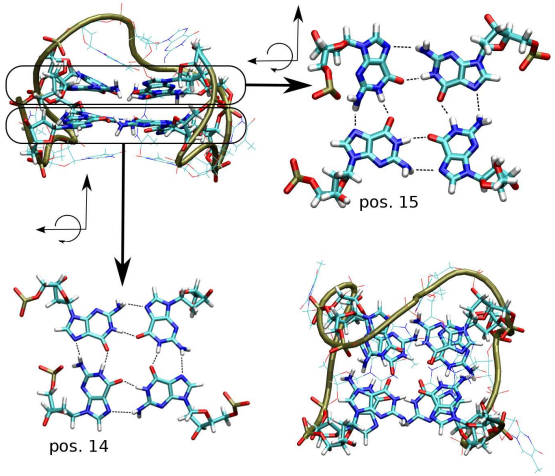
Seminaris F. Farmàcia, UB
Barcelona, 18 Març 2014

Oligonucleòtids: Aplicacions a la Biotecnologia Farmacèutica

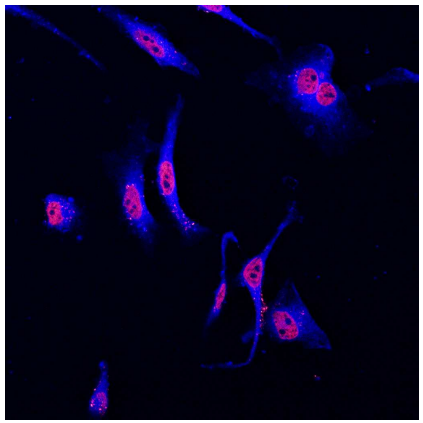
Ramon Eritja
IQAC-CSIC, Barcelona
CIBER-BBN

Nucleic Acids Chemistry Group

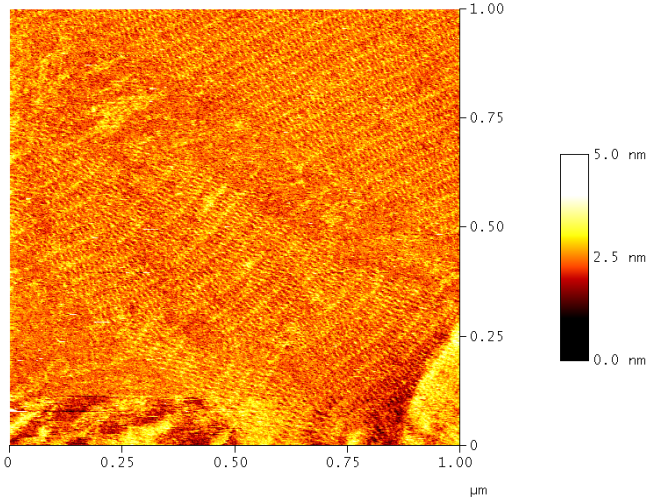


DNA structure: G-quadruplex, Triplex, Aptamers

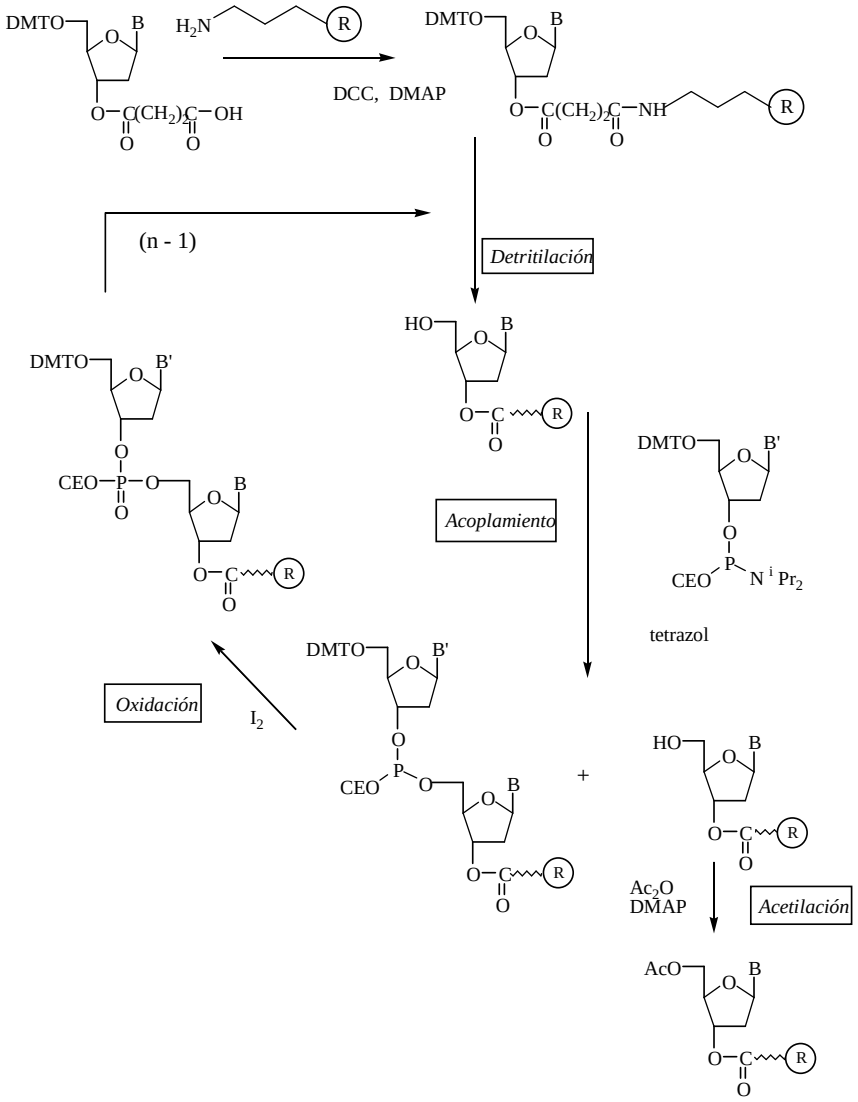
siRNA antisense



DNA nanotechnology
DNA origami

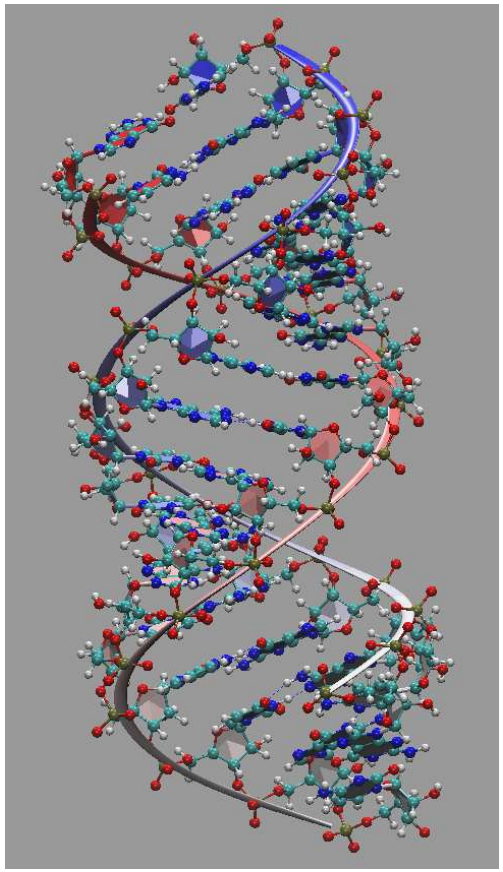


Sintetitzados automàtics de DNA i RNA



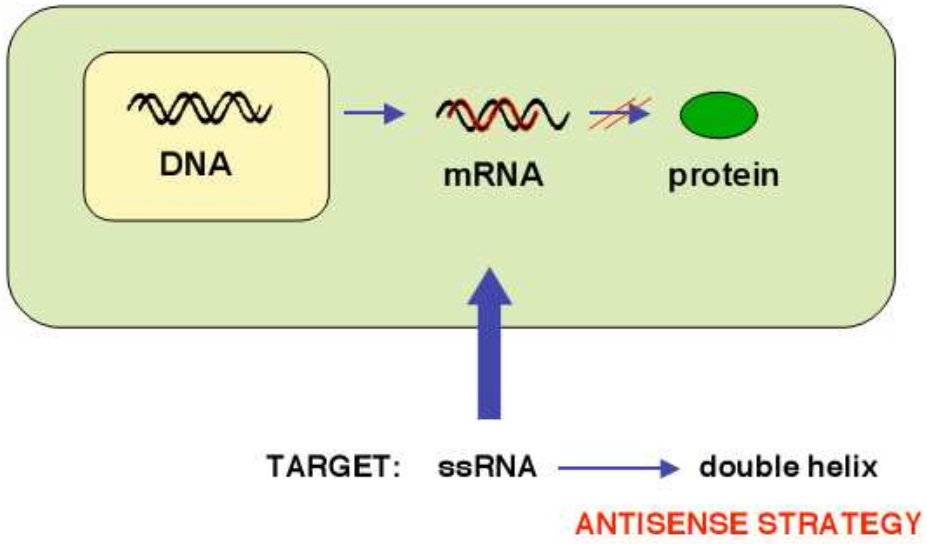
Els àcids nucleics, medicaments dels futur?

- **Antisense**
- **Ribozims**
- **Triplex, pinces (diana dsDNA)**
- **Aptamers (diana proteïnes)**
- **RNA de interferencia, siRNA**
- **Antigomirs, microRNA mimetics (diana miRNA)**
- **Esquè (Decoys, diana proteïnes d'unió a DNA)**



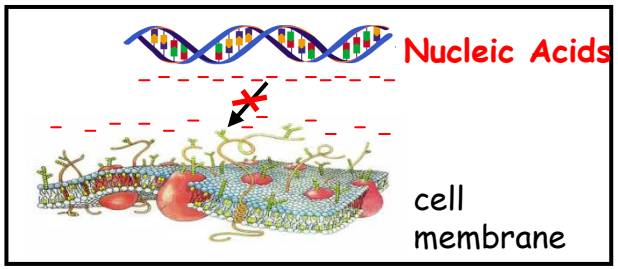
ANTISENSE strategy :

Oligonucleotides of 15-25 nucleotides complementary to a specific mRNA

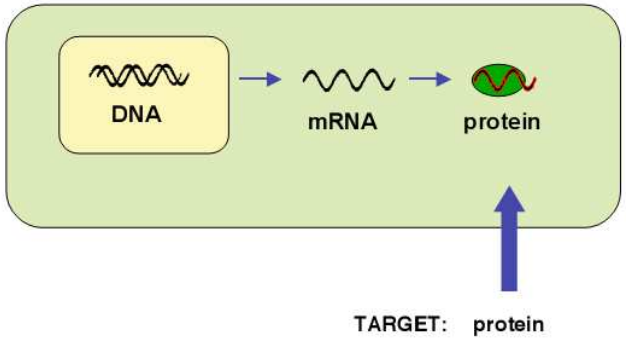


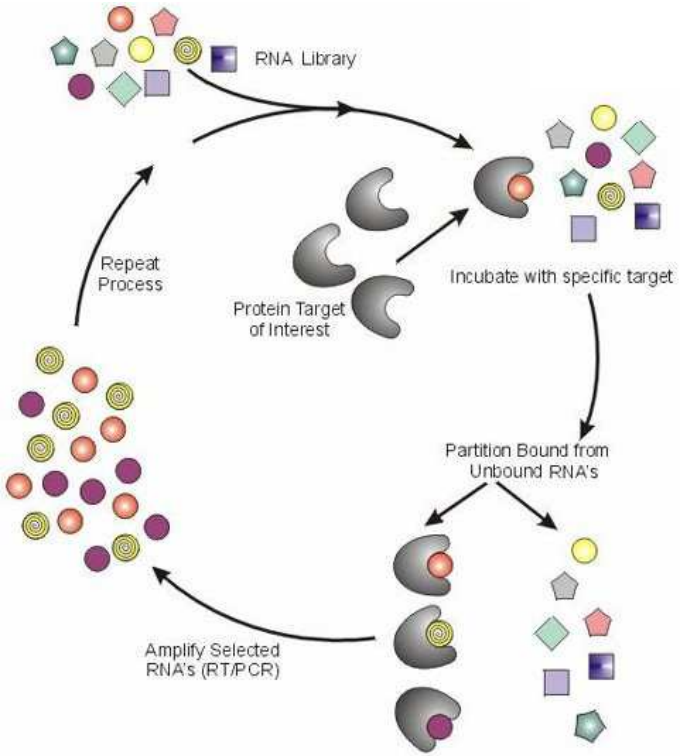
Pros: Specificity is given by the sequence. Universal and simple design

Cons: degradation by nucleases, poor cellular uptake, immunostimulation

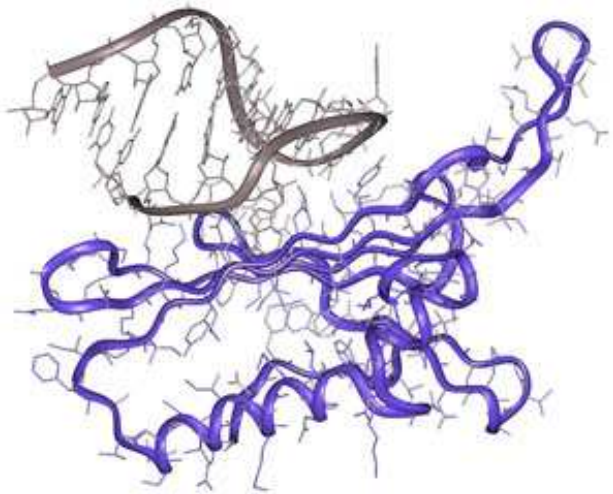


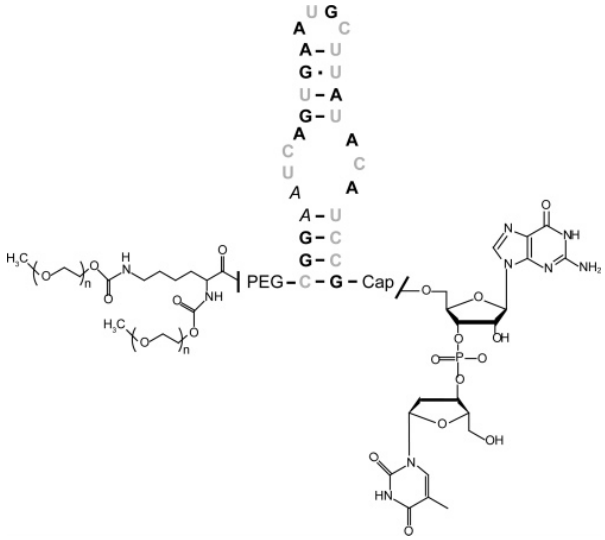
APTAMERS: Nucleic Acids that have the property of binding to a specific protein. They are obtained by selection (SELEX)





