



Synthesis and properties of modified oligonucleotides

Synthetic oligonucleotides are convenient tools for a large number of studies. With the aim to obtain novel compounds with new and/or improved properties, we focus on the methodology used for the synthesis of DNA and

RNA derivatives. The projects undertaken during 2009 have addressed: i) the conjugation of small molecules to DNA and RNA for potential use in DNA/RNA therapeutics; ii) the effect of modified bases in the structural and biological properties of oligonucleotides; and iii) the use of modified oligonucleotides in the assembly of nanomaterials and biosensors.

Chemical modification to control the inhibitory properties of nucleic acids over gene expression

The use of synthetic oligonucleotides to regulate gene expression has triggered the search for new oligonucleotide derivatives with improved therapeutic potential. In these cases, nucleic acids are used to inhibit a specific gene by blocking gene translation or gene transcription or by stimulating the degradation of a particular messenger RNA (mRNA). Several strategies are possible. In the antisense strategy, synthetic oligonucleotides complementary to the mRNA of a given gene are used to inhibit the translation of mRNA to protein. In the siRNA strategy, small RNA duplexes complementary to mRNA bind to a protein complex named RISC. siRNA duplexes contain two strands: the antisense or guide strand, which binds to RISC and the sense or passenger strand, which is released as a result of the interaction of the siRNA duplex with RISC. The complex formed by the antisense or guide RNA strand and the protein complex RISC catalyses the efficient degradation of a specific mRNA, thereby lowering the amount of target protein.

This year has witnessed the completion of a 3-year study on siRNA aimed to answer whether it is possible: i) to increase siRNA stability without affecting RISC recognition; ii) to increase cellular uptake/biodistribution without affecting RISC recognition; iii) to modulate RISC recognition by chemical modification of the guide strand; iv) to prevent off-target effects by chemical modification of siRNA duplexes; and v) to fabricate a simple pharmaceutical formulation based on siRNA to cure a disease. We used luciferase and TNF- α as target genes. The latter was selected because it is a major mediator of apoptosis as well as inflammation and immunity and it has been implicated in the pathogenesis of a wide spectrum of human diseases. Luciferase was selected because it can be measured by chemiluminescence. In the dual luciferase assay, cells were transfected with two plasmids, one with the firefly luciferase gene and the other carrying the Renilla luciferase gene. One of the genes was inhibited by specific siRNA duplexes while the other was used as a control. Using this assay, it is possible to measure the inhibition of gene expression of one luciferase gene by means of chemiluminescence.

i) The question of stability versus inhibitory properties has been addressed by modifying siRNA duplexes with bicyclohexane pseudo-sugars. The pucker of the furanose ring is a crucial structural parameter in DNA/RNA. In standard B-DNA, the pucker is 2'-endo or 'South' (S) whereas A-DNA and RNA are characterised by 3'-endo or 'North' (N) pucker of N-type conformation. Several authors have focused on the synthesis of novel nucleosides as potential therapeutic agents that are biased toward one specific ring puckering. We have studied the effect of the N-type derivatives in RNA interference experiments. A few pseudo-nucleoside modifications either at guide or passenger strands have a strong stabilising effect towards degradation without decreasing the inhibitory properties of siRNA duplexes. These compounds have been prepared by Víctor Márquez (National Institutes of Health, USA).

ii) To address cellular uptake, we designed several siRNA conjugates carrying peptides, lipids, steroids, intercalating agents, carbohydrates and so on. More than 30 new siRNA duplexes have been produced. Jose Carlos Perales' group (University of Barcelona) assist us in evaluating the inhibitory properties of the conjugates. The first manuscript derived from this work has been published. In that study, we describe the synthesis of RNA carrying nucleoplasmine and the efficient delivery of these siRNA duplexes to HeLa cells. These conjugates entered the RNAi pathway to silence gene expression as efficiently as unmodified and 3'-cholesterol modified siRNA duplexes (Aviñó *et al*, 2009).

iii) The modulation of the affinity of siRNA to RISC has been addressed by synthesising siRNA duplexes with guide strands carrying several groups designed to fit on a hydrophobic pocket of RISC. We have observed that the inhibitory properties of siRNA duplexes carrying modified guide strands are affected by the size of the group at the 3'-end while the same modification on the passenger strand does not yield any change in activity. This observation is relevant for the design of new siRNA derivatives with higher potency.

iv) We have tested the effect of small chemical modifications on the passenger strand on the innate immunostimulation described

as a source of undesired off-target effect. Some modifications were shown to produce reduced immunostimulation and a strong and prolonged specific inhibitory action.

v) We have started a preclinical study on a mouse model. Modified siRNA duplexes have been tested in a mouse model of inflammatory bowel disease. Biomedical markers of this disease were clearly improved with one of the modified siRNA duplexes. This research has been performed in collaboration with José Carlos Perales (University of Barcelona) and Esther Fernández (Autonomous University of Barcelona).

