

Nucleic acids chemistry

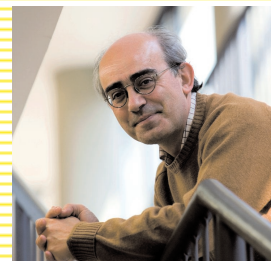
Principal Investigator
Ramon Eritja (CSIC)

Lab Technician
Roger Ramos

Postdoctoral Fellows
Anna Aviñó
Clara Caminal

Visitors
Stefania Manzini (Italy)

PhD Students
Sandra M Ocampo
Margarita Alvira



Ramón Eritja

Nucleic acids (DNA and RNA) formed by long chains of nucleotides play an important role in the genetic inheritance. In the lab, small versions of nucleic acids known as oligonucleotides, can be prepared. Our research focuses on the methodology used for the preparation of oligonucleotides and related compounds as well as the study of their properties.

Synthesis of oligonucleotide conjugates

Oligonucleotides are essential tools for DNA detection and manipulation. At the end of the eighties, the discovery of the polymerase chain reaction (PCR) and the use of oligonucleotides as inhibitors of gene expression triggered a high demand for oligonucleotide derivatives with new, tailored properties, in addition to their hybridisation properties. Most of these new properties were achieved by the addition of special molecules to oligonucleotides, which results in oligonucleotide conjugates.

Synthesis of oligonucleotide-peptide conjugates

The preparation of oligonucleotide-peptide conjugates poses an interesting challenge since conventional protection schemes are not compatible. In this regard, all standard protection schemes in solid-phase peptide synthesis use acid treatments which could cause partial depurination of DNA. Two strategies that overcome these problems have been described: 1) the post-synthetic conjugation approach; and 2) the stepwise solid-phase synthesis approach.

In the stepwise approach, these conjugates are prepared by stepwise addition of amino acids and nucleobases in solid phase on the same solid support. This is usually performed by first assembling the peptide by means of *t*-butoxycarbonyl (Boc)-protected amino acids with base labile groups, such as fluoren-9-ylmethyl (Fm), fluoren-9-ylmethoxycarbonyl (Fmoc), and trifluoroacetyl (TFA) groups, to protect side chains. Although these protocols generate oligonucleotide-3'-peptide, they cannot be used to incorporate peptides at the 5' end or in the middle of the oligonucleotide sequence. We have developed an efficient method to synthesise short oligonucleotide-5'-peptide conjugates via stepwise synthesis using

commercially available Fmoc-protected amino acids (Ocampo *et al*, 2005). Although the Fmoc-removal conditions are not orthogonal to the base-labile cyanoethyl protection of DNA phosphotriester moieties, we found that the presence of unprotected phosphate groups did not hinder the assembly of small peptide sequences on oligonucleotide supports. Groups labile to mild acidic conditions (such as 1% trifluoroacetic acid) were used to protect the side chains of N α -Fmoc-protected amino acid (Ocampo *et al*, 2005).

Synthesis of oligonucleotides conjugates carrying lipids, steroids and carbohydrates

Modified oligonucleotides are being used to inhibit gene expression. In order to enhance the activity of oligonucleotides and their analogues, they have been covalently linked to intercalating, alkylating, photocrosslinking and radical-generating reagents. In addition to increasing affinity for the target sequence, some of these compounds promote the uptake of oligonucleotides by cells and improve their resistance to nucleases. Lipid moieties, such as cholesterol to oligonucleotides, enhance the antisense activity of these compounds as well as the silencing properties of small interfering RNA (siRNA). This year we have prepared several oligonucleotide (DNA and RNA) conjugates carrying lipids, steroids and carbohydrates. The properties of these new compounds are currently being studied. We are collaborating with several groups in this field. The group led by Jose Carlos Perales is working on the evaluation of the inhibitory properties of conjugates *in vivo*, while that of Juan Carlos Morales is preparing the carbohydrate derivatives needed for solid-phase synthesis. We are also involved in a collaborative project with Syntentis-PharmaMar. Oligonucleotide-steroid conjugates are also being used for the development of bioanalytic

devices for anti-doping and food control of illegal steroidal anabolic hormones, in collaboration with Pilar Marco and Josep Samitier.