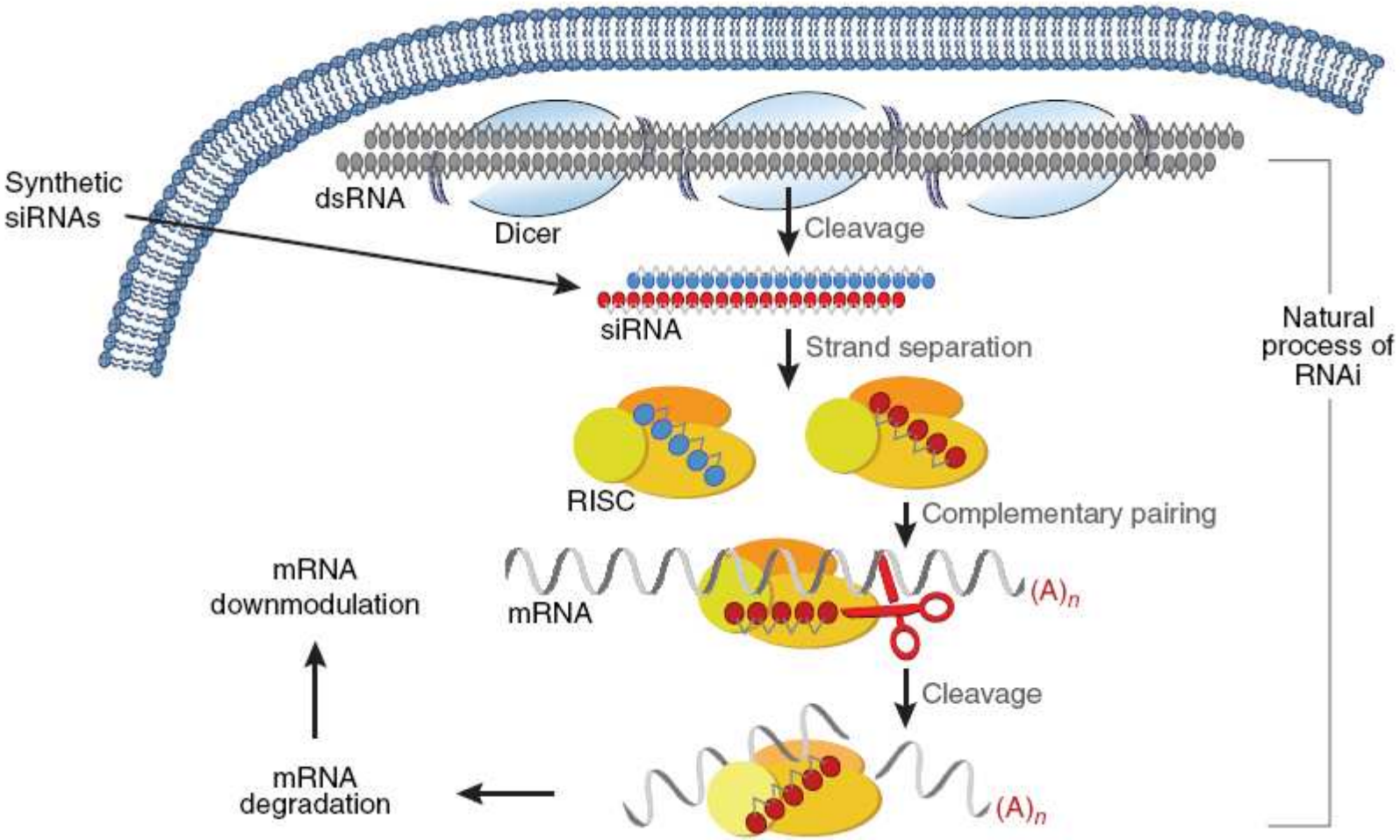
SELEX



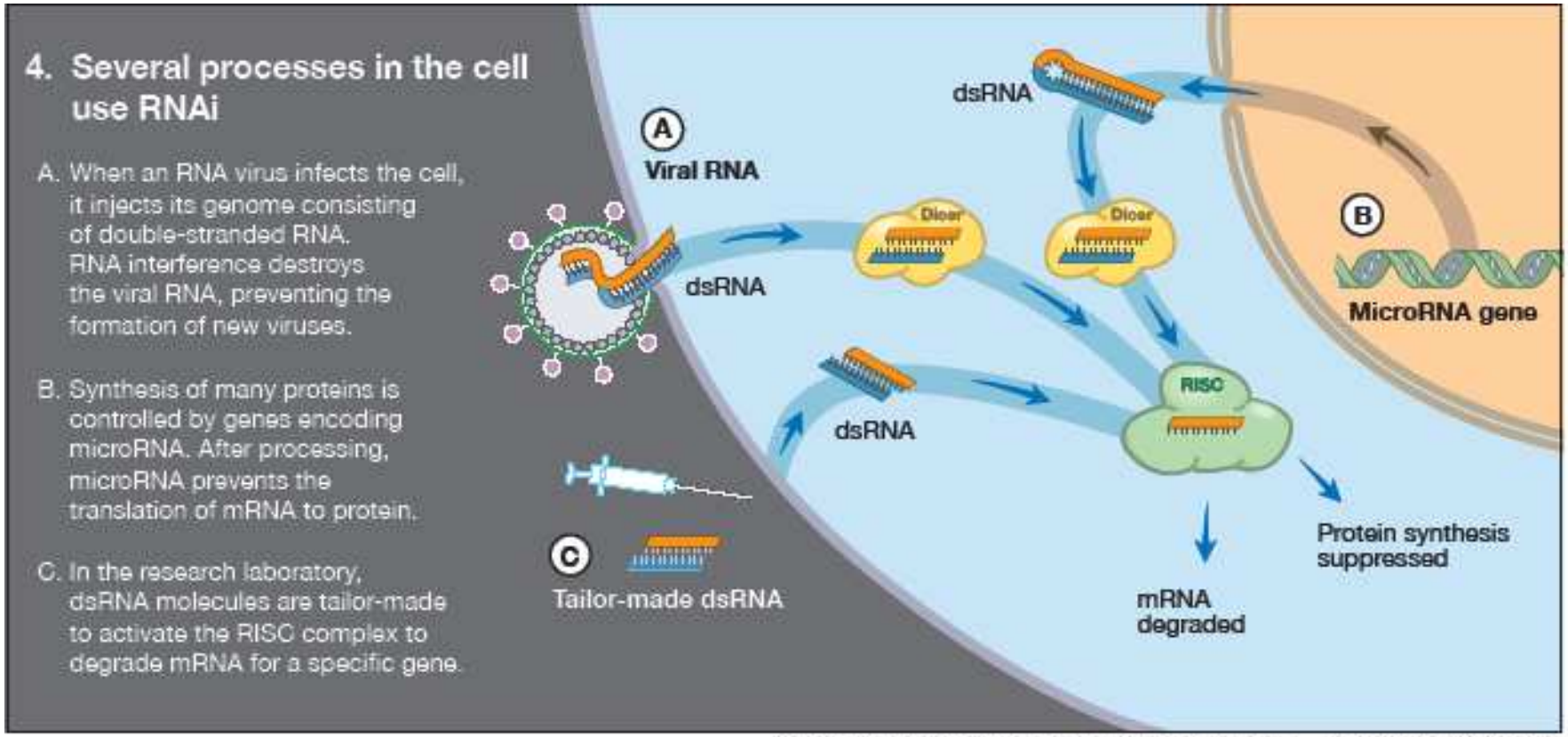


MACUGEN:
treatment of macular degeneration

RNA interference

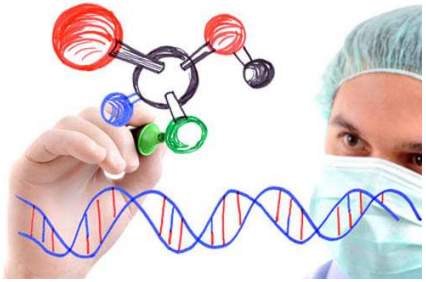


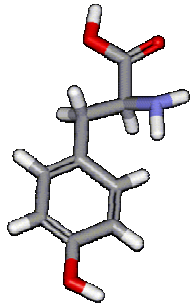
1990. RNA interference observed in Petunias by Jorgensen
1998. Discovery of RNA interference in *C. elegans* by Fire & Mello
1999. siRNA as posttranscriptional gene silencing by Baulcombe
2001. Use of synthetic siRNA by Tuschl
2006. Nobel Prize for Physiology or Medicine to Fire & Mello



Development of modified siRNA as potential drugs

- Design of nuclease-resistant siRNA
- Development of formulations for local and intravenous administration
- Improvement of cellular uptake and delivery





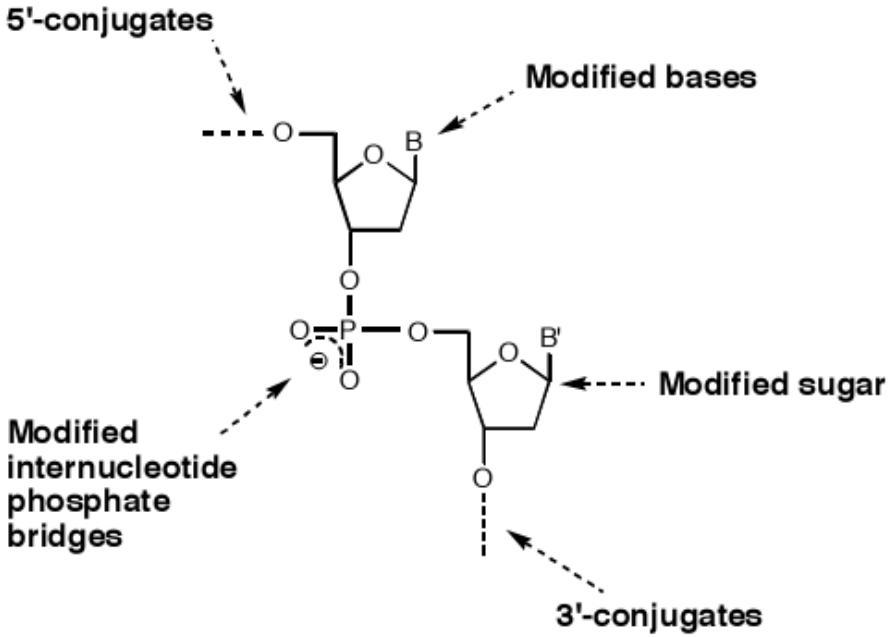
CHEMICAL MODIFICATION OF siRNA/ antisense

DESIGN OF ANTISENSE OLIGONUCLEOTIDES

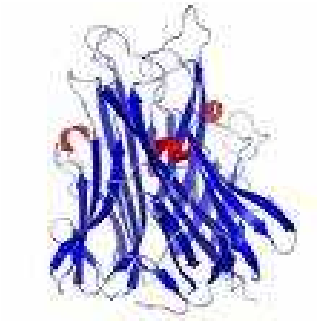
- REQUIREMENTS

Half-life { duplex (stable complex)
oligonucleotide (stability to nucleases)

Cell uptake (lipophilic cell membrane)



Inhibition of TNF-α expression by chemically modified siRNAs



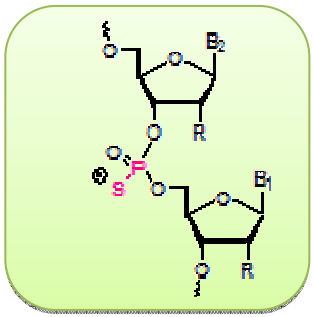
Why TNF-α ?

- Because overexpresssion of this gene is found a large number of diseases: cancer, autoimmune diseases: rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, psoriasis, refractory asthma…..
- These disorders are treated with TNF inhibitors such monoclonal antibodies
- There are siRNA that have good inhibitory activity (Sioud et al.)
- Possibility of external adminitration
- Inconvenient: Innate immune response

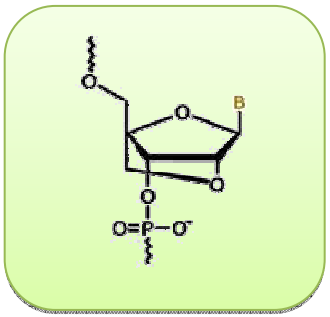
Synthesis (IQAC-CSIC, IRB Barcelona):
Clara Caminal, Anna Aviñó,Santiago Grijalvo, Alvaro Somoza
Analysis (Facultat de Medicina. Universitat de Barcelona, Bellvitge)
Sandra M. Ocampo, Francesc X. Blasco, José C. Perales
Animal model (Universitat Autónoma de Barcelona, Bellatera)
Ester Fernández, Carolina Romero, Joan Burgueño

Modified siRNA (modifications on sense strand)

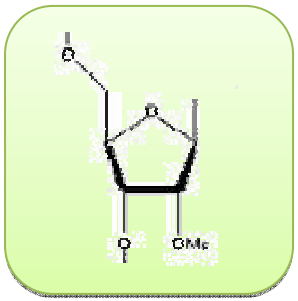
Modificación	siRNA	Secuencia
Antisense	As	5’-GAGGCUGAGACAUAGGCAC-dT-dT-3’
Sense	S	5’-GUGCCUAUGUCUCAGCCUC-dT-dT-3’
Sense OMe-Propanediol	OMe-Prop	5’-guGCCUAUGUCUCAGCCUC-dT-dT-$(CH_2)_3$-OH-3’
Sense PS-Propanediol	PS	5’-GUGCCUAUGUCUCAGCCUC-dT*dT*$(CH_2)_3$-OH-3’
Sense LNA-Propanediol	LNA	5’-GUGCCUAUGUCUCAGCCUC-***T***-***T***-$(CH_2)_3$-OH-3’
Sense-Propanediol	Prop	5’-GUGCCUAUGUCUCAGCCUC-dT-dT-$(CH_2)_3$-OH-3’



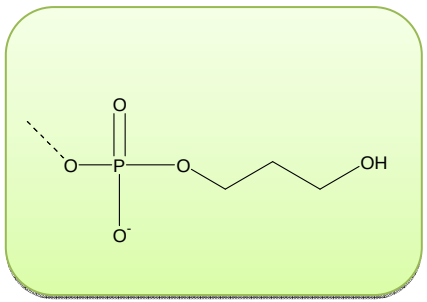
Phosphorothioate



LNA

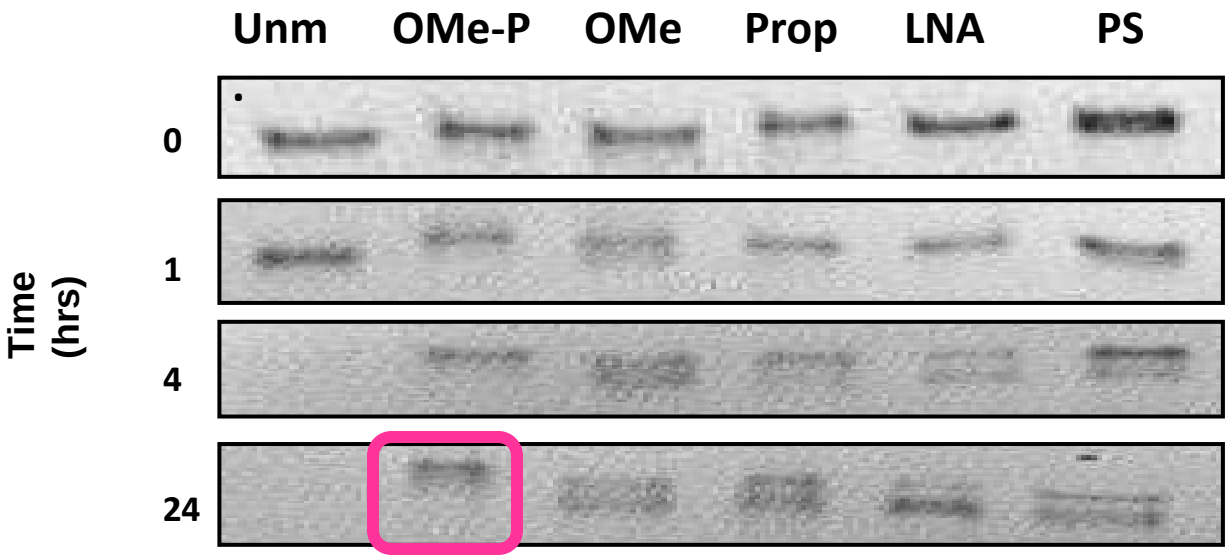


2’-OMe



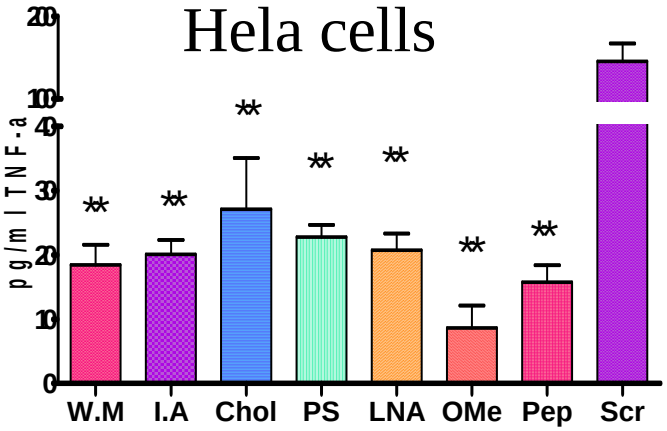
Propanediol

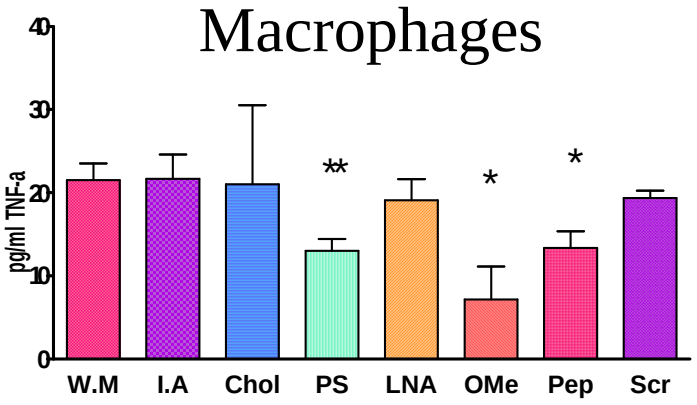
Nuclease resistance (sera)

