

Functionalized-Cucurbit[*n*]urils: Advanced Drug Carriers

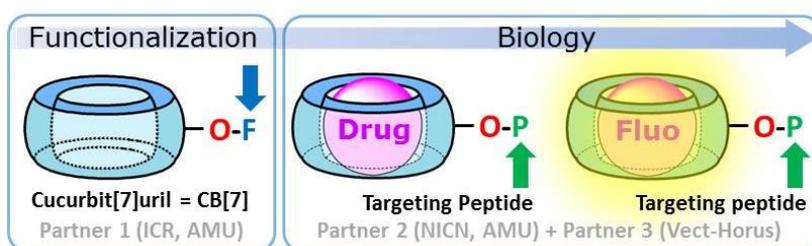
1. DESCRIPTION OF THE PHD THESIS PROJECT

1.1 OBJECTIVES OF THE PROJECT BASED ON THE CURRENT STATE OF THE ART

This PhD thesis project will involve 2 academic partners from Aix-Marseille University and 1 biotech company located in Marseille:

- 1) The Free Radical Chemistry Institute (Institut de Chimie Radicale - ICR, UMR 7273, CNRS-AMU, Marseille, France),
- 2) The Neurobiology of Cellular Interactions and Neurophysiopathologies Research Unit (Neurobiologie des Interactions Cellulaires et Neurophysiopathologies – NICN, UMR 7259, CNRS-AMU, Marseille, France),
- 3) The biotechnology company Vect-Horus (Marseille, France).

Aim. Our objective is to use functionalized CB[7] as a molecular cargo for the construction of advanced nanoscale delivery systems comprising targeting peptides and benefiting from the versatile cavity of CB[7] to encapsulate various drugs and fluorescent compounds.



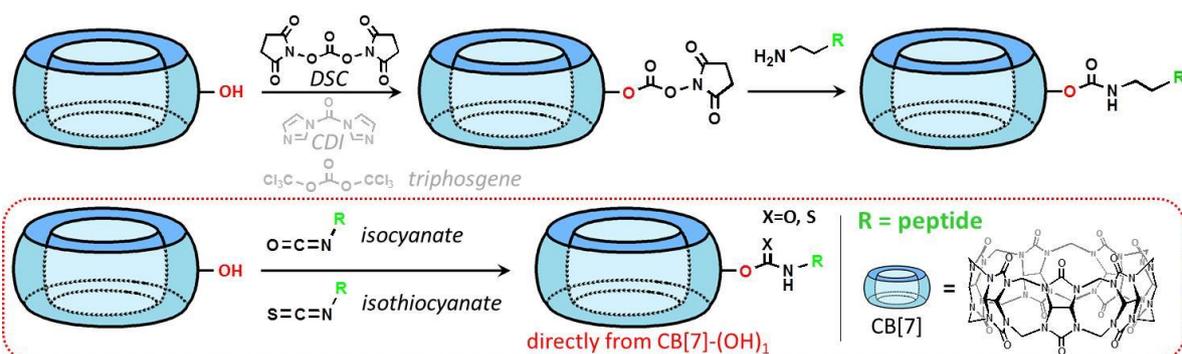
After the discovery and the setting of the principal rules of the supramolecular chemistry in the 1960-1980's that was recognized through the awarding of the 1987 Nobel Prize for Chemistry, the first molecular machines,

constructed after having mastered the synthesis of macrocycles, rotaxanes and catenanes have led to tremendous developments. These structures are the basic building-blocks of molecular machines and the construction of molecular shuttles, elevators, pumps and rotational machines have just been awarded the 2016 Nobel Prize for Chemistry. This recognition acknowledges the pioneering work of precursors that have shed the basis for the control of molecular motion at the nanoscale. However, there is still a huge gap before the first molecular machines are applied in nanotechnologic applications for biology. Yet, nature marvelously managed to set outstanding molecular machines, working out of equilibrium, to perform tasks pivotal for many crucial biological functions. One particular challenge for biology is to reliably deliver relevant compounds to sites of interest. To this aim, several approaches have been developed. One of these use functionalized-cyclodextrins (F-CDs) as molecular cargos to transport drugs to relevant targets by means of targeting groups directly grafted on the CDs. However, the binding constants offered by CDs toward the inclusion of guests of interest only range in the 10^2 to 10^5 M^{-1} regime which is problematic due to the detrimentally large