Synthesis of oligonucleotide clamps for triplex formation

Oligonucleotides interact in a sequence-specific manner to 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 that 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 stabilised under acidic conditions. In the antiparallel triplexes, the third strand, composed of 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, has found that the introduction of an amino group at position 8 of the Watson-Crick purine produces a high stabilisation of parallel triplexes. The triplex-stabilisation 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. We have found that the stability of triple helices is enhanced by replacing natural bases with several modified bases, such as 8-aminopurine residues.

This same strategy has been proven to generate stable antiparallel triplexes using purine-rich clamps as templates (*ie*, antiparallel-clamps). Antiparallel clamps

formed by a G,A- or G,T-Hoogsteen sequences linked to a polypurine sequence carrying 8-aminopurines have also been prepared and shown to form more stable triplexes if 8-aminoguanine is present in the purine Watson-Crick position.

The increased binding properties of clamps carrying 8-aminopurines may be of particular interest for the development of applications based on triple-helix formations. Specifically, we have developed capture probes for the detection of bacteria in food. Figure 1 shows an outline of the capture assay. In a previous study, we observed that binding of oligonucleotide clamps was prevented by the target's secondary structure. This problem was solved with a new strategy based on the addition of a tail sequence to a Watson-Crick strand, such that modified clamps formed triple helices with structured DNA or RNA molecules. This tail-clamp strategy overcame structural interferences, while simultaneously greatly increasing the stability of triplex formation. We synthesised parallel tail-clamps designed to bind to *Listeria innocua iap* mRNA sequences containing a polypyrimidine track (Nadal *et al*, 2006). Our aim was to obtain optimum conditions for the triplex affinity capture of these sequences in order to develop new detection methods for pathogens, by means of the specific identification of their nucleic acids. In our study, we explored the effects of pH on the interaction of parallel tail-clamps with their target by UV thermal melting analysis. In addition, we optimised a triplex affinity capture assay capable of recovering *iap* mRNA molecules from a total RNA solution purified from *L. innocua* cells in a sequence-specific manner. Optimal results were obtained with tail-clamps carrying 8-aminoadenine moieties under neutral pH conditions: 45% of the *iap* mRNA molecules from a total RNA solution were captured (Nadal *et al*, 2006).

Oligonucleotides and nanotechnology

There is a considerable interest in the use of biopolymers (peptides, proteins and nucleic acids) for the assembly of nanomaterials. Moreover oligonucleotides linked to nanoparticles are being used to monitor DNA hybridisation as well as to detect a nucleic acid sequence of interest. We are collaborating actively with several groups in this area. The following results have been obtained.

A method for the fabrication of gold nanostructures using oligonucleotide derivatives

From the fabrication of the first chips in the 1950s to the present the density of the functional units per surface unit has doubled every 3-4 years. This has been possible thanks to technological advances that allow the miniaturisation of circuits. However, the current technologies derived from photolithography

are reaching their physical limits while, conversely, chemists and biologists are working with larger and larger biomolecules. For these reasons, it is believed that in the near future, circuitry may be prepared using biomolecules that will order the nanomaterials in solution. The resulting structures will then be settled into the spaces produced by photolithography.

One promising biomolecule is DNA since it forms linear structures composed of two strands with known self-assembling properties. Moreover, there is a solid methodology to prepare DNA strands of up to 100 bases (about 30 nm of length).

One line of research of particular interest is the preparation of synthetic DNA derivatives designed to assemble a molecular wire between gold electrodes, which are required to address individual nanoparticles from macroscopic electrodes. We have used synthetic oligonucleotides to prepare the molecular wires, thereby offering the possibility to introduce modifications at any predetermined position.