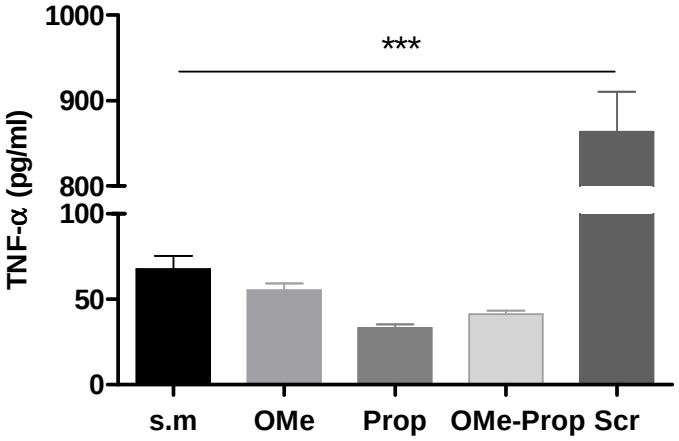


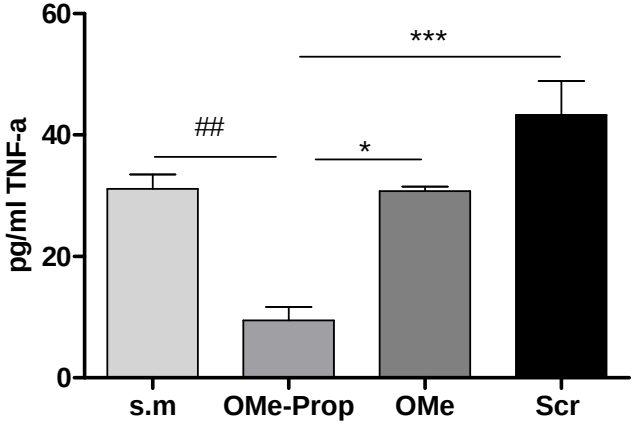
Ocampo et al. 2013

Effect of the modifications in the sense strand

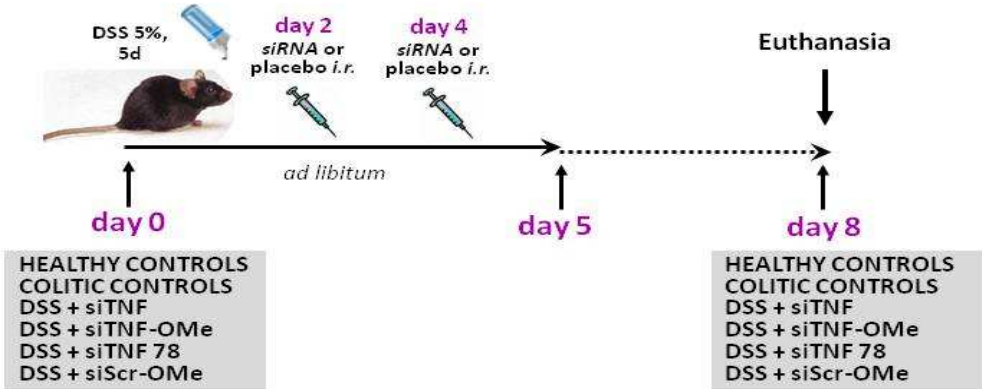




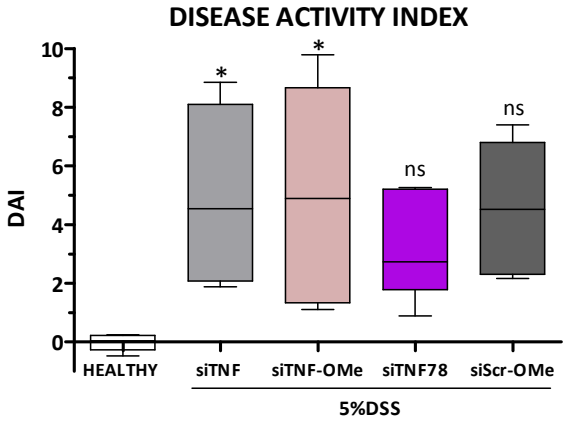




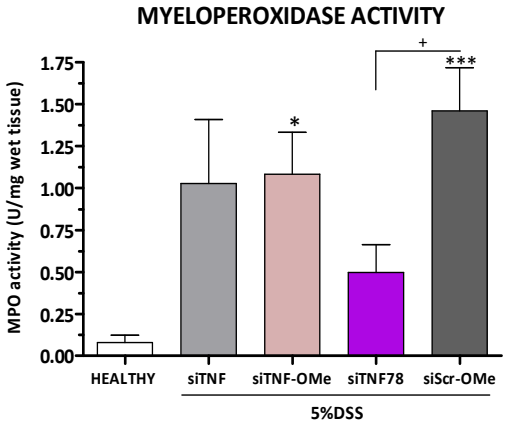
Ocampo et al. 2013



IN VIVO: siTNF78(OMe-Prop) AMELIORATES MURINE DSS COLITIS IMPROVES CLINICAL & BIOCHEMICAL INDEXES



*** P<0.001, Kruskal-Wallis.
* P <0.05 *vs.* HEALTHY CONTROL. (Dunn's post-test).



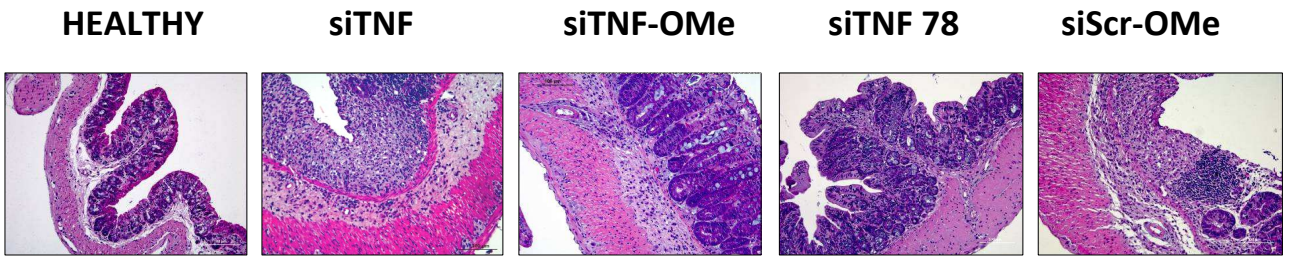
***P<0.001, 1 Way ANOVA.*P<0.05 and ***P<0.001 *vs.* Healthy Control.
+P<0.05 siScr-OMe *vs.* siTNF78. (Bonferroni's post-test).

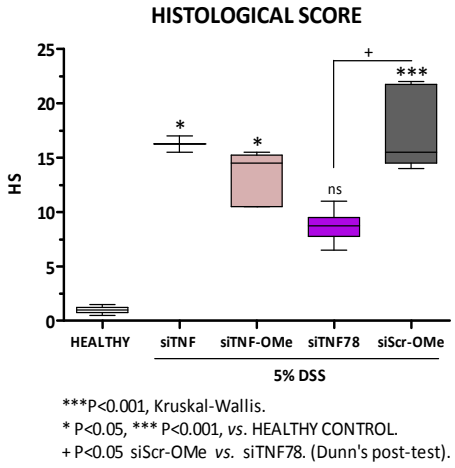
Ester Fernandez et al.

IN VIVO: siTNF78 (OMe-Prop) AMELIORATES MURINE DSS COLITIS

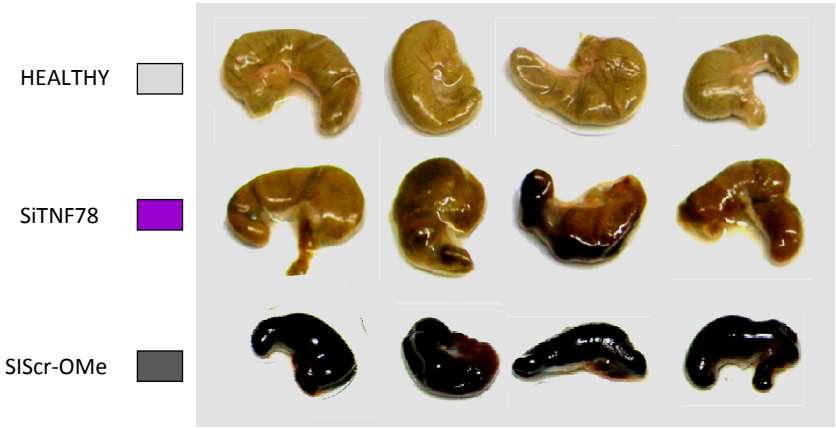
IMPROVES CLINICAL & BIOCHEMICAL INDEXES

IMPROVES CLINICAL & HISTOLOGICAL SCORE



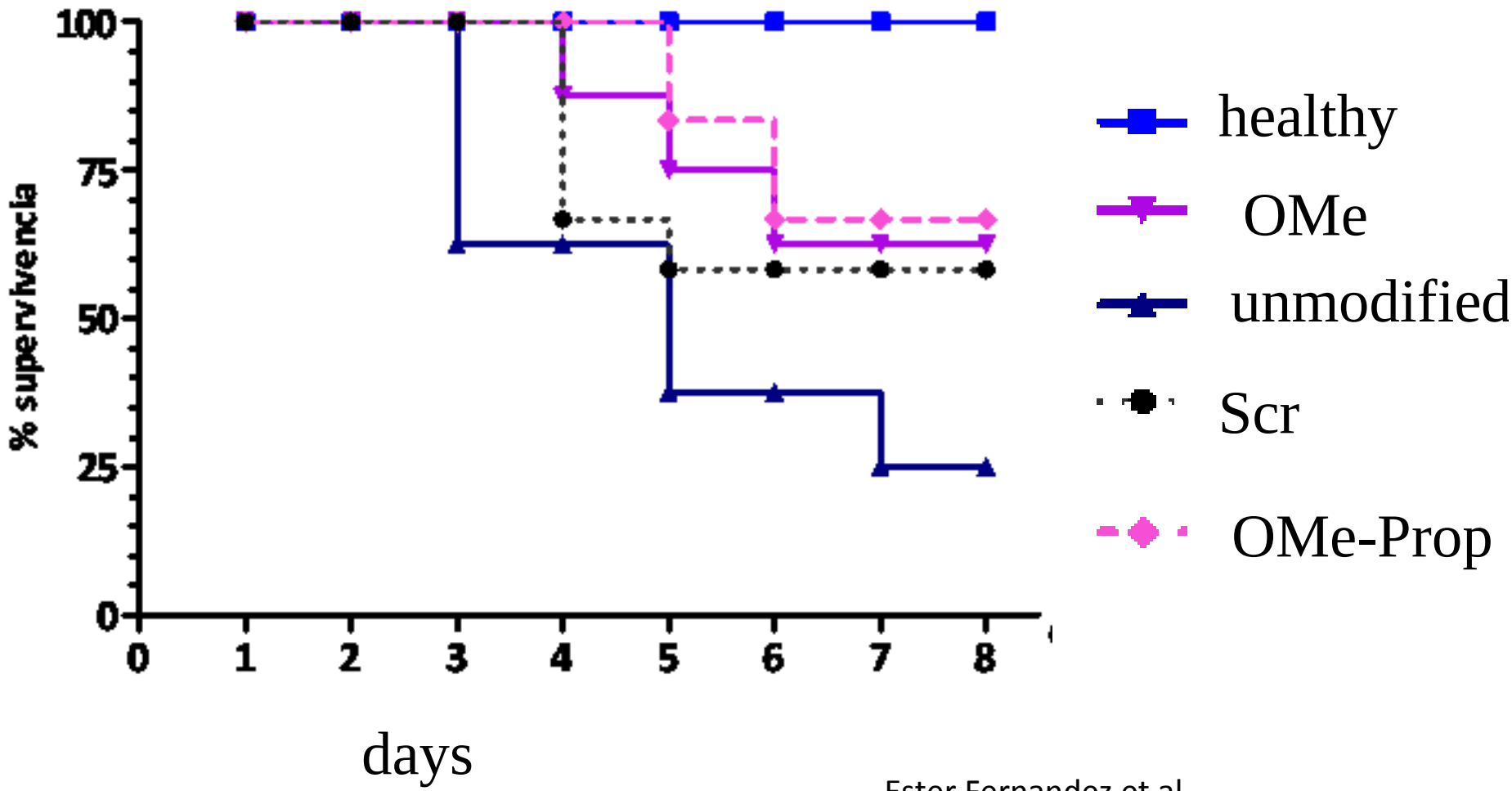


***P<0.001, Kruskal-Wallis.
* P<0.05, *** P<0.001, *vs.* HEALTHY CONTROL.
+ P<0.05 siScr-OMe *vs.* siTNF78. (Dunn's post-test).



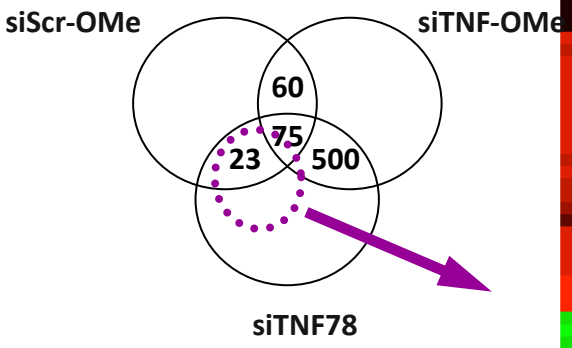
Ester Fernandez et al.

Survival curves



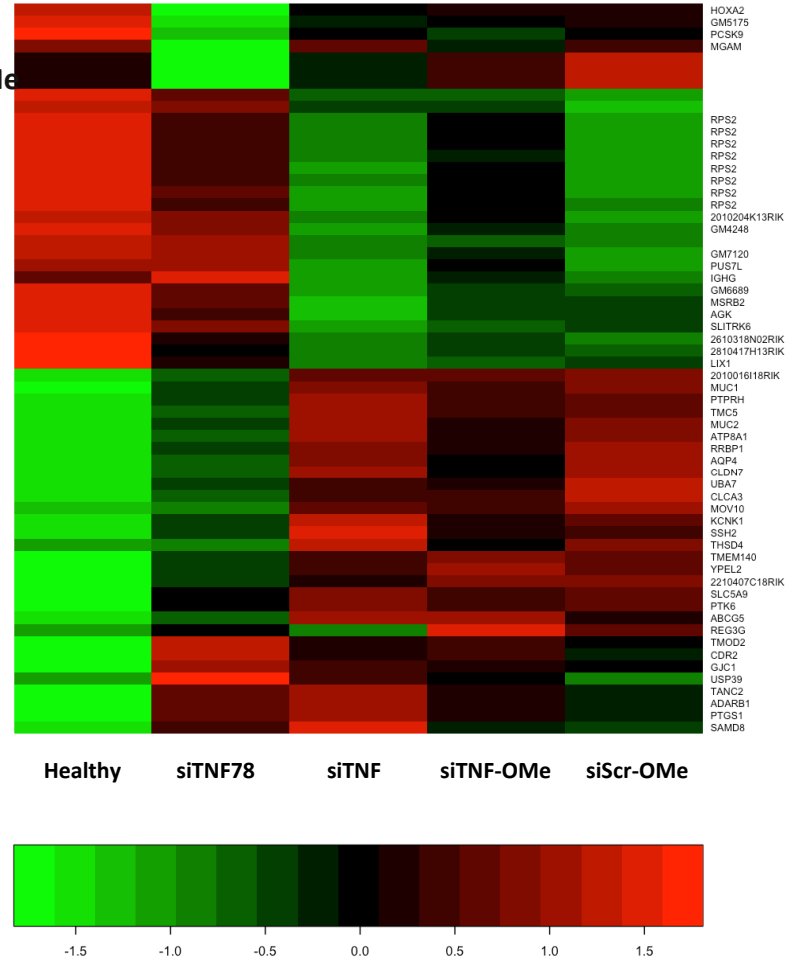
Ester Fernandez et al.

MICROARRAY ANALYSIS (25000 genes)



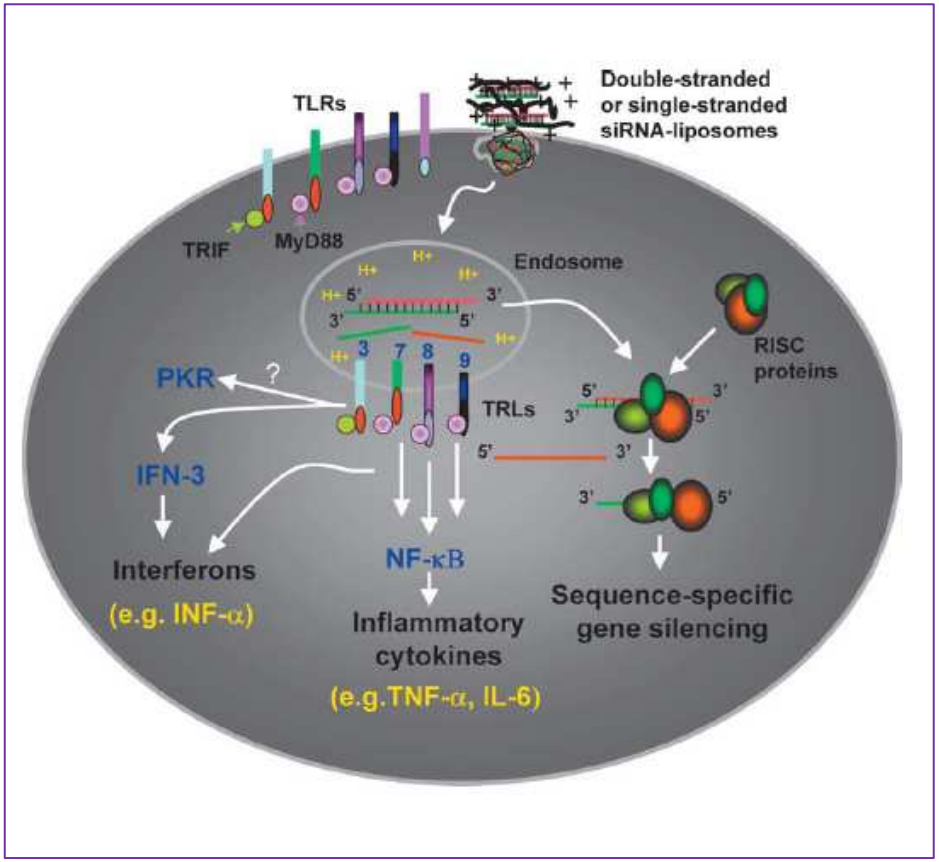
Increased expression of genes related to tissue repair and normal colon function

Decreased expression of genes related to inflammation and innate immunity



Ester Fernandez et al.

Off-target effects. Estimulation of innate immune response

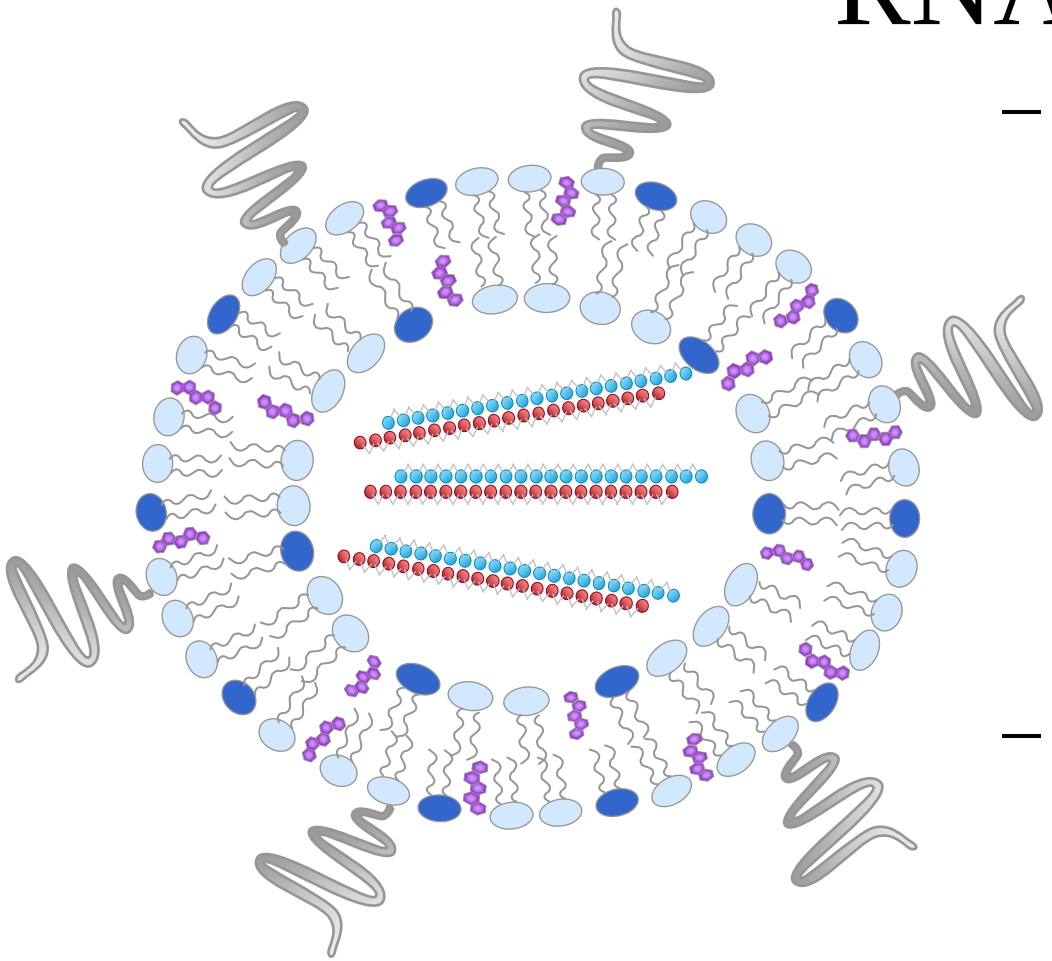


Several modified RNA have been demonstrated that are able to reduce immune response