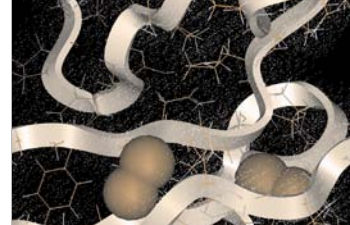
Synthesis of oligonucleotides carrying base analogues

Aberrant DNA methylation is common in cancer. Several drugs that inhibit this process 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 that lacks the 4-amino group of the other cytosine analogues, has been shown to inhibit DNA methylation and may have activity against cancer. To carry out a detailed comparison of the interaction between purified DNA methyltransferases (bacterial M.HhaI and mammalian Dnmt1) and oligonucleotides, we synthesised oligonucleotides containing either 5-azacytosine or 2-(1H)-pyrimidinone in place of the cytosine targeted for methylation. This study, performed by Judith Christman (University of Omaha, USA), supports the hypothesis that the efficacy of zebularine as an inhibitor of DNA methylation *in vivo* is dependent on its capacity to be incorporated into DNA (van Bommel *et al*, 2009).

In addition, we have examined the base-pairing properties of 2-thio- and 4-thiothymidine derivatives. Previous results cited in the literature suggested that the replacement of carbonyl oxygen atoms by sulphur atoms leads to dramatic changes in the tautomeric properties of these pyrimidine derivatives. We have shown that the presence of thiothymines induces only mild changes in DNA structure, stability and fidelity. Thus thiothymines are excellent molecules to introduce thiolated nucleosides into DNA (Faustino *et al*, 2009).

Oligonucleotides and nanotechnology

A remarkable development in the field of DNA nanotechnology was the use of stable DNA Holliday junctions with addressable sticky ends to form two-dimensional DNA crystals.