The proposed structure is shown in Figure 2 and consists of three elements with distinct roles: anchoring, extension, and recognition. Two anchoring elements are located at each end, both with disulfide groups

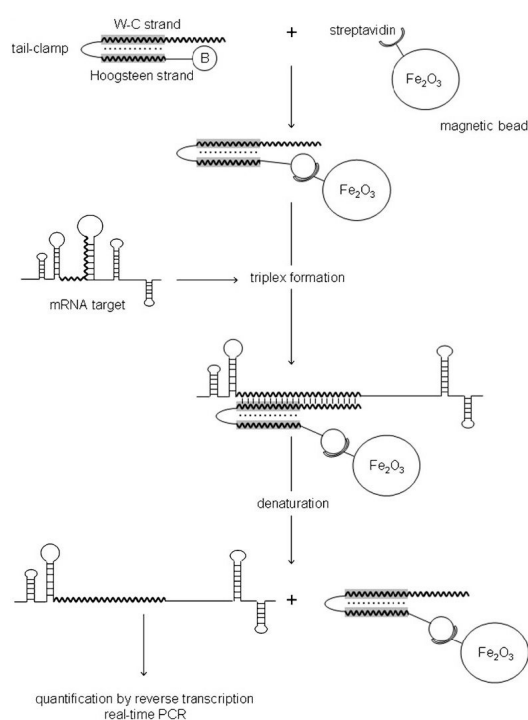


Figure 1. Triplex-mediated capture of *Listeria* RNA (Nadal et al., 2006)

that allow the wires to be attached to the electrodes. The centre of the structure is a chimeric compound with a DNA segment that positions the element in the middle of the structure. It also contains biotin as a recognition group, isolated from the DNA by a spacer molecule comprising two hexaethyleneglycol units. This recognition element is used to direct a nanoparticle into the middle of the structure as well as to connect the two branches. The size of the whole structure is determined by the extension elements between the recognition and the anchoring elements.

The extension elements (100 bases long) were prepared using protocols to produce long oligonucleotides. Special protocols were developed for the preparation of the recognition elements since the polarity of the DNA strands is reversed in the middle of the molecule, thereby providing symmetry to the central assembly. The two-armed recognition element was prepared by sequentially adding 10 different phosphoramidites. Starting from the 3'-end, the first half of the sequence was assembled using the four standard phosphoramidites. Subsequently, hexaethyleneglycol and biotin-tetraethyleneglycol phosphoramidites were added. Finally, the second half of the molecule was assembled using the four reversed phosphoramidites. Synthesis of the oligonucleotides carrying three branches was conducted in similar fashion although a symmetric branching molecule was added. First, the 20 mer sequence was built in the 3'->5' direction using standard phosphoramidites and the hexaethyleneglycol phosphoramidite. Biotin-tetraethyleneglycol was then added. Afterwards, a symmetric branching phosphoramidite was added to the sequence. Finally, the rest of the desired sequence was assembled in the 5'->3' direction using reversed phosphoramidites and the hexaethyleneglycol phosphoramidite.

Using these oligonucleotides the DNA-templated assembly of a protein-functionalised 10 nm gap electrode, from suitably modified gold nanoparticles on a silicon wafer substrate, was achieved (Stanca et al., 2006). This protein-functionalised electrode was recognised and selectively bound by a suitably modified gold nanoparticle that was localised in the 10 nm gap (Stanca et al., 2006). These findings are of interest for the fabrication of next-generation electronic devices.

Immunodetection of oligonucleotide-peptide conjugates by induced-coupled plasma mass spectroscopy (ICPMS)

ICPMS has been used to study the atomisation of nanoparticles for the development of a novel non-isotopic immunoassay by coupling this technique with the sandwich-type immunoreaction. ICPMS is an out-

standing method for trace element determination and is gaining wide acceptance due to its extremely high sensitivity and element specificity. In collaboration with Arben Merkoçi, we have explored for first time the use of ICPMS for DNA monitoring through the detection of metallic nanoparticles. The enhanced DNA signals obtained by ICPMS of gold tags are combined with the high specificity of oligonucleotide-peptide conjugate interactions with anti-c-myc monoclonal antibody followed by immunoreaction with the secondary antibody (Anti-mouse IgG) conjugated to gold nanoparticles. Although in a very early phase, the proposed ICPMS-linked DNA assay may have significant potential as a non-isotopic DNA detection method for the simultaneous determination of various sequences by labelling several kinds of inorganic nanoparticles and also by taking advantage of the recent development of ICPMS technique. This study will also be of interest for the development of novel genosensors and DNA chips based on multiple labelling by the specific immunoreactions with the peptide sequences introduced into the DNA probes (Merkoçi *et al*, 2005).