Beneficial effects of OMe-Prop siRNA

- Increase stability to nucleases (modification at both 5’ and 3’ ends)
- The modification on the 5’-end directs the guide strand to RISC (through phosphorylation of guide strand).
- Less activation of innate immunity

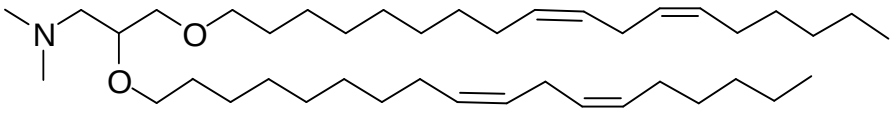
Liposomal Formulations for Systemic RNAi



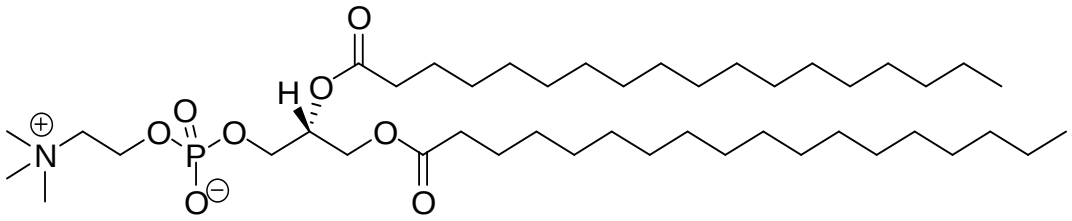
- Multi-component lipid formulation
  - Cationic lipid
  - Fusogenic lipid
  - PEG lipid
  - Cholesterol
- Highly efficient for liver delivery
  - Hepatocyte-specific gene silencing achieved

SNALP Formulation

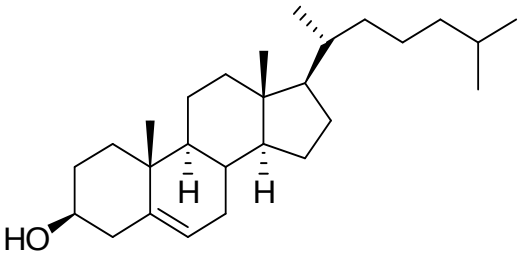
DLinDMA:DSPC:mPEG2000-C-DMA:Cholesterol (40:10:2:48 molar percent)



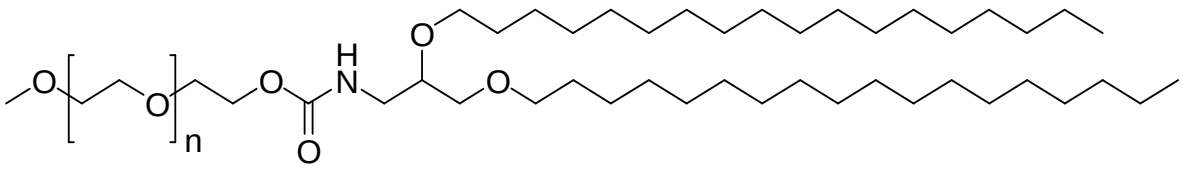
1,2-dilinoleyloxy-*N,N*-dimethyl-3-aminopropane (DLinDMA)



DSPC



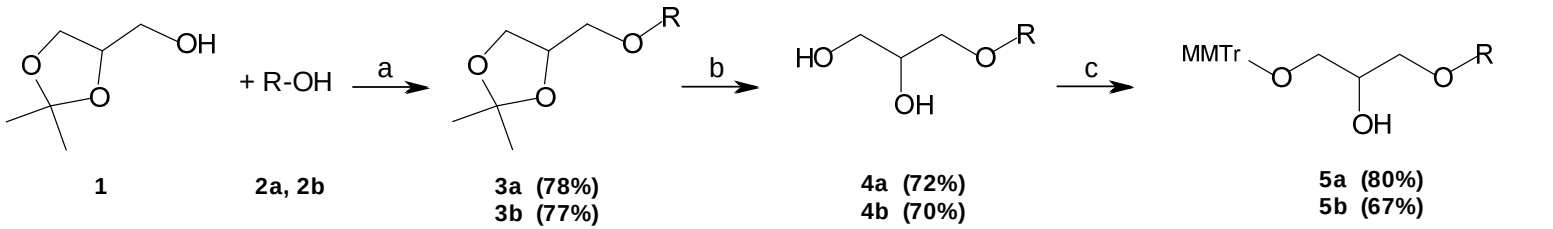
Cholesterol

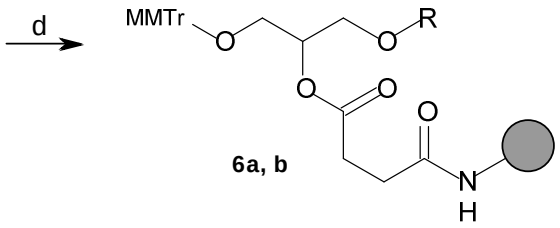


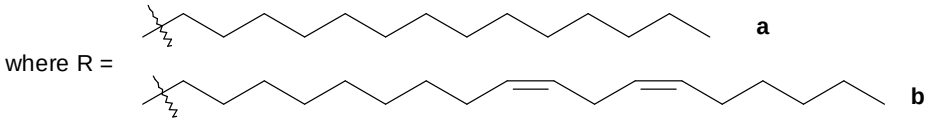
mPEG2000-C-DMA

Synthesis of Lipid-oligonucleotide conjugates

Synthetic strategy for the introduction of lipid at the 3'-end

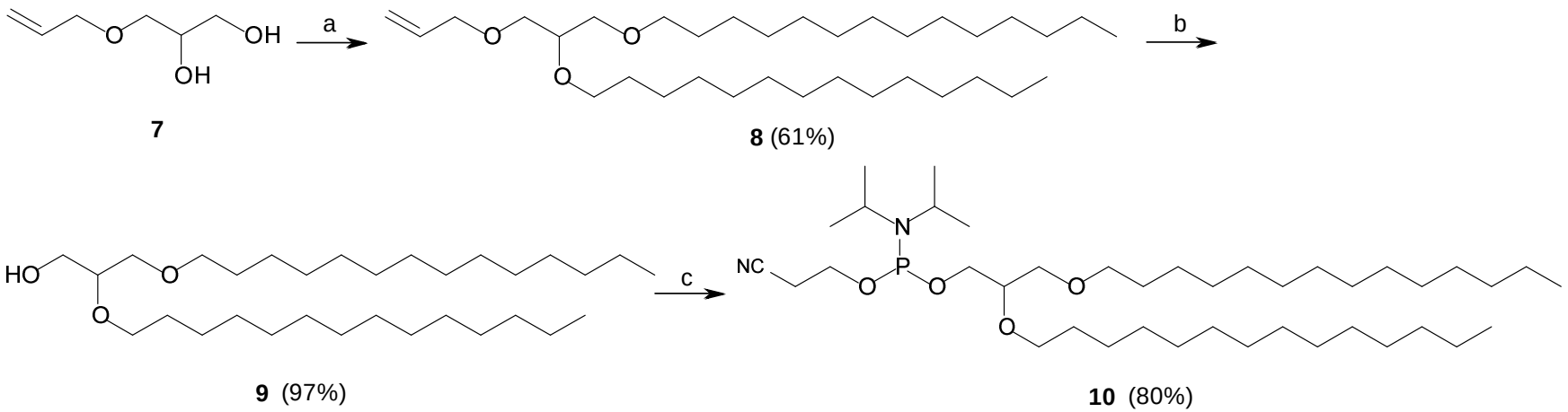




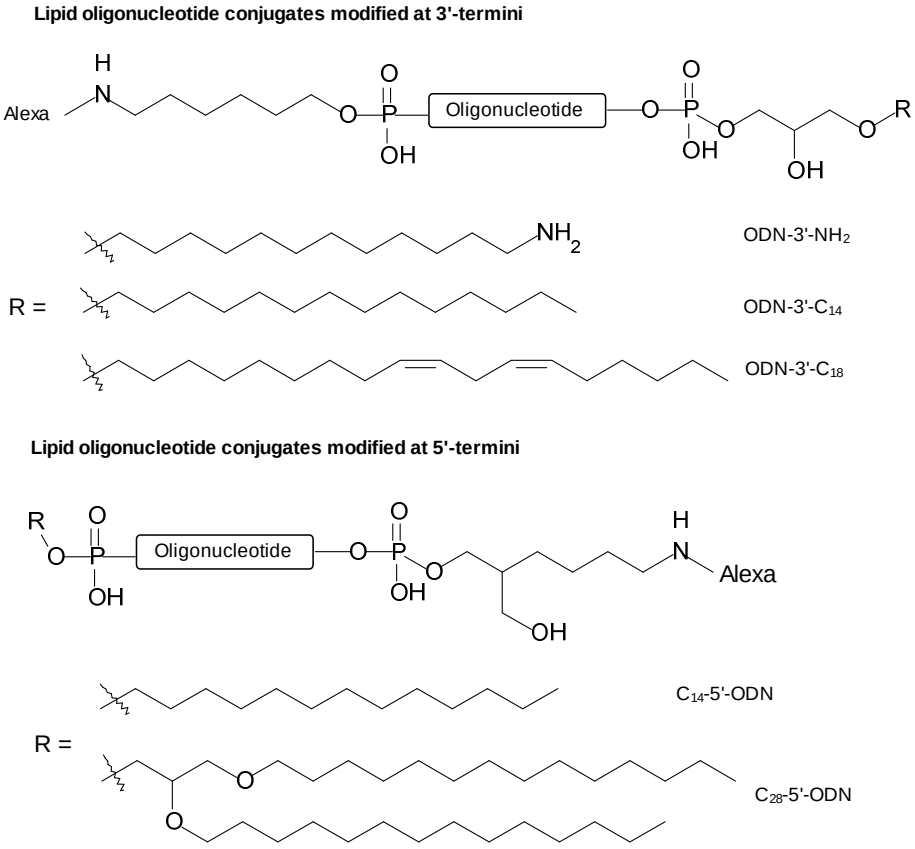


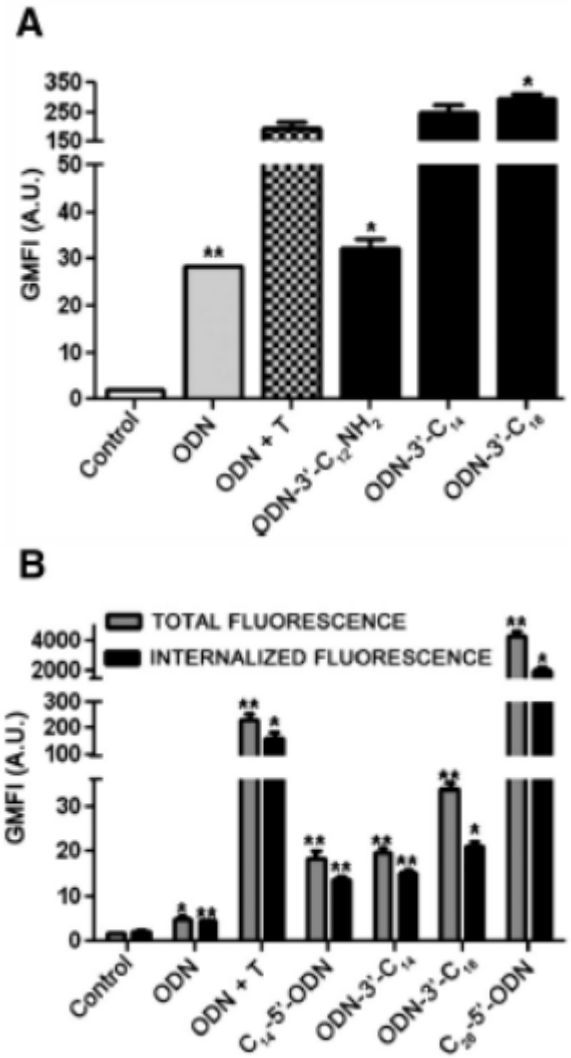
Synthesis of Lipid-oligonucleotide conjugates

Synthetic strategy for the introduction of lipid at the 5’-end



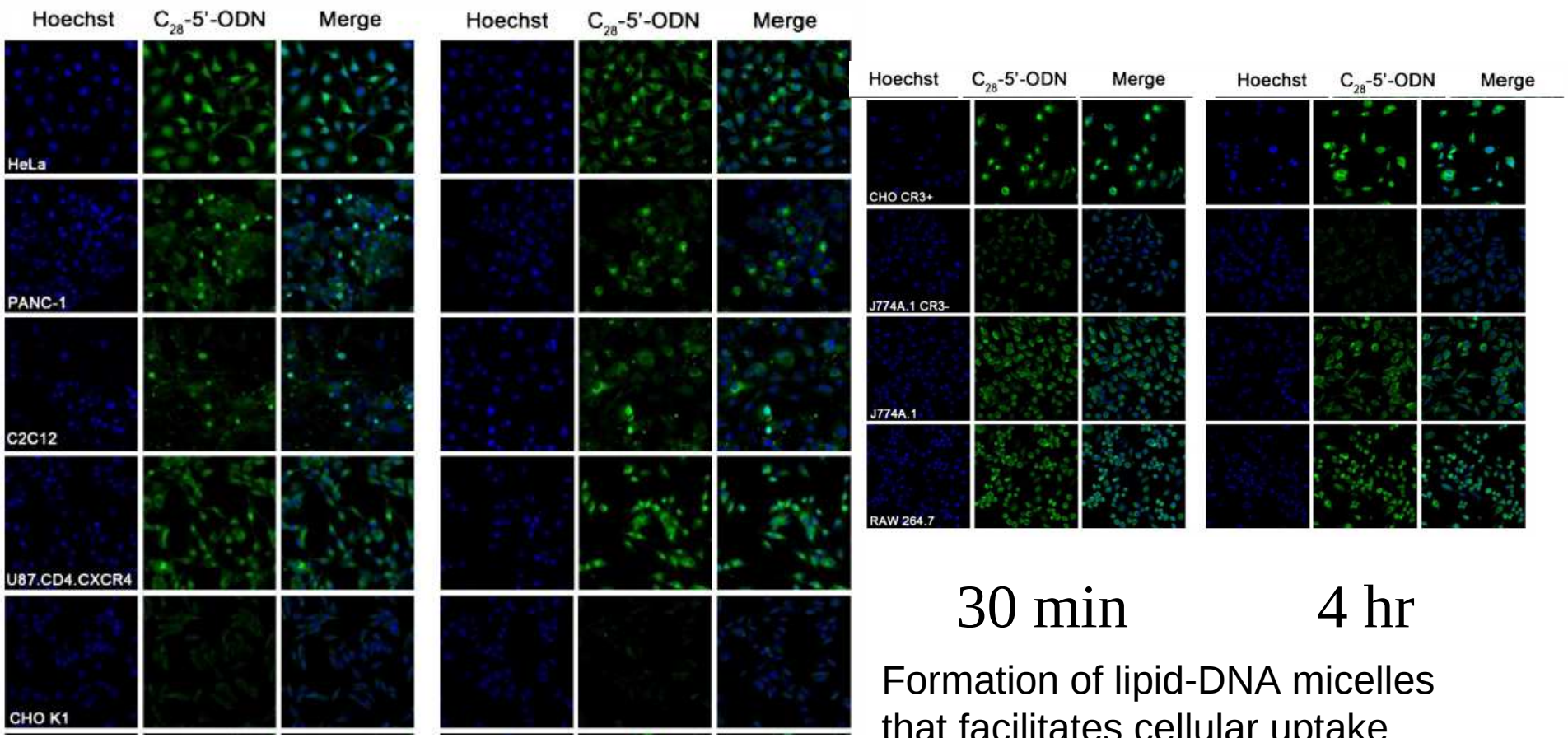
Efficient cellular uptake of lipid modified DNA/RNA