Research Group Members

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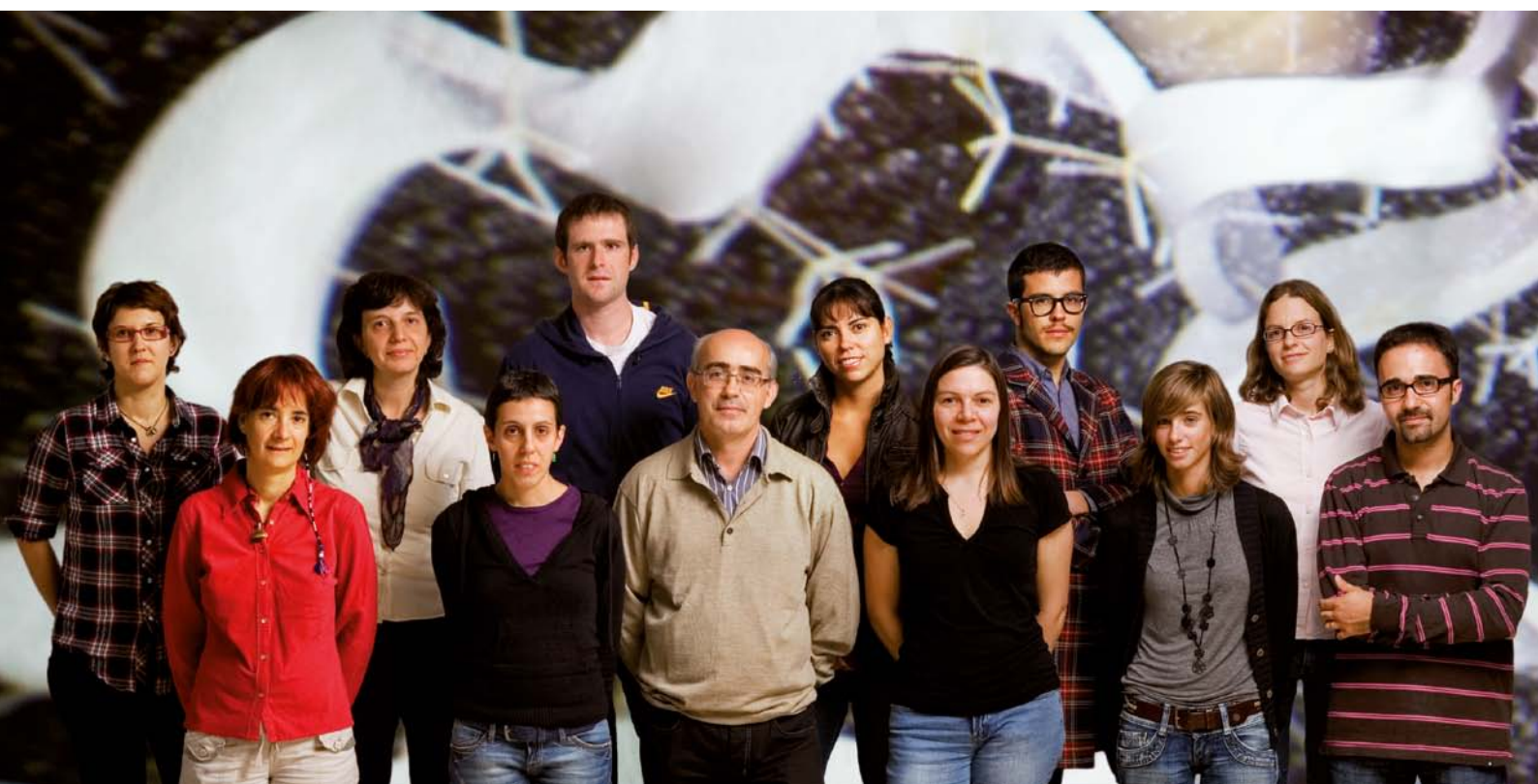
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We used the principles of construction described by Seeman and adapted then to generate systems with fine control of shape and function. For example, we transformed large DNA lattices into highly regular two-dimensional (2D) DNA networks on surfaces that provide templates for the deposition of gold nanoparticles. Our interest has focused on the preparation of thiolated 2D DNA arrays because the special reactivity of the thiol group will allow the functionalisation of 2D DNA arrays. Thiol groups have a strong affinity for gold surfaces and can also be used to introduce peptides and proteins as well as large number of molecules that have been functionalised with maleimido groups or bromo- and iodo-acetyl groups. We inserted reactive thiol groups at the nucleobase of specific sites of a well-characterised bidimensional DNA lattice to study the formation of the DNA lattices on gold, a surface that allows electrical contacts. We have demonstrated that DNA lattices carrying a single thiol derivative in each topological hairpin marker can be prepared and deposited on mica substrates. However, and most importantly, we have also shown that, in contrast to unmodified 2D DNA arrays, these thiolated 2D DNA arrays are readily deposited on gold surfaces (Garibotti *et al*, 2009; Figure 1).

In addition, we have developed a new photolithographic method that uses photolabile DNA hairpins to make patterns on silicon oxide wafers. The method described offers an attractive option for the fabrication of patterned surfaces of potential interest in the electronics and biosensor sectors (Ramos *et al*, 2009 and Manning *et al*, 2009).

In the framework of the strategic action on nanotechnology, we have provided modified oligonucleotides to M^a Teresa Martínez (CSIC, Zaragoza) to perform a study of DNA hybridisation on carbon nanotube field-effect-transistors (CNTFETs) at the Molecular Foundry of Lawrence Berkeley National Laboratory (Berkeley,

