in each experiment. We believe that these properties will lead to the development of a broad number of applications in the field of drug- and protein-nucleic acid interactions (Mazzini *et al*, 2008).

Oligonucleotides and nanotechnology

There is a considerable interest in the use of biopolymers (peptides, proteins and nucleic acids) for the assembly of nanomaterials. Also oligonucleotides linked to nanoparticles are being used to monitor DNA hybridization as well as to detect a particular nucleic acid sequence of interest. Our group collaborates actively with several teams working in this field. This line of research has been summarized in a review (Eritja, 2007) and a progress report (Eritja *et al*, 2007).

Development of new drugs that bind DNA

Drug development has traditionally focused on active sites of proteins, and on identifying molecules, such as inhibitors, that bind to the active sites of proteins and directly block interactions with natural substrates. In addition to this direct mode of enzymatic regulation, it is possible to use drugs that interact with nucleic acids. These compounds have become relevant antibiotic, antiviral and anticancer agents. In collaboration with the group led by Fernando Albericio (IRB Barcelona) and Crystax Pharmaceuticals, we are designing and synthesising new drugs that interact with DNA. In this project, we are applying the knowledge acquired from peptide and oligonucleotide synthesis to obtain new and larger molecules in order to increase affinity for a particular DNA site.

In collaboration with the group directed by Raimundo Gargallo (University Barcelona), we have studied the interaction of actinomycin D (ACTD) with its target DNA sequences: 5'-CAAAGCTTTG-3', 5'-CATGGCCATG-3' and 5'-TATGGCCATA-3'. We have observed the formation of an interaction complex with a stoichiometry 1:1 (ACTD:duplex) and log of formation constants 5.1 ± 0.3 , 6.4 ± 0.2 , and 5.6 ± 0.2 , respectively. An additional interaction complex at higher temperatures has been detected in the case of AGCT and GGCC_G sequences, which has been related to the formation

of a hairpin structure stabilized by the terminal G-3' base (Vives *et al*, 2007).

Synthesis of oligonucleotides carrying DNA methyltransferase inhibitors

Aberrant DNA methylation is common in cancer. Several drugs that inhibit DNA methylation are active against some malignancies. The cytosine analogues, 5-azacytidine and 5-aza-2'-deoxycytidine, are the most frequently studied inhibitors of DNA methylation. Zebularine (1-(β -D-ribofuranosyl)-1,2-dihydropyrimidin-2-one), another pyrimidine analogue which lacks the 4-amino group of the other cytosine analogues, inhibits DNA methylation and may have activity against cancer. Zebularine has the advantage of being highly stable, and it can be administered orally. However, the disadvantage of this drug is that it is a less potent inhibitor of DNA methylation compared to azacytosine derivatives. It is believed that zebularine is not metabolised as efficiently to

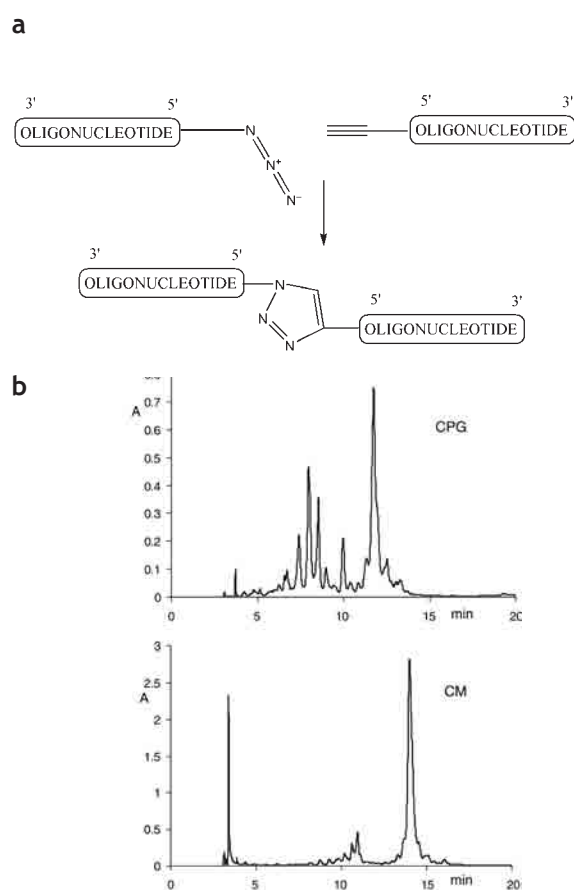


Figure 1. (a) Copper-catalysed [3+2] cycloaddition or 'Click Chemistry' between oligonucleotides carrying azido and alkyne groups. (b) HPLC profile of Cu-catalysed cycloaddition between T8-5'propargyl and 5'azido-oligonucleotides attached to controlled pore glass (CPG, above) and ChemMatrix (CM, below) supports to yield the corresponding 5'-5' linked oligonucleotides (Mazzini *et al*, 2008).