Ugarte-Uribe et al., *Biochem. Biophys. Acta* **2013**

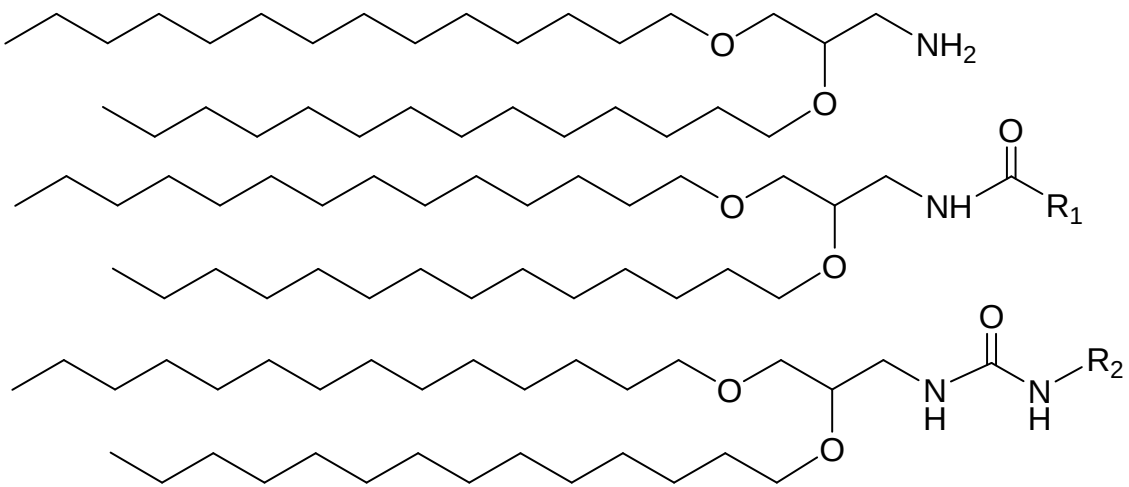
Uptake of C_{28}-lipid-DNA conjugate

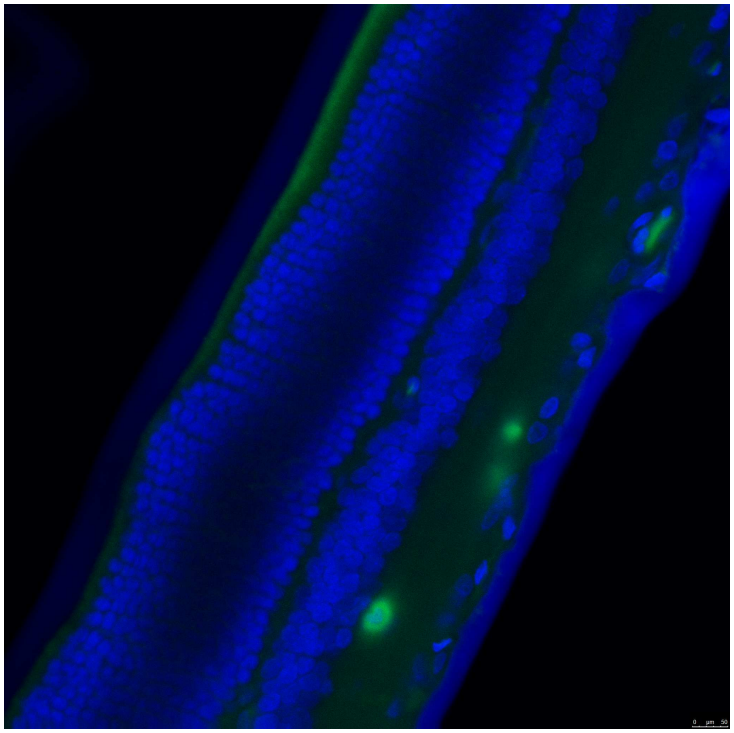


Formation of lipid-DNA micelles that facilitates cellular uptake

Ugarte-Uribe et al., *Biochem. Biophys. Acta* **2013**

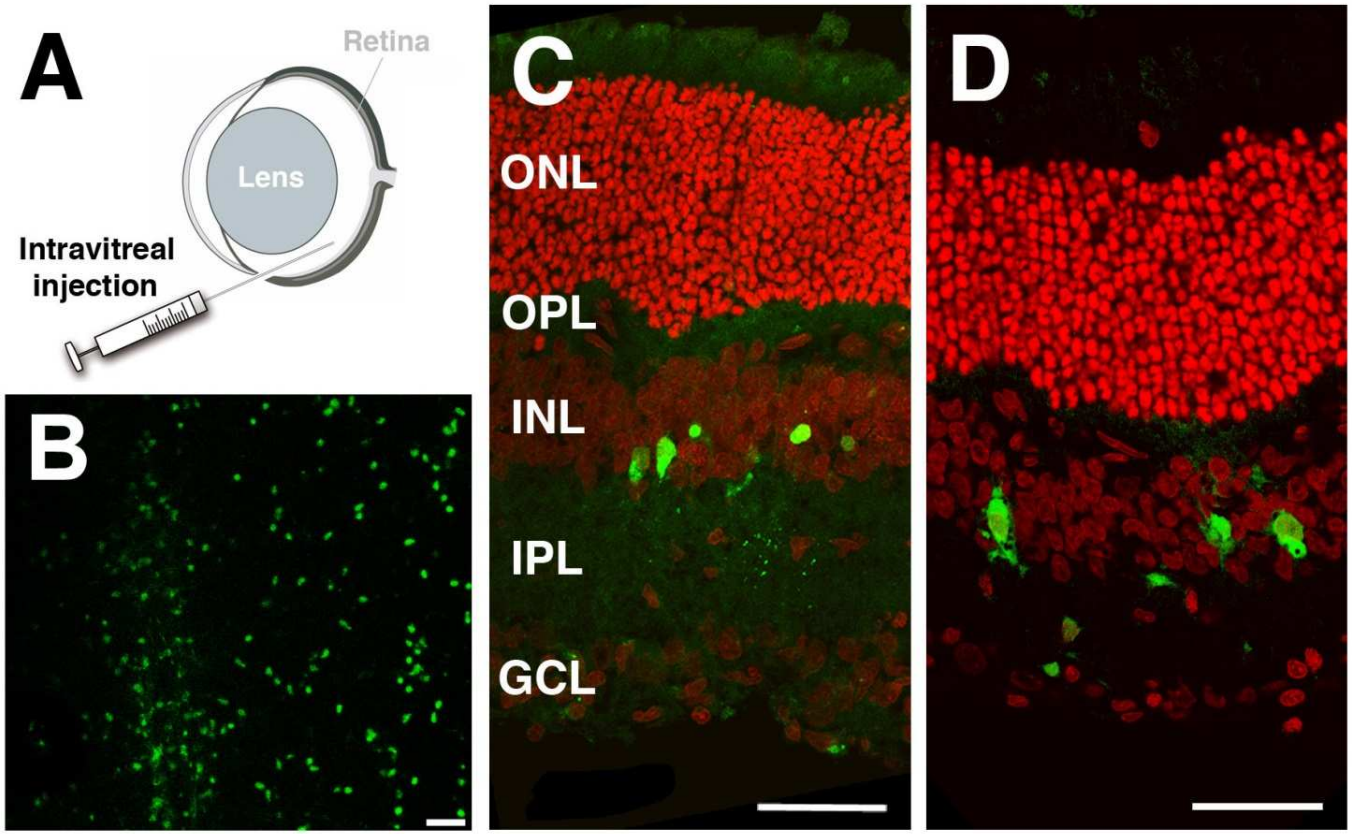
Developing transfecting agents /formulations for ocular administration





Gustavo Puras, José Luis Pedraz, UPV, Vitoria; Eduardo Fernandez, UMH

In vivo gene expression of EGFP post intravitreal injection



Puras et al. *submitted* (2014)

Conclusions

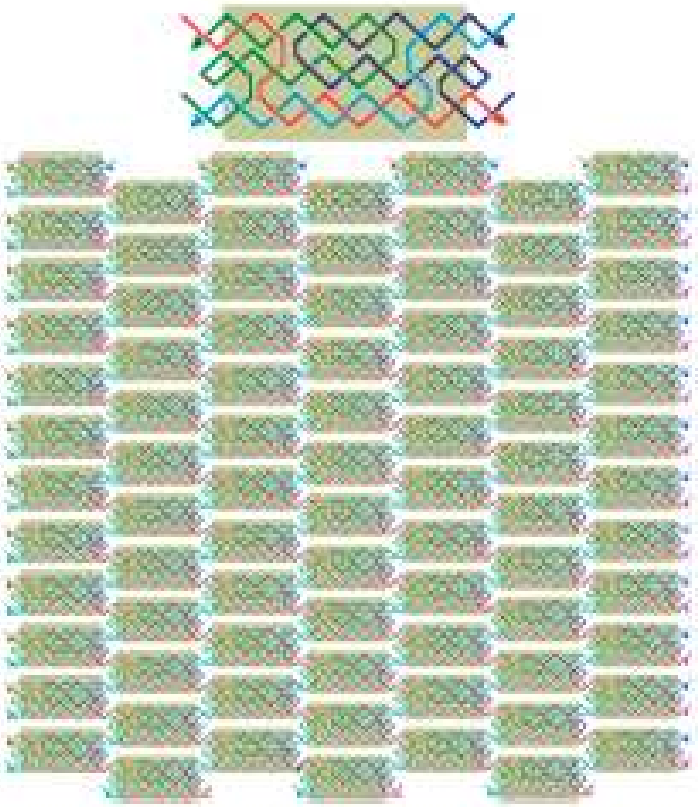
- Specific chemical modifications prevents siRNA degradation by nucleases *in serum* without affecting inhibitory properties. However, the lengthening of half-life of modified siRNAs does not imply more durable silencing effects.

- Double modification in the sense strand of a siRNA improves *in vivo* antiflammatory properties in an IBD mouse model. A combination of nuclease resistance and decrease on the innate stimulation seems to be critical for *in vivo* therapy.

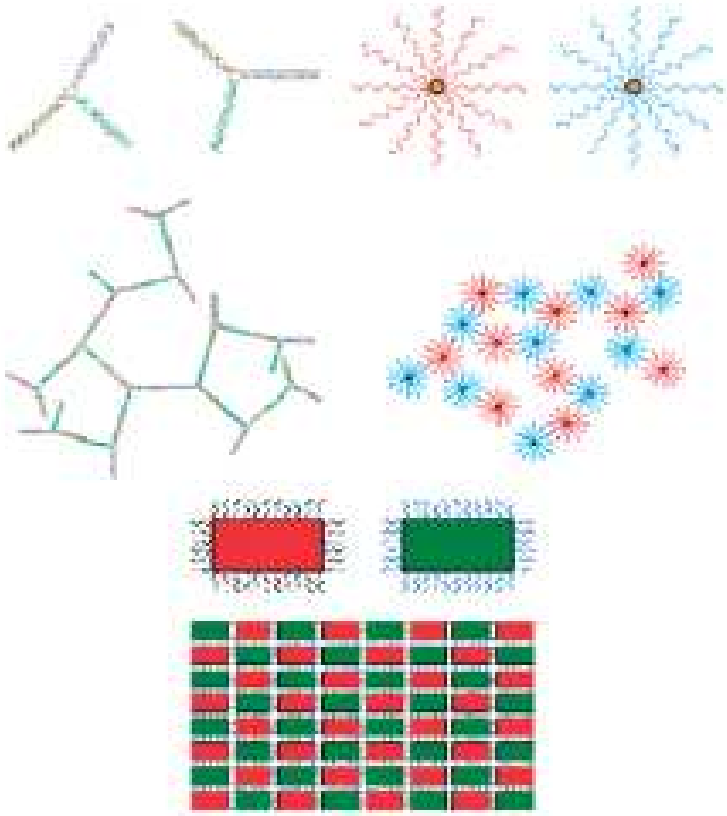
- Lipid modification seems to be the most interesting modification for improving cellular uptake. The double hydrocarbon tail derivative (C28) has interesting properties for transfecting nucleic acids in mammalian cells

DNA NANOTECHNOLOGY

High Resolution/Structural:
DNA as Bricks and Mortar

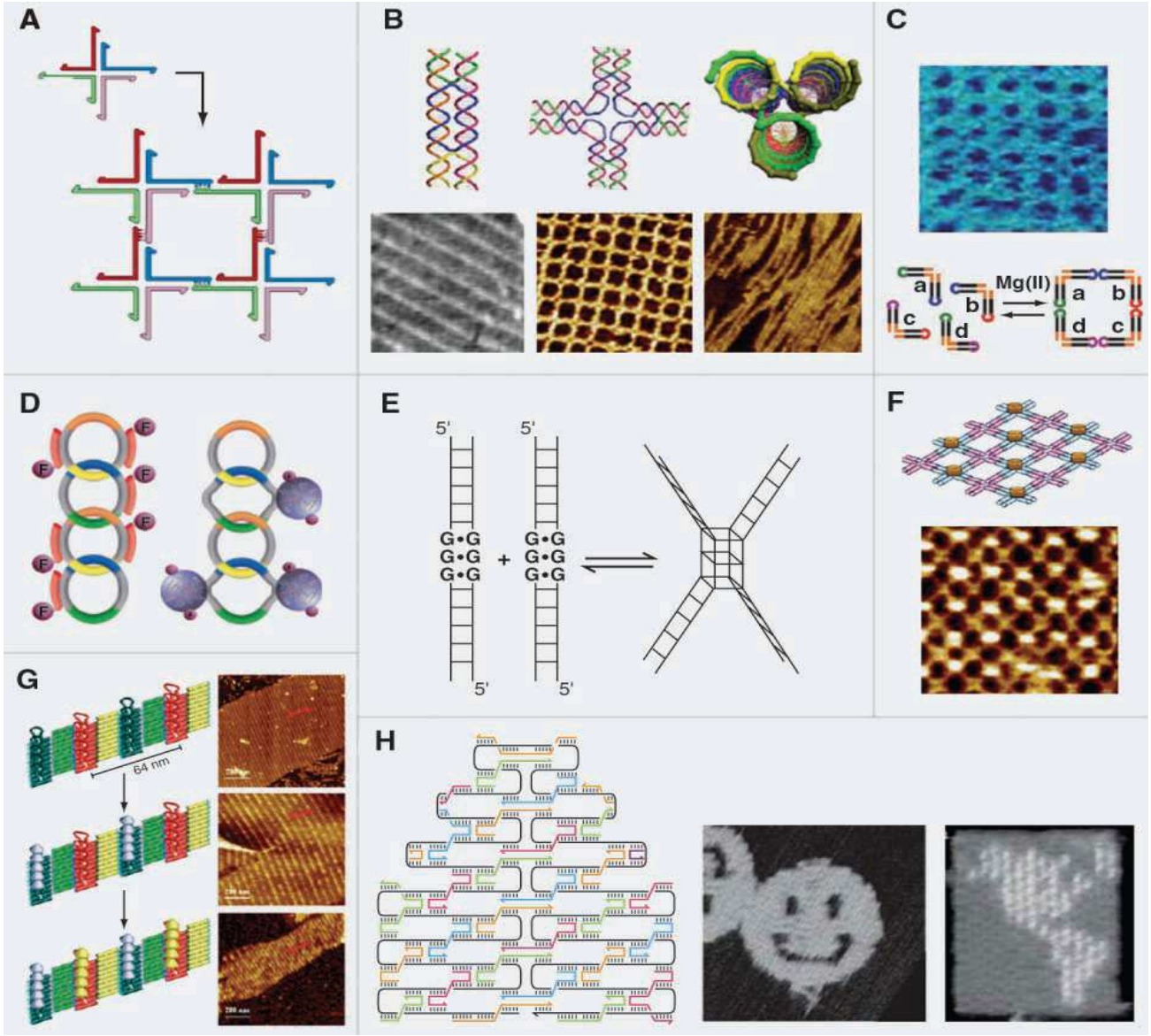


Low Resolution/Compositional:
DNA as Mortar Only



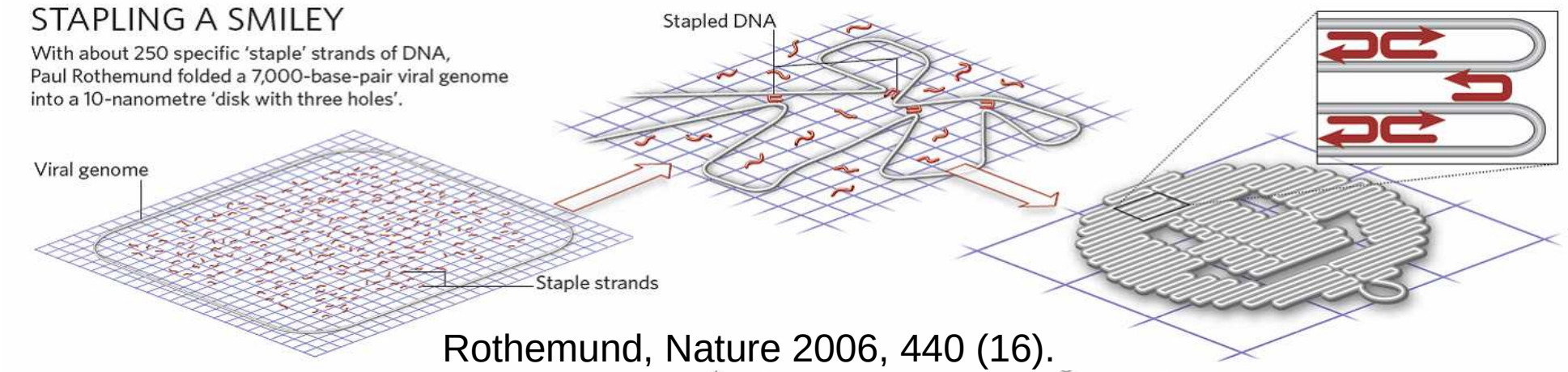
Seeman et al. 2007

DNA bidimensional arrays

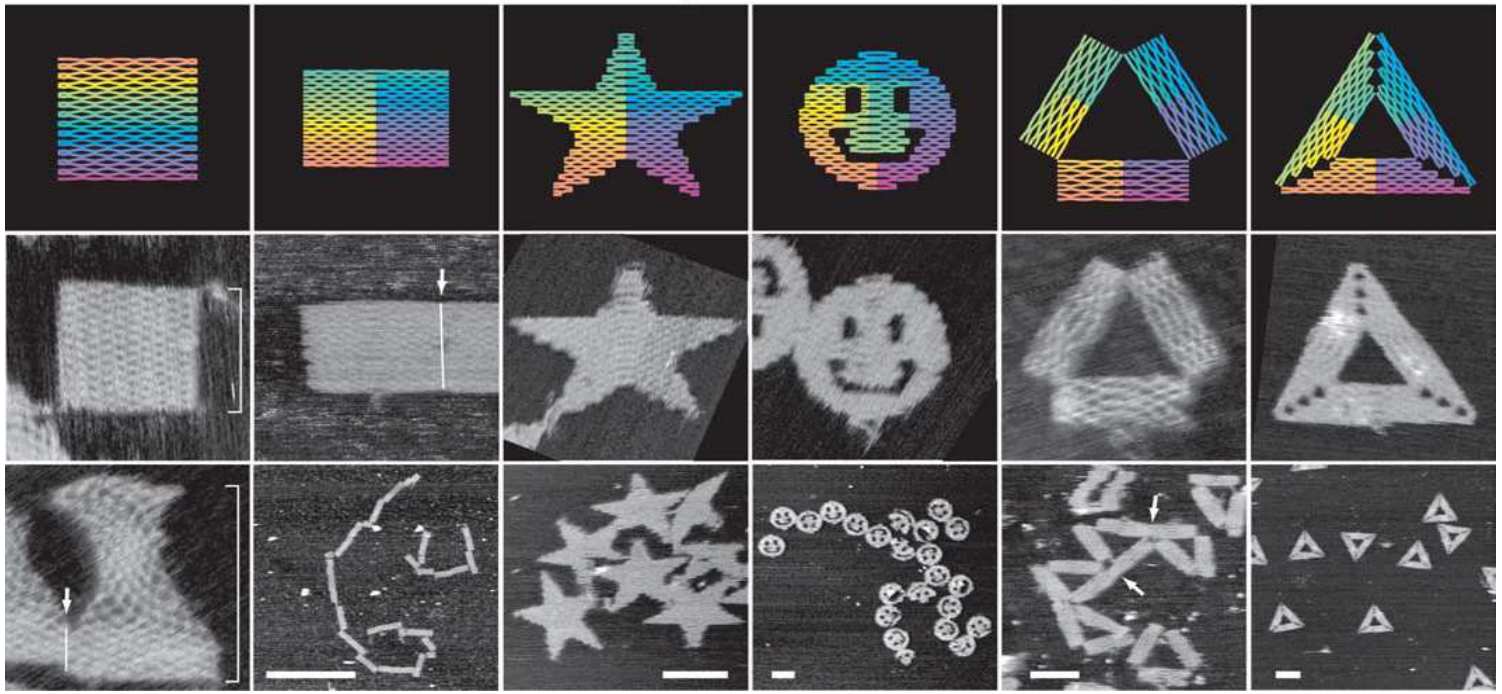


Aldaye et al., Science 2008

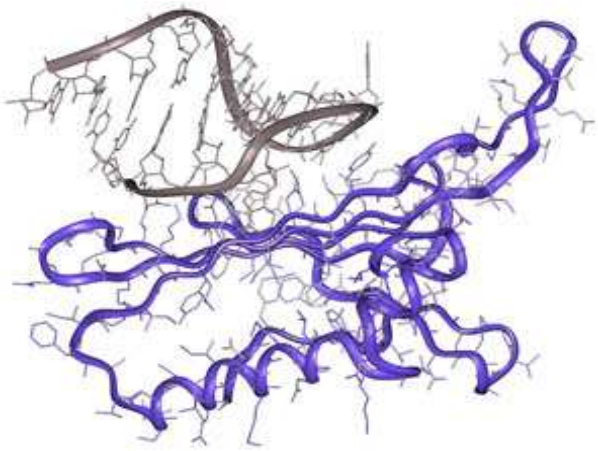
DNA Origami



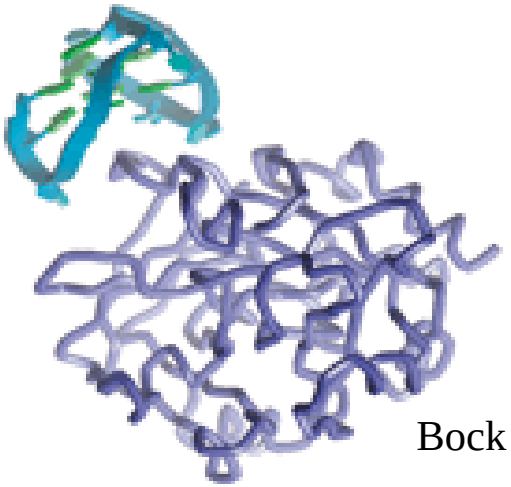
Rothemund, Nature 2006, 440 (16).