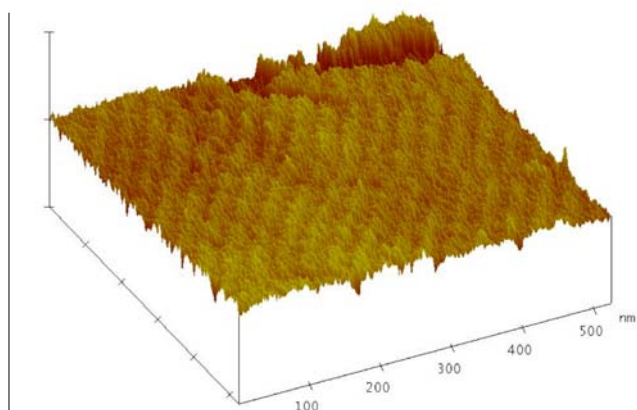


Figure 1. Topological AFM image of the thiolated A-B* DNA lattice assembled on mica. This DNA array was described by Winfree *et al* (Winfree E, Liu F, Wenzel LA and Seeman NC (1998), *Nature*, 394, 539-44). The lattice is formed by annealing of 10 oligonucleotides and subsequent deposition of DNA crystal on mica. Note the regular alignment of DNA hairpins that are used as topological markers. The functionalisation of these hairpins is the main goal pursued by our group.

USA). Using oligonucleotides and a special polymer developed by Iraidia Loinaz at CIDETEC and the facilities at the Molecular Foundry for the fabrication of CNTFETs, Martínez has achieved high precision measurements of DNA hybridisation using electrical means (Martínez *et al*, 2009).

Furthermore, in this same strategic action and in collaboration with the groups headed by Pilar Marco (CSIC, Barcelona) and Josep Samitier (Institute for Bioengineering of Catalonia, Barcelona), we have prepared oligonucleotide conjugates carrying steroids. These conjugates are being used for the development of analytic devices for anti-doping and food control of illegal steroidal anabolic hormones (Tort *et al*, 2009).

Finally, using atomic force microscopy (AFM), we have characterised peptide nanotubes formed by cyclic peptides, which were prepared by Juan Granja's group (University of Santiago de Compostela) (Reiriz *et al*, 2009).

G-quadruplex

Aptamers are oligonucleotides that were originally derived from an *in vitro* evolution process known as SELEX (systematic evolution of ligands by exponential enrichment). This process selects aptamers on the basis of their specific and tight binding affinity to a ligand of choice from a library of sequences. Through this approach, aptamers with very high affinity have been developed for diagnostic, therapeutic and other technical applications. One of the most studied aptamers is the 15-base long thrombin binding aptamer (TBA). This oligonucleotide binds specifically to thrombin at nanomolar concentrations and thus shows anti-coagulant properties of interest. TBA is characterised by a chair-like, anti-parallel quadruplex structure consisting of two G-tetrads connected by two TT loops and a single TGT loop (Figure 2). We have studied the effect of 2'-deoxyguanine (dG) residues with locked North- or South-bicyclo[3.1.0]hexane pseudo-sugars when inserted in TBA. Individual 2'-deoxyguanosines at four positions of the aptamer were replaced by these analogues where the North/anti and South/syn conformational states were confined. We conclude that locked bicyclo[3.1.0]hexane nucleosides appear to be excellent tools in the study of the role of critical conformational parameters for the formation of a stable, antiparallel G-tetrad DNA structures (Saneyoshi *et al*, 2009). This work was performed in close collaboration with the groups of Modesto Orozco (IRB Barcelona), Víctor Márquez (National Institutes of Health, USA), Stefania Mazzini (University of Milan, Italy) and Carlos González (CSIC, Madrid).

Moreover, guanine-rich sequences capable of forming G-quadruplex structures have been found in telomeres and in transcriptional regulatory regions of critical oncogenes such as *c-myc*, and *c-kit*. Ligands that selectively bind and stabilise these structures have become anticancer drugs. We have initiated the study of the G-quadruplex structures present at the initiation sites of oncogenes as well as their interaction with small drugs and with the complementary C-rich strand, which may also form a quadruplex structure known as the i-motif. This research is done in collaboration with Raimundo Gargallo's group (University of Barcelona). A detailed analysis of the equilibrium

formed by the G-quadruplex of bcl-2 and c-kit oncogenes and the corresponding complementary C-rich sequences has been made in order to determine the relative amount of duplex or separate quadruplexes that forms at a range of pH (del Toro *et al.*, 2009; Bucek *et al.*, 2009).