its triphosphate form as 5-azacytidine and 5-aza-2'-deoxyctyidine, and therefore not efficiently incorporated into DNA. In collaboration with Victor Márquez and Allen Yang, we have prepared oligonucleotides carrying 2'-deoxyzebularine in order to measure the efficiency of incorporation of the drug to DNA. When zebularine was included in the template strand, dGTP was preferentially incorporated by the Klenow fragment opposite zebularine, but dATP and dTTP were incorporated with 8.1% and 5.2% the efficiency of dGTP. In addition zebularine in the template strand inhibited DNA extension. Thus dZTP is efficiently incorporated into DNA and acts as a cytosine analogue, which is consistent with its capacity to inhibit DNA methylation (Dowd *et al*, 2007).

G-quadruplex and i-motif

G-rich oligonucleotides may form intra- or inter-molecular structures involving the formation of G tetrads. These structures are naturally present at the end of chromosomes or telomeres. Recently, G-quadruplex structures have been reported in promoter regions of oncogenes and several authors have suggested that G-quadruplex structures regulate the expression of these proteins. Given the potential biological relevance of the G-quadruplex, we have initiated the study of its structure and its complementary C-rich strand, which may also form a quadruplex structure known as the i-motif.

In collaboration with Raimundo Gargallo, we have studied the G-quadruplex/i-motif present in the B-cell lymphoma-2 (bcl-2) promoter. Bcl-2 is a potent oncoprotein that plays an essential role in cell survival and functions as an inhibitor of cell apoptosis.

The human bcl-2 gene contains a guanine-cytosine-rich region upstream of the P1 promoter that is implicated in the regulation of gene expression. We have examined the structural transitions of the guanine-rich and cytosine-rich sequence by means of acid-base, mole-ratio and melting experiments monitored by molecular absorption, circular dichroism, and NMR spectroscopies. One intramolecular i-motif structure has been detected in the pH range 3-7, with maximal formation at pH 6. At pH 7.1 most species have been associated with a hairpin involving Watson-Crick base pairs. Upon addition of the quadruplex-interacting ligand TmPyP4, both bcl-2 structures yield two identical interaction species with stoichiometries 1:1 and 1:2 (DNA : ligand) (Khan *et al*, 2007).

Delivery of oligonucleotide derivatives

One of the problems encountered when oligonucleotides are used to inhibit gene expression is cellular uptake. The group led by Rafael Gómez (University of Alcalá de Henares) has developed carbosilane dendrimers with positive charges that can be used to improve oligonucleotide delivery. Thus, we have initiated a collaboration project with the groups led by Rafael Gómez and Angeles Muñoz (Hospital General Universitario Gregorio Marañón) and Maria Bryszewska (University of Łódź). In this research, we have produced several oligonucleotides to be used to study complexation and cellular uptake. This study has shown a higher affinity of oligonucleotides to dendrimers (Bermejo *et al*, 2007). Moreover, oligonucleotides bound to dendrimers do not interact with serum albumin, thereby increasing the inhibitory properties of oligonucleotides (Shcharbin *et al*, 2007; Chonco *et al*, 2007).

Publications

Alvira M and Eritja R. Synthesis of oligonucleotides carrying 5'-5' linkages using copper-catalysed cycloaddition reactions. *Chem Biodivers*, 4, 2798-09 (2007)

Aviñó A, Grimau MG, Alvira M, Eritja R, Gargallo R, Orozco M and González C. Triplex formation using oligonucleotide clamps carrying 8-aminopurines. *Nucleosides Nucleotides Nucleic Acids*, 26, 979-83 (2007)

Bermejo JF, Ortega P, Chonco L, Eritja R, Samaniego R, Müllner M, de Jesús E, de la Mata FJ, Flores JC, Gómez R and Muñoz-Fernández A. Water-soluble carbosilane dendrimers: Synthesis, biocompatibility and complexation with oligonucleotides; evaluation for medical applications. *Eur J Chem*, 13, 483-95 (2007)