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 these active sites and directly block directly interactions with natural substrates. In addition to this direct mode of enzymatic regulation, nature makes extensive use of drug interaction with nucleic acids that have become important antibiotic, antiviral and anticancer agents. In collaboration with 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 gained from peptide and oligonucleotide synthesis to obtain new and larger molecules in order to increase affinity for a particular DNA site.

Oligonucleotides of biological and structural interest Synthesis of oligonucleotides carrying DNA methyltransferase inhibitors

Aberrant DNA methylation is a common finding 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, has been shown to inhibit DNA methylation and may have activity against cancer. Zebularine has the advantage of being very stable, and can be administered orally. However, zebularine has the disadvantage of being a less potent inhibitor

of DNA methylation compared to azacytosine derivatives. It is believed that zebularine is not metabolised to its triphosphate form as efficiently as 5-azacytidine and 5-aza-2'-deoxyctyidine, and therefore it is 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 in response to the drug. 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 was noted to inhibit 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.

Oligonucleotides and G-quadruplex

G-rich oligonucleotides may form intra- or intermolecular 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 found in promoter regions of oncogenes and several authors have proposed that the G-quadruplex regulates the expression of these proteins. Due to the potential biological relevance of the G-quadruplex, we have initiated the study of

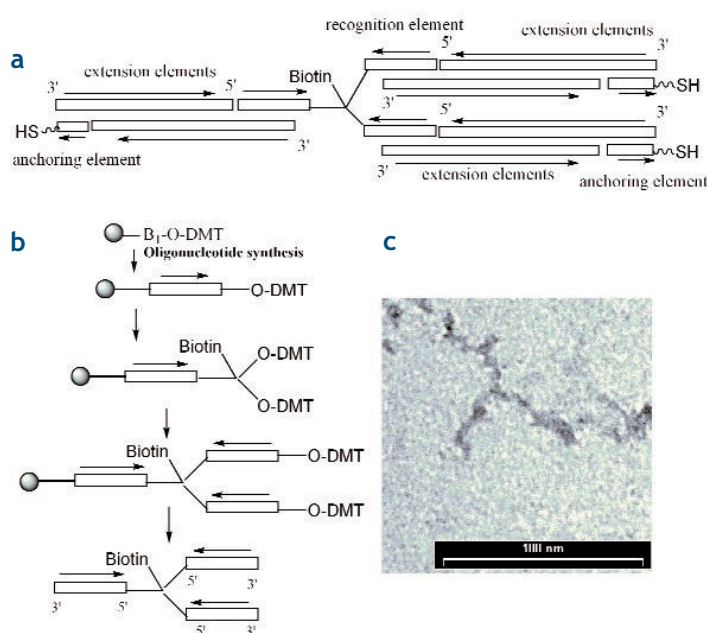


Figure 2. a) Targeted 3-branch oligonucleotide assembly used as template for gold nanostructure containing a biotin molecule used to direct a streptavidine molecule to the middle of the structure (Stanca *et al*, 2006); b) outline of the synthesis for the three-branched recognition elements; c) Transmission electron microscopy (TEM) image of three-branched DNA negative stained with uranyl acetate 1%.

these structures using synthetic oligonucleotides. In collaboration with Raimundo Gargallo, we have studied a bimolecular quadruplex. Specifically, the resolution of the dimeric intermolecular G-quadruplex/duplex competition of the telomeric DNA sequence 5'-TAG-GGT-TAG-GGT-3' and its complementary 5'-ACC-CTA-ACC-CTA-3' was examined. Melting experiments of both sequences and their mixtures were monitored by molecular absorption, molecular fluorescence and circular dichroism spectroscopy. Molecular fluorescence measurements were carried out using the molecular beacons technology, in which the 5'-TAG-GGT-TAG-GGT-3' sequence was labelled with a fluorophore and a quencher at the ends of the strand. It was observed that the parallel G-quadruplex is more stable than the antiparallel G-quadruplex. When the complementary C-rich strand is present, a mixture of both G-quadruplex structures and Watson-Crick duplex is observed, the duplex being the major species (Jaumot *et al*, 2006).