APTAMERS: Nucleic acids that have strong affinity for a protein. Obtained by selection on a library (SELEX)

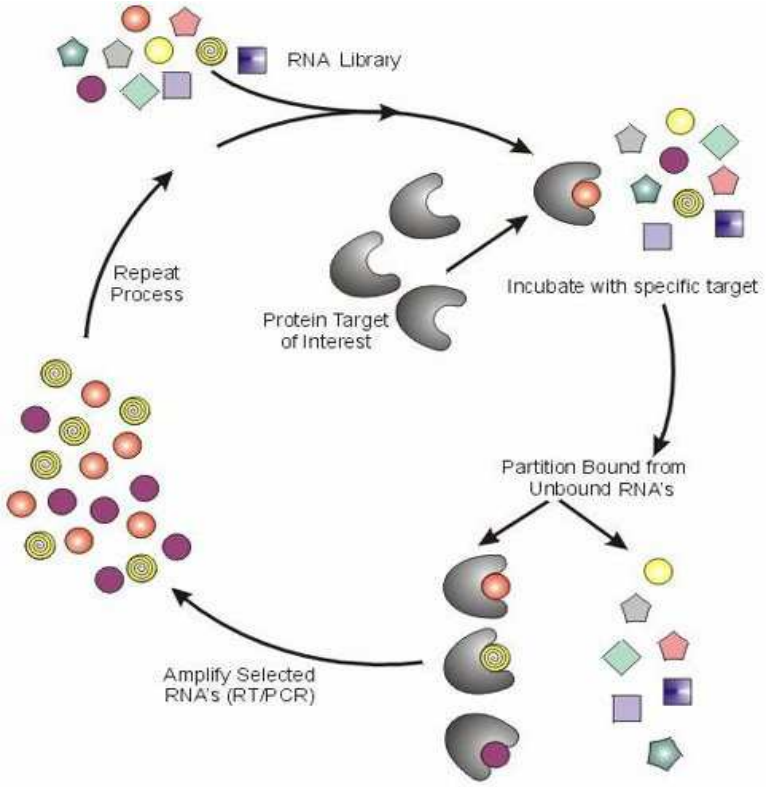


MACUGEN: VEGF, macular degeneration



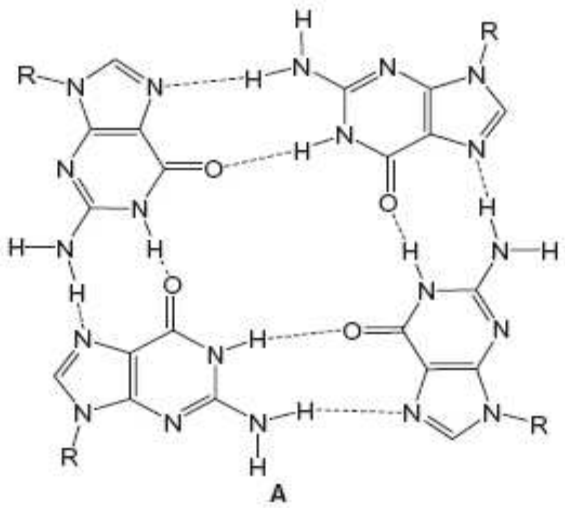
Bock et al. Nature 355, 564 (1992)

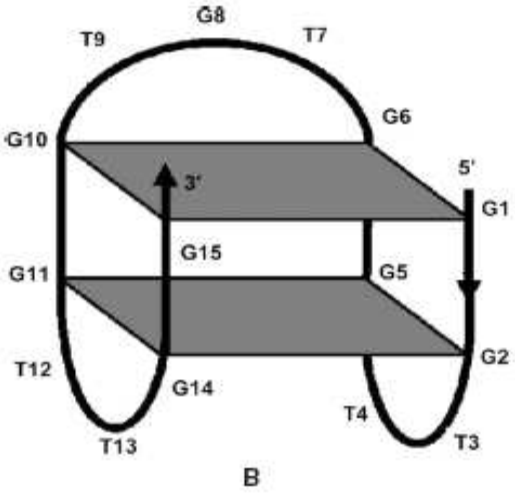
TBA: Thrombin, anticoagulant

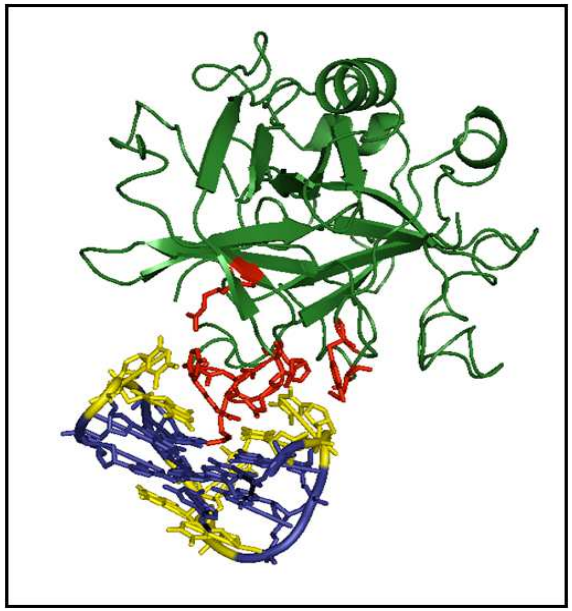


SELEX

Thrombin-binding aptamer (TBA)







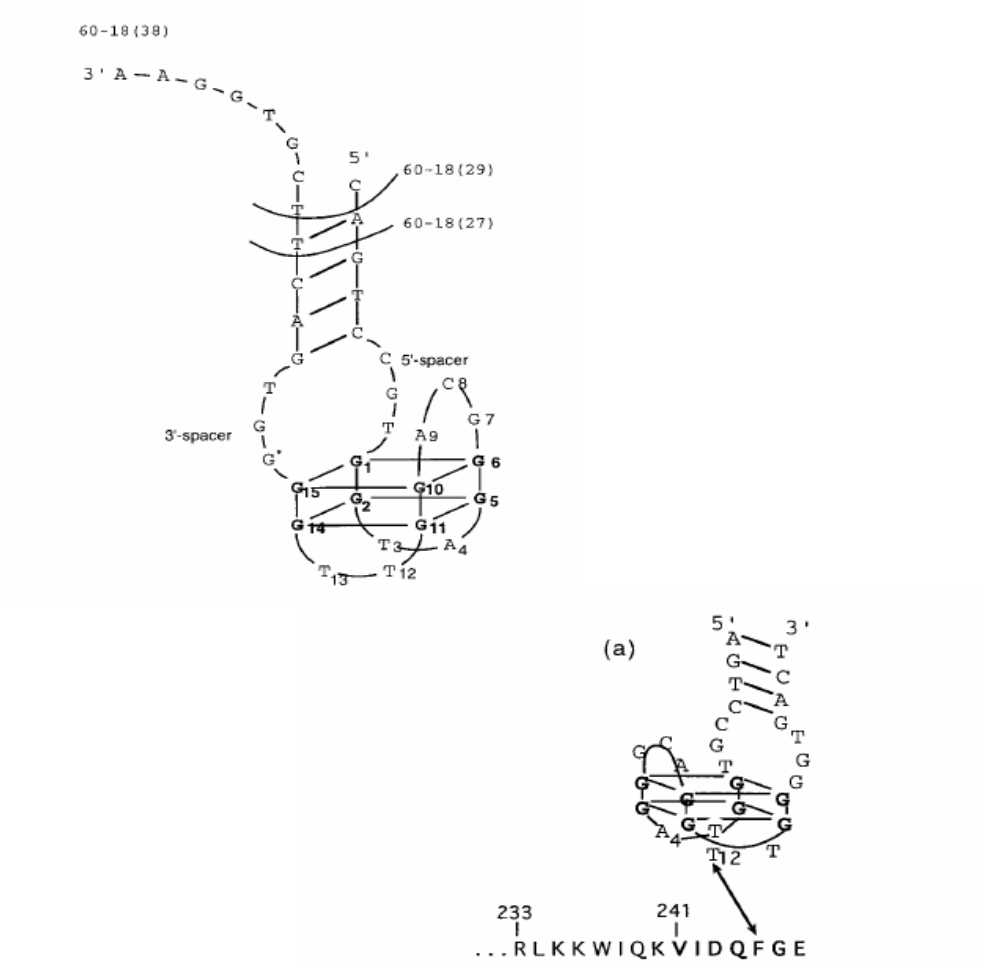
Binds to heparine exocite
Anticoagulant

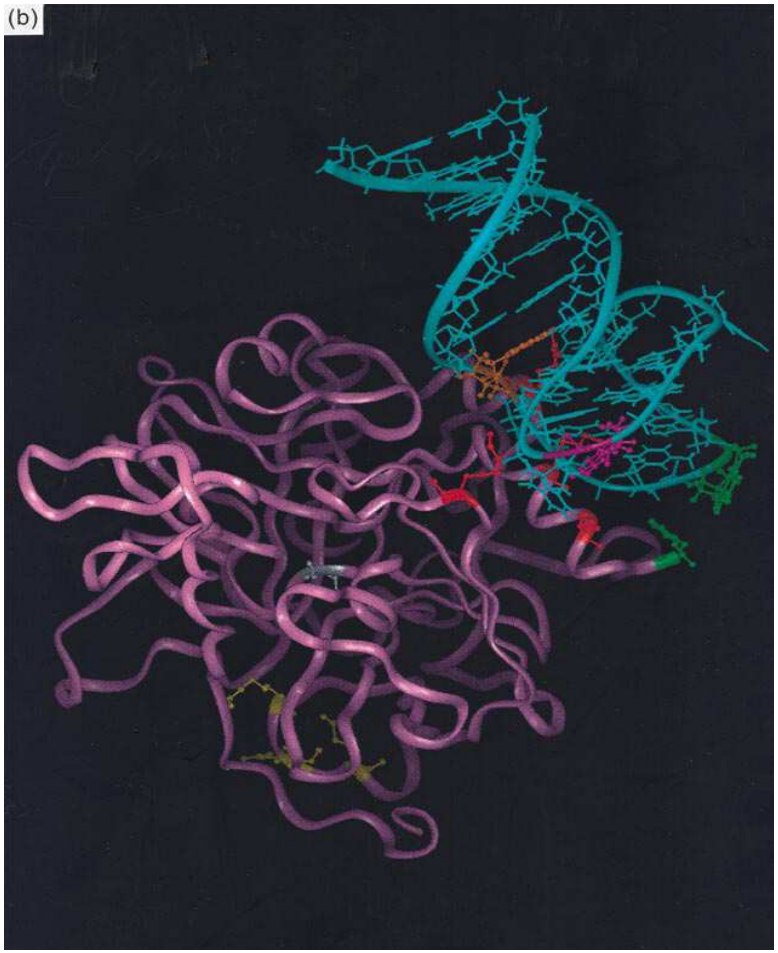
Sequence: 5’-$G^1G^2TTG^5G^6TGTG^{10}G^{11}TTG^{14}G^{15}$-3’
Antiparallel quadruplex
Discovered by SELEX

Macaya et al. PNAS 90, 3745 (1993)

A second thrombin-binding aptamer (29 b) binds to the fibrinogen exosite

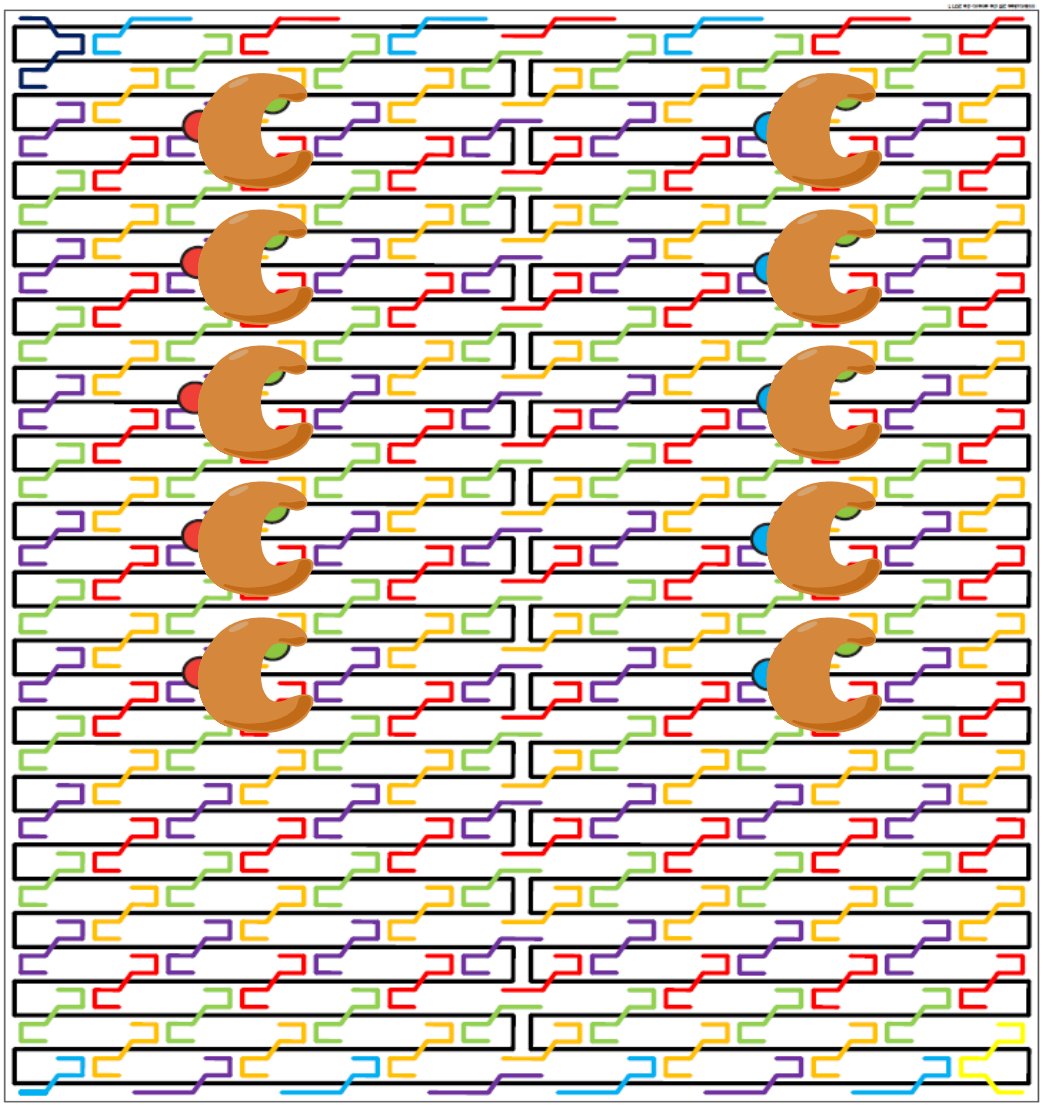
Both 15 b and 29b TBAs bind to thrombin in a cooperative way





Tasset et al. J. Mol. Biol., 1997, 272, 688

New design. Introduction of two TBA aptamers (15mer and 29mer)