Chonco L, Bermejo-Martín JF, Ortega P, Shcharbin D, Pedziwiatr E, Klajnert B, Javier de la Mata F, Eritja R, Gómez R, Bryszewska M and Muñoz-Fernández MA. Water-soluble carbosilane dendrimers protect phosphorothioate oligonucleotides from binding to serum proteins. *Org Biomol Chem*, 5, 1886-93 (2007)

Dowd CL, Sutch BT, Haworth IS, Eritja R, Marquez VE and Yang AS. Incorporation of Zebularine from its 2'-deoxyribonucleoside triphosphate derivative and activity

as a template-coding nucleobase. *Nucleosides Nucleotides Nucleic Acids*, 27, 131-45 (2008)

Eritja R. Solid-phase synthesis of modified oligonucleotides. *Int J Pept Res Ther*, 13, 53-68 (2007)

Eritja R, Aviñó A, de la Torre BG, Fitzmaurice D, Ongaro A, Stanca SE, DiSalvo A, Manning B and Iacopino D. A flexible method for the fabrication of gold nanostructures using oligonucleotide derivatives. *Nucleosides Nucleotides Nucleic Acids*, 26, 1605-09 (2007)

Khan N, Aviñó A, Tauler R, Gonzalez C, Eritja R and Gargallo R. Solution equilibria of the i-motif-forming region upstream of the B-cell lymphoma-2 P1 promoter. *Biochemie*, 89, 1562-72 (2007)

Mazzini S, García-Martín F, Alvira M, Aviñó A, Manning B, Albericio F and Eritja R. Synthesis of oligonucleotide derivatives using ChemMatrix supports. *Chem Biodivers*, 5, 209-18 (2008)

Portela C, Albericio F, Eritja R, Castedo L and Mascareñas JL. Ds-Oligonucleotide-peptide conjugates featuring peptides from the leucine zipper region of fos as switchable receptors for the oncoprotein Jun. *ChemBiochem*, 8, 1110-14 (2007)

Portela C, Mascareñas JL, Albericio F, Mazzini S, Caminal C, Ramos R, Ocampo SM and Eritja R. New developments on the synthesis of oligonucleotide-peptide conjugates. *Nucleosides Nucleotides Nucleic Acids*, **26**, 963-67 (2007)

Shcharbin D, Pedziwiatr E, Chonco L, Bermejo-Martin J, Ortega P, de la Mata F, Eritja R, Gómez R, Klajnert B, Bryszewska M and Muñoz-Fernández MA. Analysis of interaction between dendriplexes and bovine serum albumin. *Biomacromolecules*, **8**, 2059-62 (2007)

Villorbina G, Canals D, Carde L, Grijalvo S, Pascual R, Rabal O, Teixidó J, Fabrias G, Llebaria A, Casas J and Delgado A. Solid-phase synthesis of a combinatorial library of dehydroceramide analogues and its activity in human alveolar epithelial cells. *Bioorg Med Chem*, **15**, 50-62 (2007)

Vives M, Tauler R, Eritja R and Gargallo R. Spectroscopic study of the interaction of actinomycin D with oligonucleotides carrying the central base sequences XGCY- and XGGCCY- using multivariate methods. *Anal Bioanal Chem*, **387**, 311-20 (2007)

Research Networks and Grants

Design and functionality of non-linear electrochemical nanoscale devices (Dynamo)

European Commission, STREP, NEST-2004-ADV-028669-1: 2006-2008

Research Director: Kyösti Kuntturi

Development of nanobio-analytical platforms based on biomolecular recognition using optical and/or electrical detection (Nanobiomol)

Ministerio de Educación y Ciencia, Strategic action on nanotechnology, NAN2004-09415-C05-03: 2005-2008

Research Director: Josep Samitier

Development of new nanosensors functionalised with DNA
Agencia Española de Cooperación Internacional, Ministerio de Educación y Ciencia, Tunes-Spain collaborative project, A/2673/05: 2006-2007

Research Directors: Zouhair M Baccar, Abdelhamid Errachid

Group of synthesis and structure of biomolecules

Generalitat de Catalunya, Consolidated groups, 2005SGR00693: 2006-2008

Project Coordinator: Enrique Pedroso

Inhibitory carbohydrate-ARN conjugates as new antiviral agents by several mechanisms: RNA interference (siARN), antisense, aptamers and ribozymes