The formation of G tetrads is not desired when designing triplex-forming oligonucleotides (see above), because the formation of G-tetrads may

reduce the formation of triplex. It has been described that the quadruplex inhibits triplex formation, especially the antiparallel triplex, because of the presence of a large number of guanines in the oligonucleotides. This has fuelled the design of modified nucleobases that destabilise the tetraplex. However, unfortunately in most cases, these nucleobases also destabilise the triplex. Given the large triplex-stabilising effect of 8-aminoguanine, we tested whether G tetrads were affected by the presence of 8-aminoguanine residues. As a model of quadruplex-forming oligonucleotides, we selected the 15-base long thrombin aptamer 5'-GGTTGGTGTGGTTGG-3'. It was shown that this oligonucleotide adopts a monomeric chair quadruplex structure in the presence of potassium, which is characterised by a denaturation-renaturation profile that is reversible and observable by several techniques. Using this model oligonucleotide we have demonstrated that 8-aminoguanine has a unique profile as a molecule that simultaneously shows strong triplex-stabilising and quadruplex-destabilising properties (Lopez de la Osa *et al*, 2006).

PUBLICATIONS

Bahia D, Aviñó A, Eritja R, Darzynkiewicz E and Bach-Elias M (2006) Trimethylguanosine nucleoside inhibits cross-linking between Snurportin 1 and m3G-capped U1 snRNA. *Nucleosides, Nucleotides Nucleic Acids*, 25:909-923

Bermejo JF, Chonco L, Samaniego R, Fernández G, Eritja R and Muñoz-Fernández MA (2006) Comparative uptake of phosphorothioate oligonucleotides by human peripheral blood mononuclear cells from newborns and adults. *Eur J Sci Res*, 15:113-121

Bermejo JF, Ortega P, Chonco L, Eritja R, Samaniego R, Mullner M, de Jesús E, de la Mata FJ, Flores JC, Gómez R and Muñoz-Fernández A (2007) Water-soluble carbosilane dendrimers: Synthesis, biocompatibility and complexation with oligonucleotides; evaluation for medical applications. *Chemistry*, 13:483-495

Coma S, Noé V, Eritja R and Ciudad CJ (2005) Strand displacement of double-stranded DNA by triplex-forming antiparallel purine-hairpins. *Oligonucleotides*, 15:269-283

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

Jaumot J, Eritja R, Tauler R and Gargallo R (2006) Resolution of a structural competition involving dimeric G-quadruplex and its C-rich complementary strand. *Nucleic Acids Res*, 34:206-216

Lopez de la Osa J, Gonzalez C, Gargallo R, Rueda M, Cubero E, Orozco M, Aviñó A and Eritja R (2006) Destabilisation of quadruplex DNA by 8-aminoguanine. *ChemBioChem*, 7:46-48

Merkoçi A, Aldavert M, Tarrasón G, Eritja R and Alegret S (2005) Towards an ICPMS-Linked DNA assay based on gold nanoparticles immunocrosslinked through peptide sequences. *Anal Chem*, 77:6500-6503

Nadal A, Coll A, Aviñó A, Esteve T, Eritja R and Pla M (2006) Efficient sequence-specific purification of *Listeria innocua* mRNA species by triplex affinity capture with parallel tail-clamps. *ChemBioChem*, 7:1039-1047

Ocampo SM, Albericio F, Fernández I, Vilaseca M and Eritja R (2005) A straightforward synthesis of 5'-peptide oligonucleotide conjugates using N \pm -Fmoc-protected amino acids. *Org Lett*, 7:4349-4352

Pumera M, Castañeda MT, Pividori MI, Eritja R, Merkoçi A and Alegret S (2005) Magnetically triggered direct electrochemical detection of DNA hybridisation using Au67 quantum dot as electrical tracer. *Langmuir*, 21:9625-9629