TBA 15mer

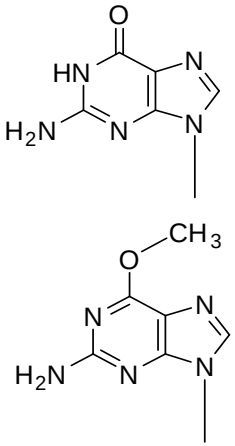
TBA 29mer

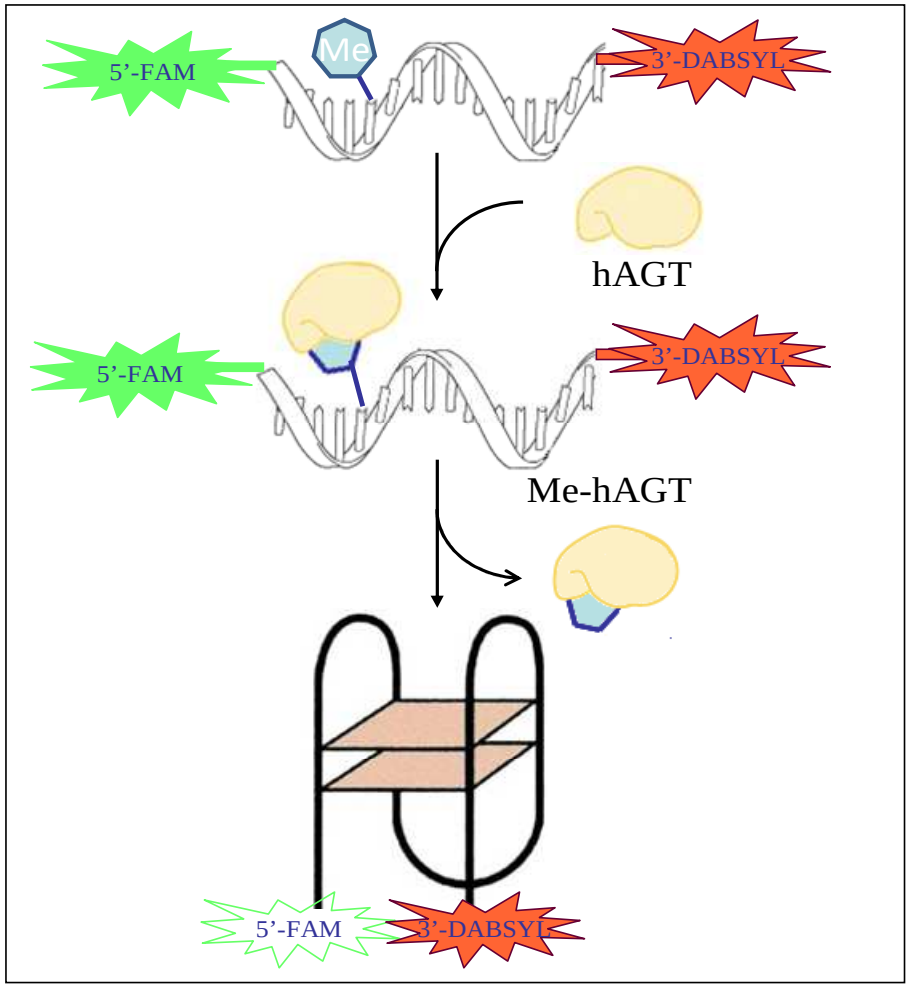
5OMeG-TBA 15mer

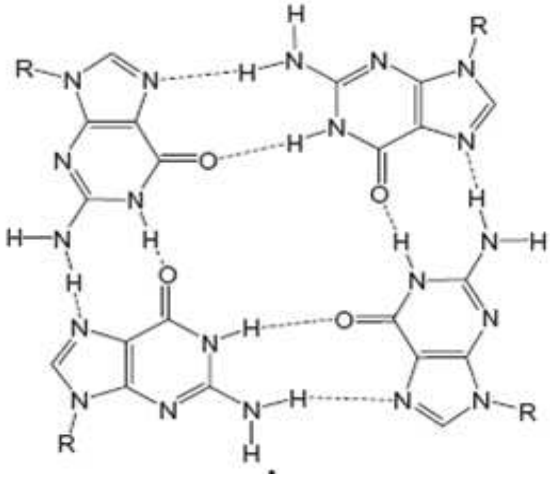
Thrombin

Rinker *et al.* Nature Nanotech. **2008**

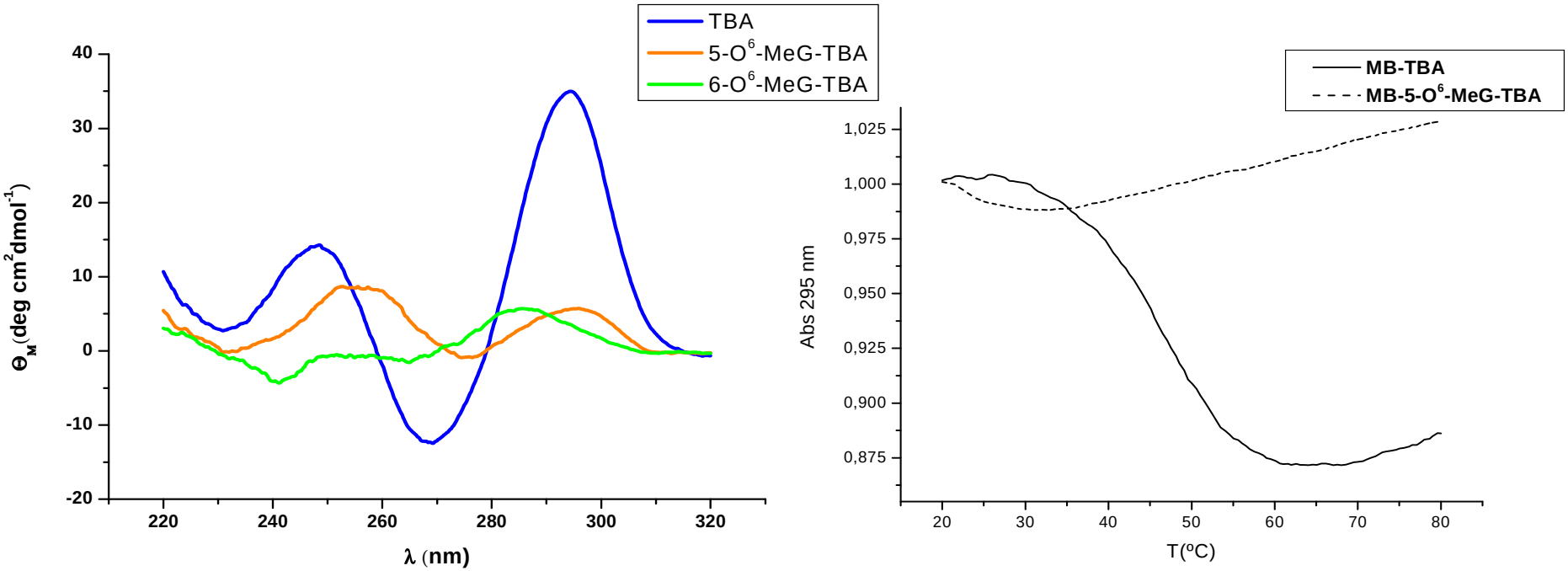
Development of a fluorescent assay for the analysis of the activity of alkyl-guanine-alkyltransferase using TBA derivatives







Methylation of one G residue prevent G-quadruplex formation



TBA: 5´-GGTTGGTGTGGTTGG-3´

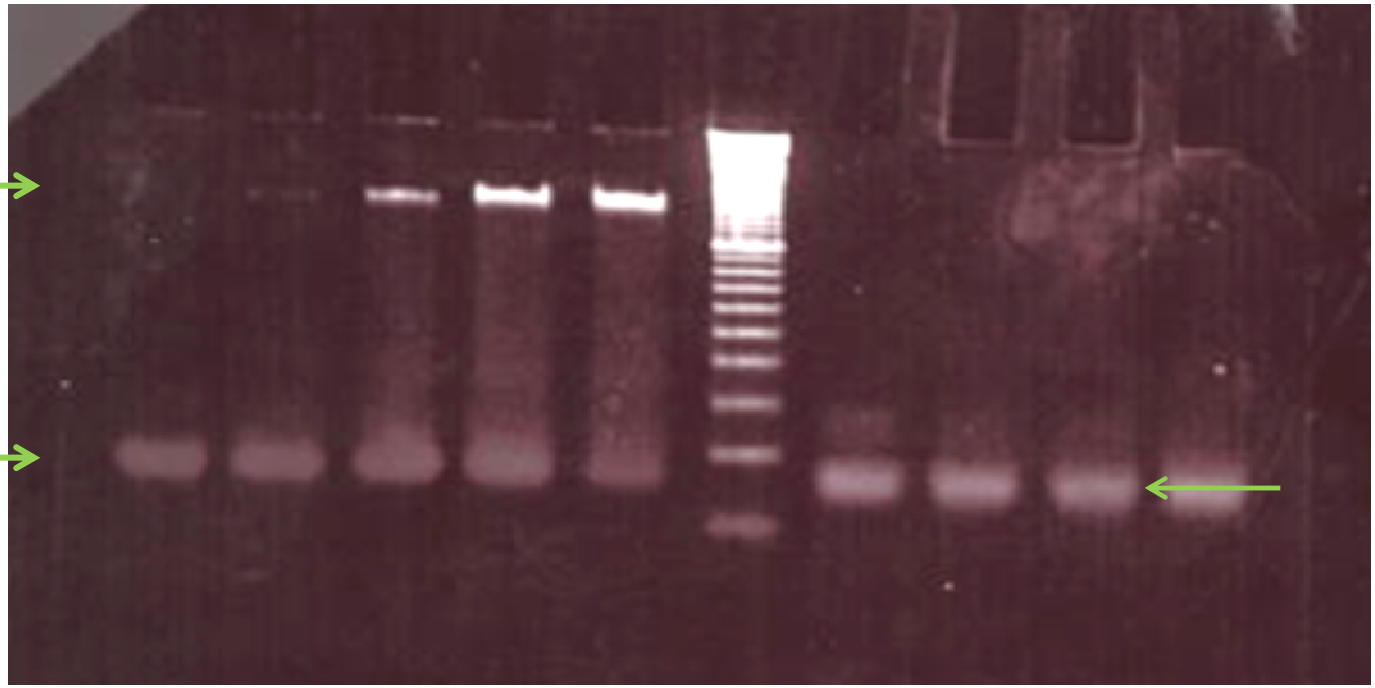
5-(O^6MeG)-TBA: 5´-GGTT(^{Me}G)GTGTGGTTGG-3´

6-(O^6MeG)-TBA: 5´-GGT TG^{Me}G TGT GGT TGG-3´

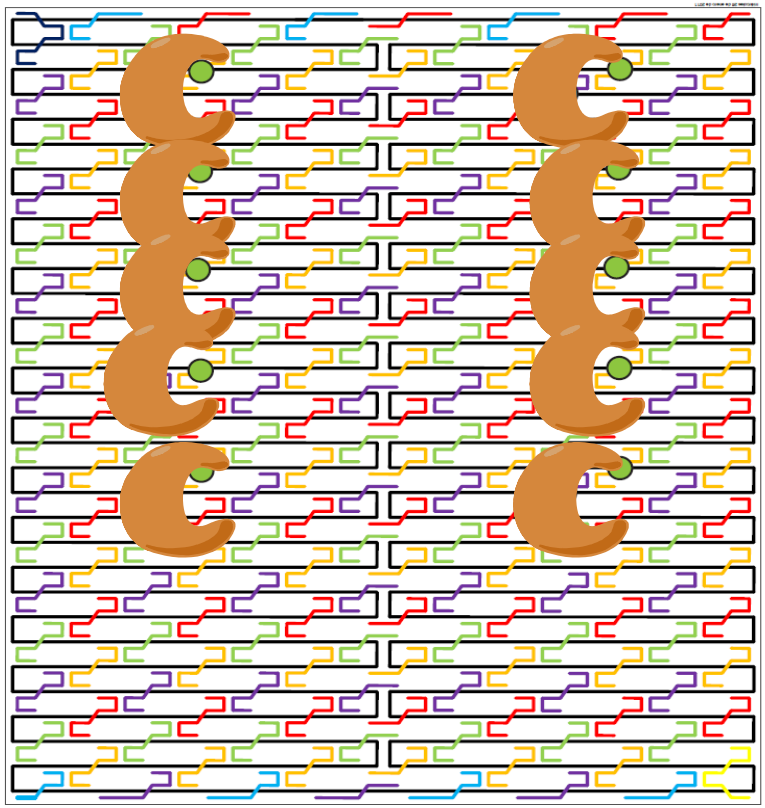
O-6-methyl guanine on TBA inhibits both quadruplex formation and thrombin binding

EMSA ASSAYS:

CONDITIONS:
-TAE Mg^{+2} 2.5x
-Thrombin buffer
-1h incubation
-3 h PAGE at 20ºC



A single methylation in one of the TBA lines of the dual system was enough to disrupt the interaction with α-thrombin.



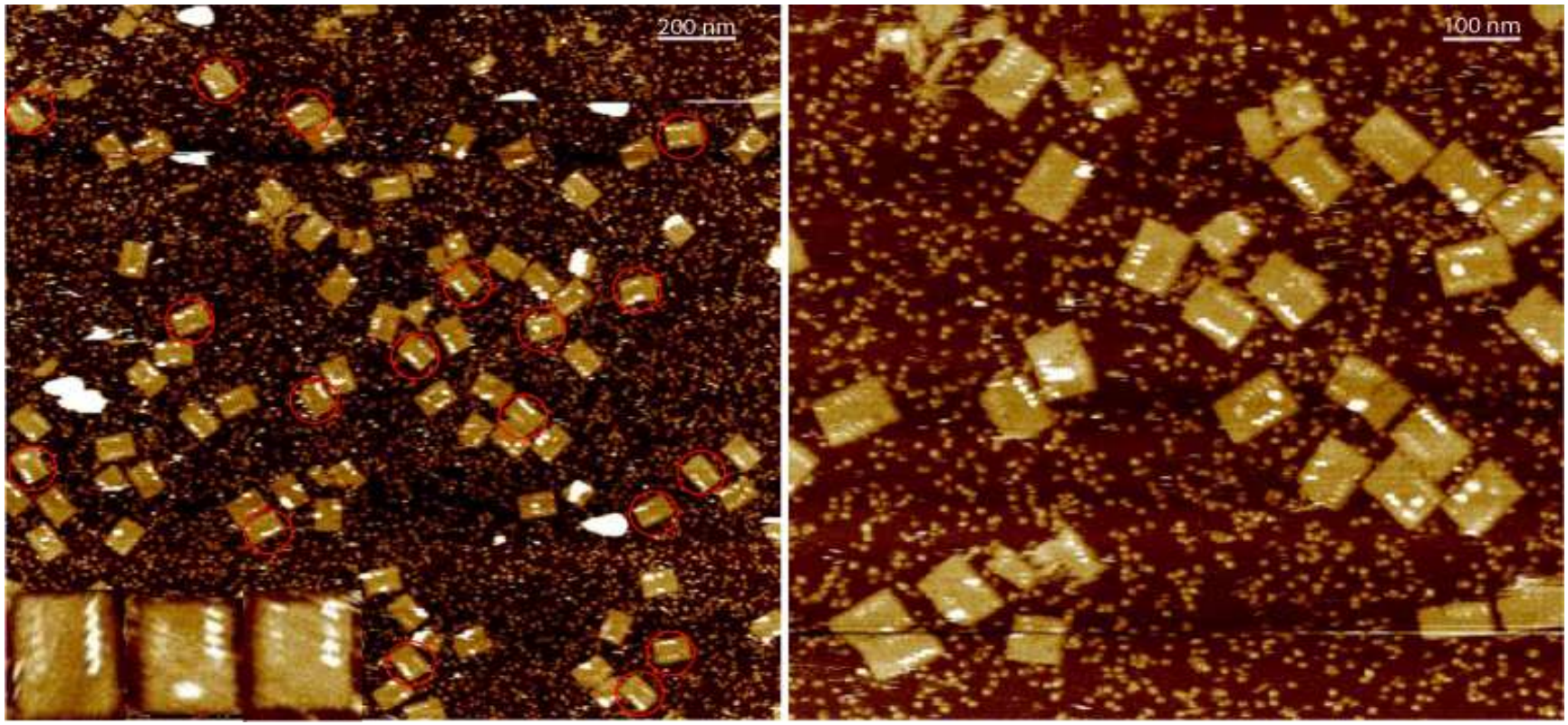
TBA 15mer

TBA 29mer

5OMeG-TBA 15mer

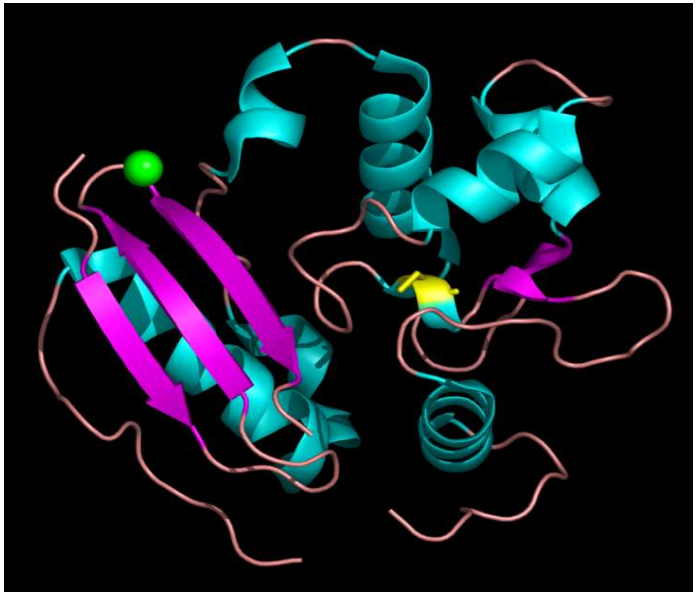
Thrombin

AFM characterization of the Thrombin- TBA-modified DNA origamis complexes

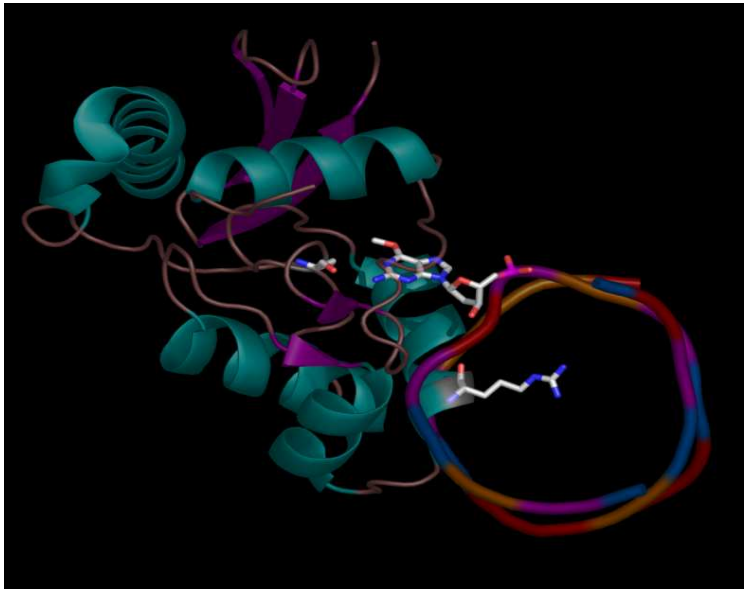


Human O^6-Alkylguanine-DNA-Methyltransferase (hAGT):

- DNA repair protein.