Consejo Superior de Investigaciones Científicas, Frontier projects, PIF06-045: 2007-2008

Project Coordinator: Juan Carlos Morales

Modified oligonucleotides for the study of triplex formation and for obtaining other structures with potential technological and structural interest

Ministerio de Educación y Ciencia, BFU-2004-02048/BMC: 2004-2007

Research Director: Ramon Eritja

Nucleic acids chemistry group

Instituto de Salud Carlos III, CIBER Network on Bioengineering, Biomaterials and Nanomedicine, CB06/01/0019: 2006-2009

Research Director: Ramon Eritja

Precision chemical nanoengineering: integrating top-down and bottom-up methodologies for the fabrication of 3-D adaptive nanostructures architectures (Nano-3D)

European Commission, STREP, NMP4-CT2005-014006: 2004-2008

Research Director: Jon Preece

Rational synthesis of molecules with affinity to double-stranded DNA constituted by several units with DNA-

intercalating properties

Office for the Transfer of Research (OTRI), PTR1995-0976-OP: 2006-2008

Research Director: Fernando Albericio

Synthesis and properties of modified oligonucleotides of biomedical and structural interest (OMIBE)

Ministerio de Educación y Ciencia, BFU2007-63287: 2007-2010

Research Director: Ramon Eritja

In addition the group is active in the following publicly funded networks:

European Platform on Nanomedicine, a network of European scientists working in nanomedicine

NanoSpain, a network of Spanish scientists working in nanosciences

Oncostem. Collaboration agreement, University of Geneva

RIBORED, a network of Spanish scientists working in the field of RNA field

Plataforma Española de Nanomedicina, a network of Spanish scientists working in nanomedicine

RANN, a network of Spanish scientists working in nucleosides, nucleotides and nucleic acids

Other Funding Sources

RNA-lipid conjugates

Research contract with Sylentis, Pharmamar: 2006-2007

Research Director: Fernando Albericio

Synthesis and properties of modified oligonucleotides with potential anticancer activity

Fundació La Caixa, BM04-52-0: 2004-2007

Research Director: Ramon Eritja

Collaborations

Research on nanosensors

Arben Merkoçi, National Center on Nanotechnology, Bellaterra (Barcelona, Spain)

Synthesis and analysis of triplex forming properties of oligonucleotide clamps

Carlos Cuidad, University of Barcelona (Barcelona, Spain), Anna Nadal, University of Girona (Girona, Spain), Modesto Orozco, IRB Barcelona (Barcelona, Spain)

Synthesis and evaluation of modified siRNA

José Carlos Perales, University of Barcelona, Bellvitge campus (Barcelona, Spain)

Synthesis and NMR characterisation of oligonucleotides

Carlos González, Institute of Structure of Matter, CSIC (Madrid, Spain)

Synthesis of new drugs that binds DNA

Joan Aymamí, Crystax Pharmaceuticals, Barcelona Science Park (Barcelona, Spain), Fernando Albericio, IRB Barcelona (Barcelona, Spain)

Synthesis of new RNA derivatives

Ana Isabel Jjiménez, Sylentis-PharmaMar (Madrid, Spain), Fernando Albericio, IRB Barcelona (Barcelona, Spain)

Synthesis of oligonucleotides active against AIDS

Maria Angeles Muñoz Fernández, Hospital Gregorio Marañón (Madrid, Spain)

Synthesis of oligonucleotide-carbohydrate conjugates

Juan Carlos Morales, Institute of Chemical Research, CSIC (Seville, Spain)

Synthesis of oligonucleotide-steroid conjugates

M^a Pilar Marco, IIQAB-CSIC (Barcelona, Spain)

Synthesis of oligonucleotide-peptide conjugates
José Luis Mascareñas, University of Santiago de Compostela
(Santiago de Compostela, Spain)

Synthesis of oligonucleotides with structural interest
Raimundo Gargallo, University of Barcelona (Barcelona,
Spain)

*Research on nanosensors functionalised with
oligonucleotides*
Josep Samitier, IBEC, Barcelona Science Park (Barcelona,
Spain)

*Synthesis of oligonucleotides carrying DNA-
methyltransferase inhibitors and conformationally-
restricted nucleosides*
Victor Marquez, National Institutes of Health (Frederick,
USA)