Design of DNA repair inhibitors in cancer chemotherapy

Chemotherapy is the main pharmacological approach used against cancer. Anti-proliferative drugs are highly cytotoxic and aggressive agents. Under attack, the biochemical repair systems of the cancer cell machinery responds, attempting to mitigate the cellular damage induced by these agents. As a result, the clinical efficacy of these drugs is often limited. High doses are required and consequently serious secondary effects are commonplace. Recent advances in the molecular biology of cancer have identified key pathways involved in the DNA repair pathways induced by chemotherapeutic agents. Regarding methylating agents, two main mechanisms have been envisaged. One involves the O6-methylguanine-DNA-methyltransferase (hAGT), which removes the methyl/alkyl group from the O6 position of guanine. A second mechanism is the base excision repair (BER) pathway, which is involved in the repair of adducts resulting from methylation of the N7 position of guanine (N7-mGs). This project seeks to develop potent inhibitors of hAGT and APE1, the latter a key endonuclease in the BER pathway. To this end, a

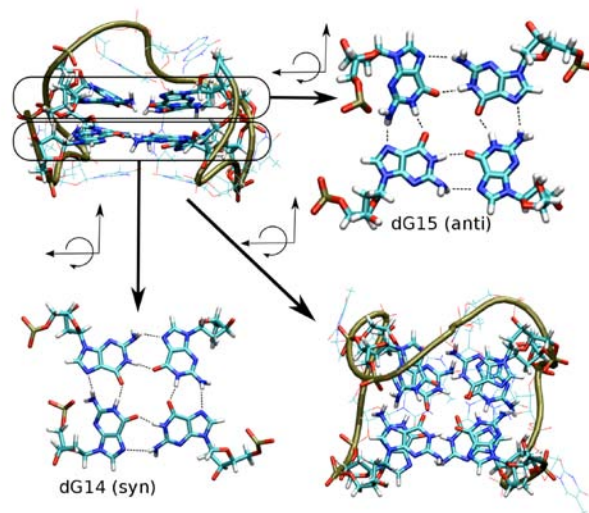


Figure 2. Structure of the thrombin binding aptamer showing the positions that were modified with locked North- or South-bicyclo[3.1.0]hexane pseudo-sugars (figure from Guillem Portella and Modesto Orozco).

combination of X-ray crystallography and *in silico* virtual screening of chemical libraries is being performed. This new research line is supervised by Carme Fàbrega.

Scientific output

Publications

Aviñó A, Ocampo SM, Caminal C, Perales JC and Eritja R. Stepwise synthesis of RNA conjugates carrying peptide sequences for RNA interference studies. *Mol Divers*, **13**(3), 287-93 (2009)

Aviñó A, Pérez-Rentero S, Garibotti AV, Siddiqui MA, Márquez VE and Eritja R. Synthesis and hybridization properties of modified oligodeoxynucleotides carrying non-natural bases. *Chem Biodivers*, **6**(2), 117-26 (2009)

Bucek P, Jaumot J, Aviñó A, Eritja R and Gargallo R. pH-Modulated Watson-Crick duplex-quadruplex equilibria of guanine-rich and cytosine-rich DNA sequences 140 base pairs upstream of the c-kit transcription initiation site. *Chemistry*, **15**(46), 12663-71 (2009)

Del Toro M, Bucek P, Aviñó A, Jaumot J, González C, Eritja R and Gargallo R. Targeting the G-quadruplex-forming region near the P1 promoter in the human BCL-2 gene with the cationic porphyrin TMPyP4 and with the complementary C-rich strand. *Biochimie*, **91**(7), 894-902 (2009)

Faustino I, Aviñó A, Marchán I, Luque FJ, Eritja R and Orozco M. Unique tautomeric and recognition properties of thioketothymines? *J Am Chem Soc*, **131**(35), 12845-53 (2009)

Garibotti AV, Sisquella X, Martínez E and Eritja R. Assembly of two-dimensional DNA crystals carrying N4-[2-(tert-Butyldisulfanyl)ethyl]cytosine residues. *Helv Chim Acta*, **92**(8), 1466-72 (2009)

Jaumot J, Eritja R, Navea S and Gargallo R. Classification of nucleic acids structures by means of the chemometric analysis of circular dichroism spectra. *Anal Chim Acta*, **642**(1-2), 117-26 (2009)

Manning B, Pérez-Rentero S, Garibotti AV, Ramos R and Eritja R. Modified oligonucleotides for biosensing applications. *Sensor Lett*, **7**(5), 774-81 (2009)

Martínez MT, Tseng YC, Ormategui N, Loinaz I, Eritja R and Bokor J. Label-free DNA biosensors based on functionalized carbon nanotube field effect transistors. *Nano Lett*, **9**(2), 530-36 (2009)