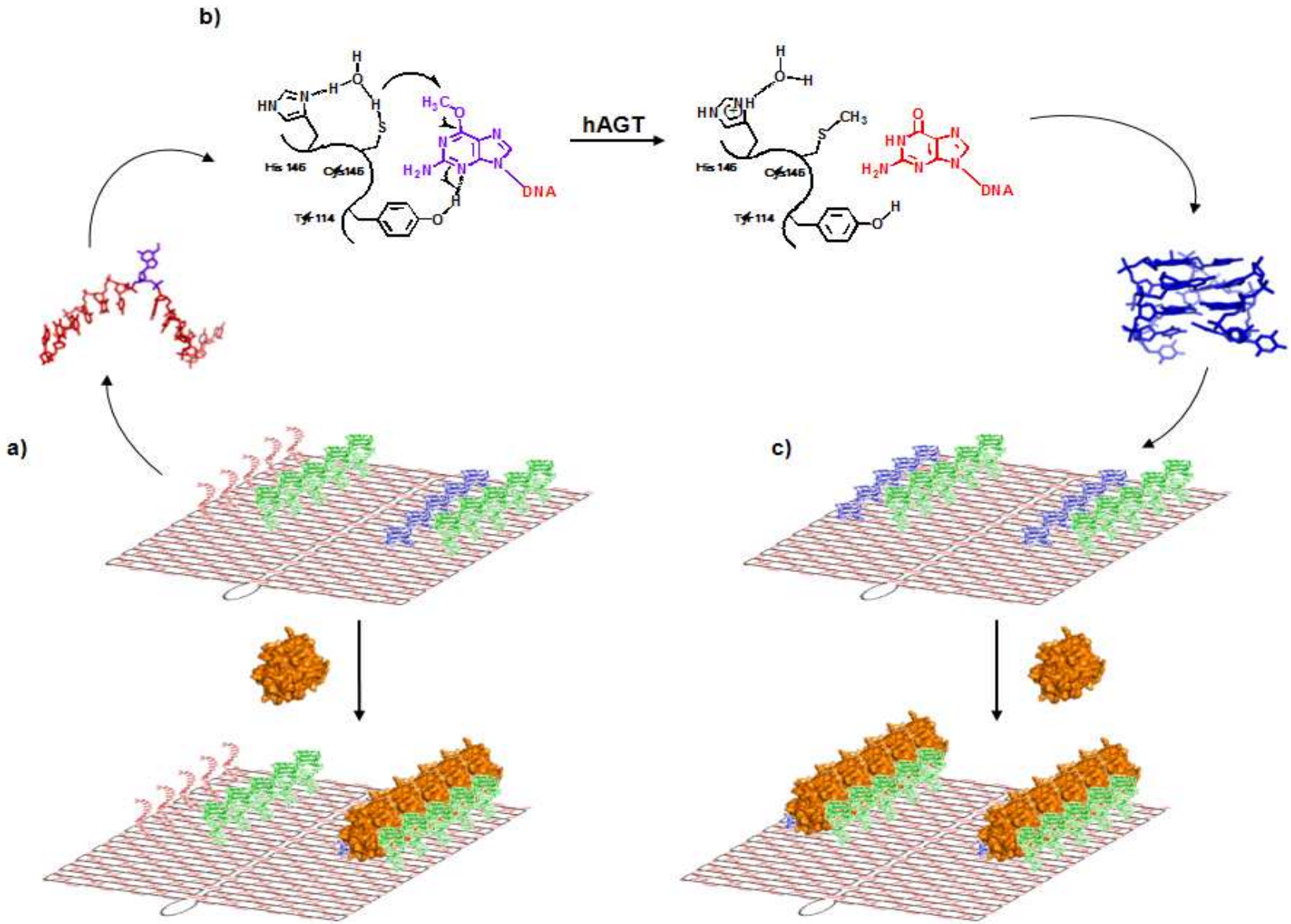
Daniels et al., EMBO J., 19(7) 1719-1730, 2000



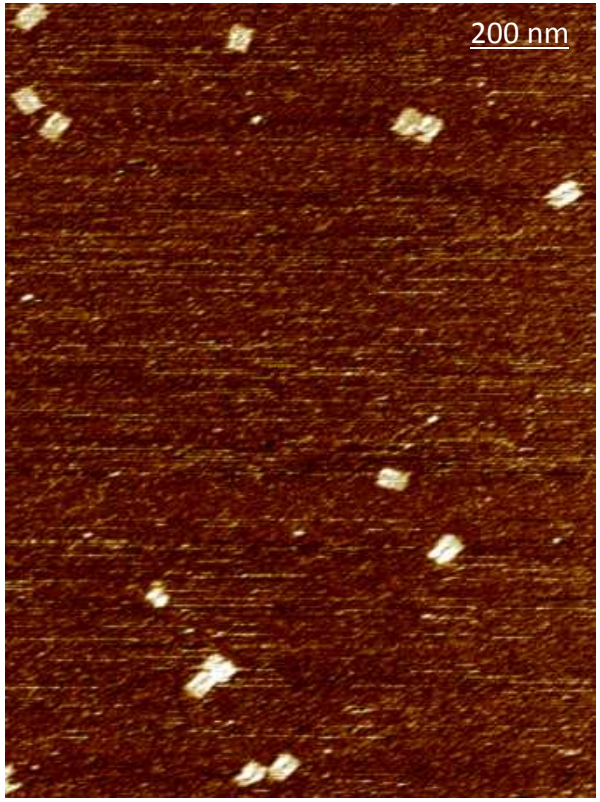
Daniels et al.,NSMB, 11(8) 714-720, 2004

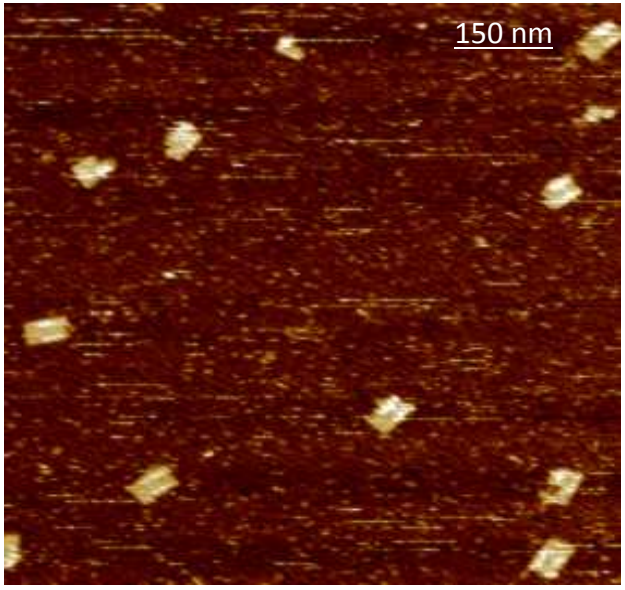
- Overexpressed in tumoral cells.
- Inverse correlation between survival and hAGT levels in patients with malignant gliomas.

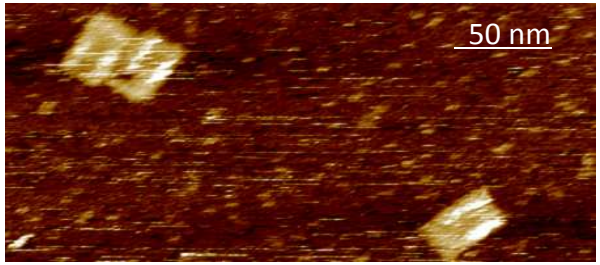
hAGT REPAIR OF THE METHYL-TBA-ORIGAMI

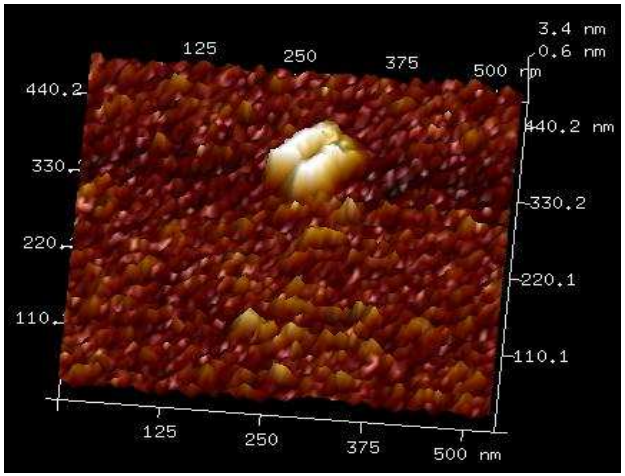


hAGT REPAIR OF THE METHYL-TBA

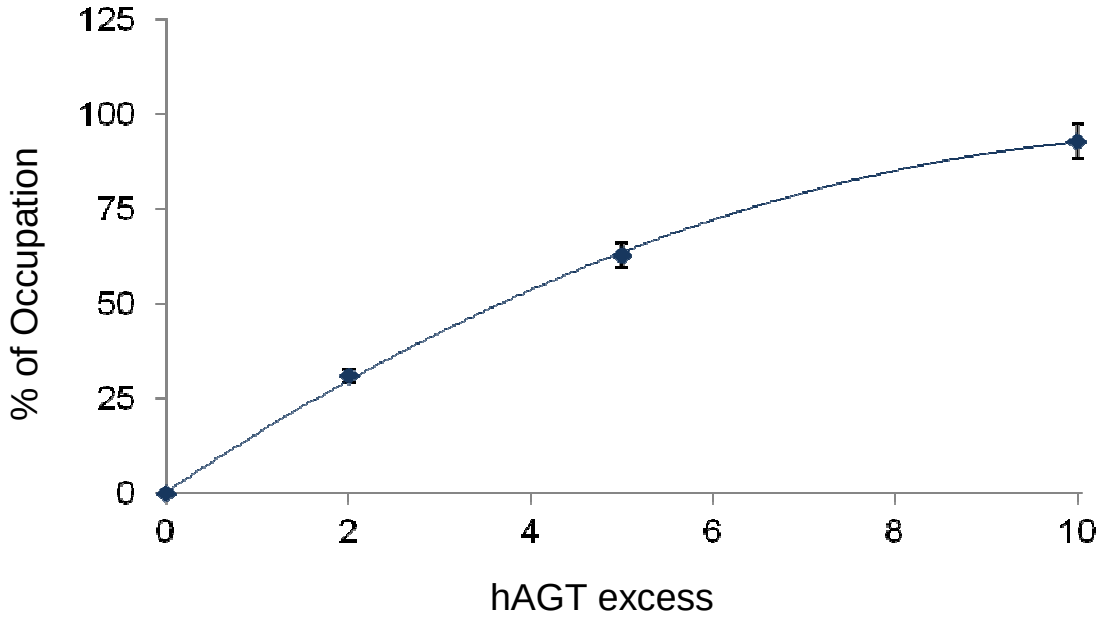


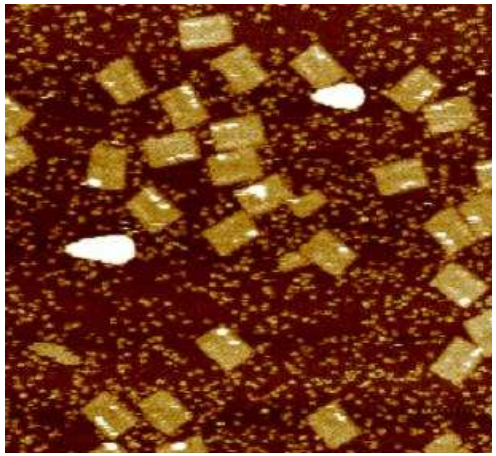




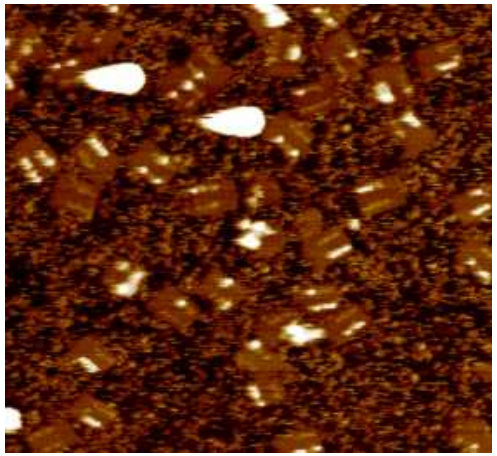


hAGT titration

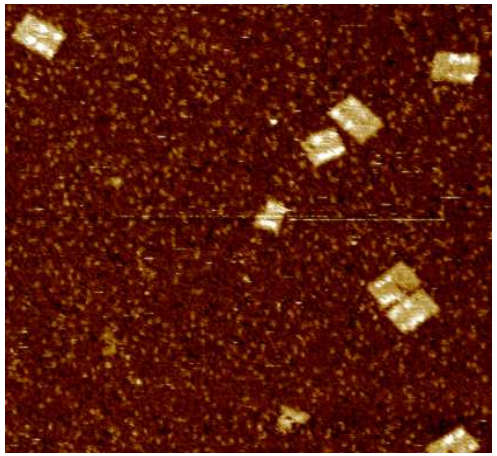




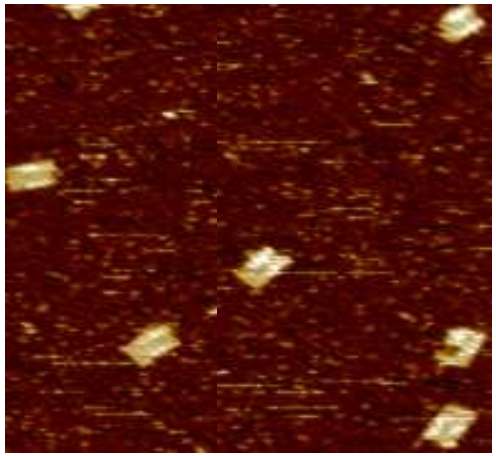
hAGT 0x



hAGT 2x



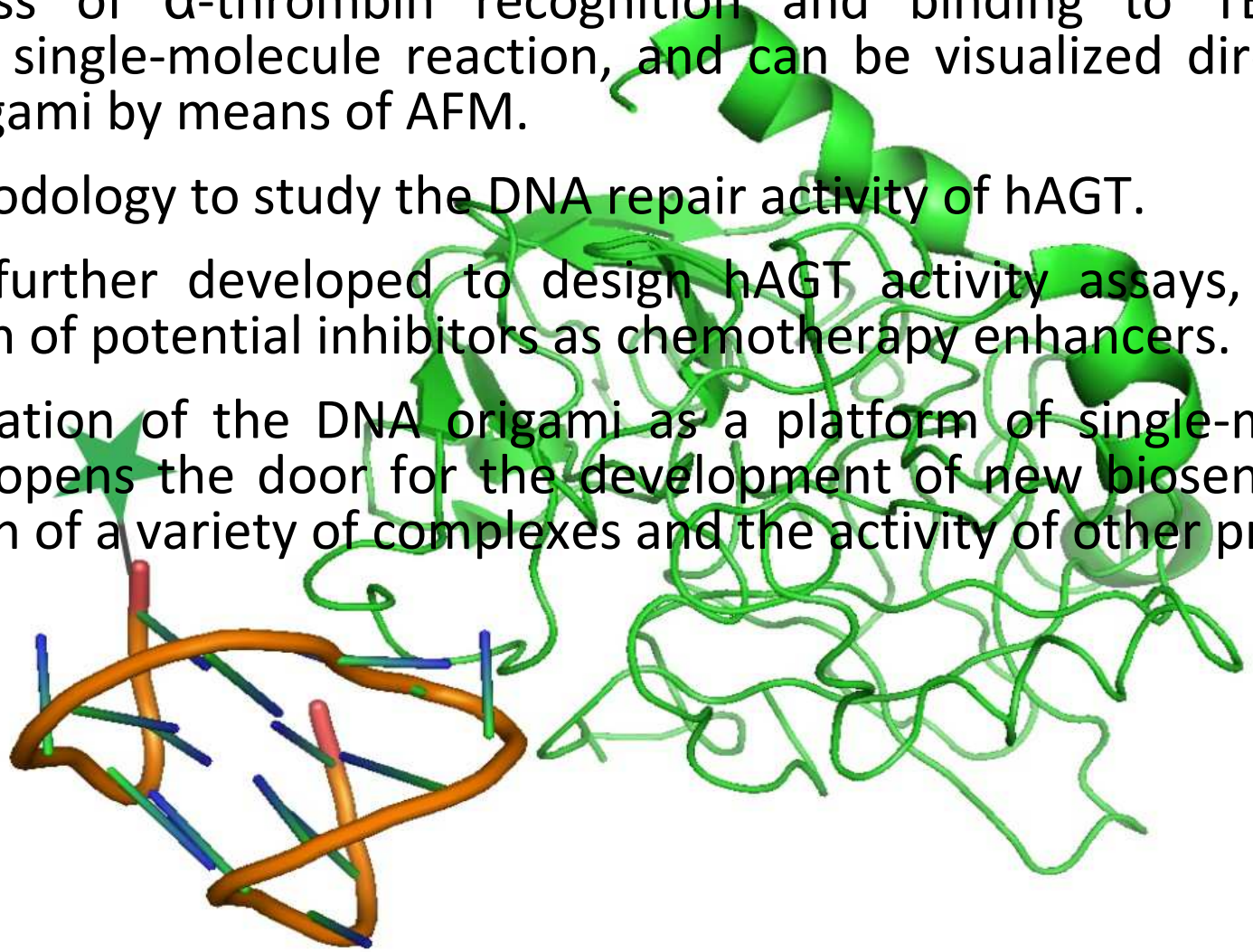
hAGT 5x



hAGT 10x

CONCLUSIONS

- The process of α-thrombin recognition and binding to TBA is a quantitative single-molecule reaction, and can be visualized directly on the DNA origami by means of AFM.
- New methodology to study the DNA repair activity of hAGT.
- It can be further developed to design hAGT activity assays, for the identification of potential inhibitors as chemotherapy enhancers.
- The application of the DNA origami as a platform of single-molecule recognition opens the door for the development of new biosensors for the detection of a variety of complexes and the activity of other proteins.



Thank you for your attention

- IQAC-CSIC:
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  - Carme Fàbrega
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  - Rubén Ferreira
  - Maria Tintoré
  - Adele Alagia
  - Clara Caminal
- UMH, Elche:
  - Eduardo Fernández

UB Bellvitge:

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UAB:

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Begoña Uribe-Ugarte

Félix Goñi

Ester Fernández

UPV, Vitoria:

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