Stanca SE, Eritja R and Fitzmaurice D (2006) DNA-templated assembly of nanoscale architectures for next-generation electronic devices. *Faraday Discuss*, 131:155-165

Vives M, Tauler R, Eritja R and Gargallo R (2007)
Spectroscopic study of the interaction of actinomycin D with oligonucleotides carrying the central base sequences XGCY- and XGGCCY- using multivariate methods. *Anal Bioanal Chem*, 387:311-320

RESEARCH NETWORKS AND GRANTS

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: 2004-2007

Project Coordinator: Jon Preece

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

Ministry of Education, Strategic action on nanotechnology, NAN2004-09415-C05-03: 2005-2008
Project Coordinator: Josep Samitier

Research groups of Catalonia. Group of synthesis and structure of biomolecules

Comission of University and Research, Autonomous Govern of Catalonia (2005SGR00693): 2006-2008

Project Coordinator: Enrique Pedroso

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

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

Project Coordinator: Kyösti Kuntturi

Development of new nanosensors functionalised with DNA

AECI-MEC, Tunez-Spain collaborative project (A/2673/05): 2006

Project Coordinators: ZM Bacca and A Errachid

In addition the group is active in the following networks:

- RIBORED, a network of Spanish scientist working in RNA
- RANN, a network of Spanish scientists working in nucleosides, nucleotides and nucleic acids
- NANOSPAIN, a network of spanish scientists working in nanosciences
- PLATAFORMA ESPAÑOLA DE NANOMEDICINA, a network of Spanish scientists working in nanomedicine
- EUROPEAN PLATFORM ON NANOMEDICINE, a network of European scientists working in nanomedicine

OTHER FUNDING SOURCES

Synthesis and properties of modified oligonucleotides with potential anticancer activity

Fundación La Caixa (BM04-52-0): 2004-2007

Project Coordinator: Ramón Eritja

Modified oligonucleotides for the study of triplex formation and for obtaining other structures with

potential technological and structural interest

Ministry of Education, BFU-2004-02048/BMC:

2004-2007

Project Coordinator: Ramón Eritja

COLLABORATIONS

Synthesis of oligonucleotides with structural interest

Raimundo Gargallo (University of Barcelona, Spain)

Synthesis and analysis of triplex forming properties of oligonucleotide clamps

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

Synthesis and analysis of triplex forming properties of oligonucleotide clamps

Anna Nadal (University of Girona, Spain)

Research on nanosensors

Arben Merkoçi (National Centre on Nanotechnology, Barcelona, Spain)

Synthesis of oligonucleotides active against AIDS

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

Synthesis of oligonucleotides

José Carlos Perales (University of Barcelona, Spain)

Synthesis of oligonucleotide-carbohydrate conjugates

Juan Carlos Morales (Institute of Chemical Research, Sevilla, Spain)

Synthesis of oligonucleotide-peptide conjugates

José Luis Mascareñas (University of Santiago de Compostela, Spain)

Synthesis of oligonucleotide-esteroid conjugates

Pilar Marco (IIQAB-CSIC, Spain)

Research on nanosensors functionalised with oligonucleotides

Josep Samitier (IBEC, Parc Científic de Barcelona, Spain)

Synthesis and analysis of triplex forming properties of oligonucleotide clamps

Carlos Cuidad (University of Barcelona, Spain)

Synthesis of oligonucleotides carrying methyltransferase inhibitors and conformationally-restricted nucleosides

Victor Marquez (National Institutes of Health, Bethesda, USA?)

Synthesis of oligonucleotides for the assembly of nanomaterials

Donald Fitzmaurice (University College Dublin, Ireland)

Synthesis of oligonucleotide-peptide conjugates, synthesis of new drugs that binds DNA, synthesis of new RNA derivatives

Fernando Albericio (IRB Barcelona, Spain)

Synthesis and analysis of triplex forming properties of oligonucleotide clamps
Modesto Orozco (IRB Barcelona, Spain)

Synthesis of new RNA derivatives
Sylentis-PharmaMar (Madrid, Spain)

Synthesis of new drugs that bind DNA
Crystax Pharmaceuticals (Barcelona, Spain)



Ramón Eritja's group, March 2006.