Ramos R, Manning B, Aviñó A, Gargallo R and Eritja R. Photocleavage of peptides and oligodeoxynucleotides carrying 2-nitrobenzyl groups. *Helv Chim Acta*, **92**(4), 613-22 (2009)

Reiriz C, Brea RJ, Arranz R, Carrascosa JL, Garibotti A, Manning B, Valpuesta JM, Eritja R, Castedo L and Granja JR. Alpha,gamma-peptide nanotube templating of one-dimensional parallel fullerene arrangements. *J Am Chem Soc*, **131**(32), 11335-37 (2009)

Saneyoshi H, Mazzini S, Aviñó A, Portella G, González C, Orozco M, Márquez VE and Eritja R. Conformationally rigid nucleoside probes help understand the role of sugar pucker and nucleobase orientation in the thrombin-binding aptamer. *Nucleic Acids Res*, **37**(17), 5589-601 (2009)

Tort N, Salvador JP, Eritja R, Poch M, Martínez E, Samitier J and Marco MP. Fluorescence site-encoded DNA addressable hapten-microarray for anabolic androgenic steroids. *Trac Trends Anal Chem*, **28**(6), 718-28 (2009)

Van Bommel DM, Brank AS, Eritja R, Marquez VE and Christman JK. DNA (Cytosine-C5) methyltransferase inhibition by oligodeoxyribonucleotides containing 2-(1H)-pyrimidinone (zebularine aglycon) at the enzymatic target site. *Biochem Pharmacol*, **78**(6), 633-41 (2009)

Research networks and grants

Ajuts a grups de recerca reconeguts

Agency for Administration of University and Research Grants (AGAUR), 2009SGR-208 (2009-2012)
Principal investigator: Ramon Eritja

CIBER Bioingeniería, Biomateriales y Nanomedicina

Carlos III Health Institute, CIBERBBN (2006-2013)
Principal investigator: Ramon Eritja

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

European Commission, STREP-NEST-2004-ADV-028669-1 (2007-2009)
Principal investigator: Ramon Eritja

Diseño combinado de inhibidores de los mecanismos de reparación del ADN hAGT y BER como terapia contra el cáncer

Carlos III Health Institute, PI061250 (2006-2010)
Principal investigator: Carme Fàbrega

Multi-scale formation of functional nanocrystal-molecule assemblies and architectures (FUNMOL)

European Commission, STREP-NMP-2007-213382 (2008-2011)
Principal investigator: Ramon Eritja

Self-assembly guanosine structures for molecular electronic devices

European Commission, COST action MP0802 (2008-2012)
Principal investigator: Ramon Eritja

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

Spanish Ministry of Science and Innovation, BFU2007-63287 (2007-2010)
Principal investigator: Ramon Eritja

Synthesis of RNA interference linked to lipids

Research contract with Sylentis SAU
Principal investigator: Ramon Eritja

Collaborations

Characterization of peptide nanotubes

Juan Granja, University of Santiago de Compostela (Santiago de Compostela, Spain)

Synthesis and characterization of DNA quadruplex structures

Stefania Mazzini, University of Milan (Milan, Italy)

Synthesis and characterization of oligonucleotides carrying non-natural bases

Modesto Orozco, IRB Barcelona (Barcelona, Spain)

Synthesis and evaluation of modified siRNA

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

Synthesis and NMR characterization of oligonucleotides

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

Synthesis of new RNA derivatives

Ana Isabel Jiménez, Sylentis SAU (Madrid, Spain)

Synthesis of oligonucleotide-carbohydrate conjugates

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

Synthesis of oligonucleotides carrying DNA-methyltransferase inhibitors and conformationally-restricted nucleosides

Victor Márquez, National Institutes of Health (Frederick, USA)

Synthesis of oligonucleotides with cell penetrating peptides

Fernando Albericio, IRB Barcelona (Barcelona, Spain); Miriam Royo, Barcelona Science Park (Barcelona, Spain)

Synthesis of oligonucleotides with structural interest

Raimundo Gargallo, University of Barcelona (Barcelona, Spain)

Awards and honours

25 years at CSIC

Consejo Superior de Investigaciones Científicas (2009)

Awardee: Ramon Eritja