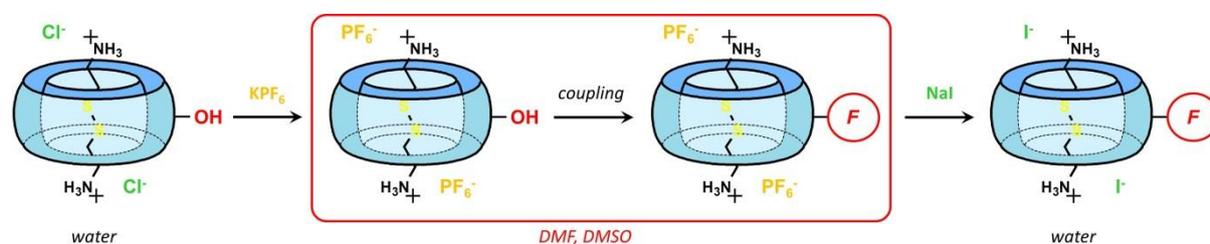
number of possible competitors in vivo. On the other hand, cucurbit[*n*]urils (CB[*n*]), a recently discovered class of macrocyclic compounds have shown outstanding properties of molecular recognition, often outperforming cyclodextrins in many prospects. Toxicity tests and in vivo tests on small animals (zebrafish) have shown the high potential of CB[7] as a potent surrogate of CDs for drug transportation. The binding constants are largely reported to be in the 10^4 to 10^9 M⁻¹ range thus enabling the use of lower doses of active compounds with more confidence that they will be delivered at the target site. However, CB[*n*] are recognized to be difficult compounds to functionalize. One possibility relies on a building-block synthetic sequence that has recently enabled CB[7] to be functionalized with a target unit. However, even though very elegant, this method is still limited by the number of synthetic steps, small overall yields and hard purifications. Since 2013, very few other examples have been reported. **Here we propose to investigate a totally new approach of functionalizing CB[7]**, starting from a method developed recently by Partner 1 enabling to get several grams of CB[7]-(OH)₁ in two days. Our aim is to **graft a cell-surface targeting peptide (Trojan Horse) on functionalized CB[7] and check**, with the aid of Partner 2, **the ability of the nano-sized CB-peptide conjugate to keep its targeting function toward the Low-Density Lipoprotein (LDL) Receptor (LDLR) used for imaging or therapeutic applications** such as cancer treatments, Blood Brain Barrier (BBB) crossing and brain delivery. Several drugs and fluorescent compounds, know to bind inside CB[7] will be tested on several models. This project will simultaneously support the commitment of Partner 1 in this rising and almost unexplored research field, and also stimulate further developments toward the amenability of CB[*n*] as highly potent components of a new generation of smart nanoscale drug delivery agents. Partner 2 will benefit from the binding abilities of CB[7] toward using a versatile cargo for targeting means. Indeed, the CB-*peptide* conjugate will be a versatile platform enabling (i) to encapsulate many compounds, (ii) without the needs for further chemical ligations and (iii) binding unmodified drugs thus avoiding altering their biological properties. The local biotechnology company Vect-Horus has shown interest in functionalized CB[*n*] and support our initiative toward new functional CB[*n*] derivatives. Clearly, there remains an enormous body of opportunities for those who will manage to gain more control over (i) targeting issues of molecular cargos and (ii) the release profiles of encapsulated drugs in optimized macrocycles. Finally, polyfunctional compounds featuring 2 and 3 targeting units will also be developed to benefit from multivalency effects in order to improve the strength toward target receptors. These systems are expected to reach 2 to 6 nm and possible self-assemblies will be studied by DLS and AFM prior to biological measurements. With a world market for cyclodextrins now evaluated > \$600 million featuring > 25 pharmaceutical products, we anticipate that CB[*n*] will face similar developments in the near future and that they can play crucial roles in upcoming technological innovations especially in the burgeoning field of nanomedicine.

1.2 METHODOLOGY

CB[*n*] are routinely produced in our laboratory in large scales (several tens of grams) and will be used for reaching several grams or tens of grams of monohydroxy-CB[7] (CB[7]-(OH)₁). The PhD candidate will be in charge of synthesizing CB[7]-(OH)₁ and developing and optimizing new synthetic approaches to enable conjugation on CB[7].



Several preliminary results have pointed to the possibility to graft several compounds on CB[5]-(OH)₁ and CB[6]-(OH)₁. For this, we use different approaches. As CB[5] is sizably DMSO-soluble, we did direct couplings and managed to get a triphenylphosphonium compound conjugated on CB[5]. CB[7] share similar solubility with CB[5] and similar couplings will be first tried to get conjugated CB[7]. For CB[6], the solubility is limited to water provided a suitable solubilizing agent has been introduced. We used a combination of (i) a cationic guest and (ii) an anion metathesis to induce CB[6]-(OH)₁ solubilization in DMF (see below). This technique allowed to graft a new compound on CB[6].

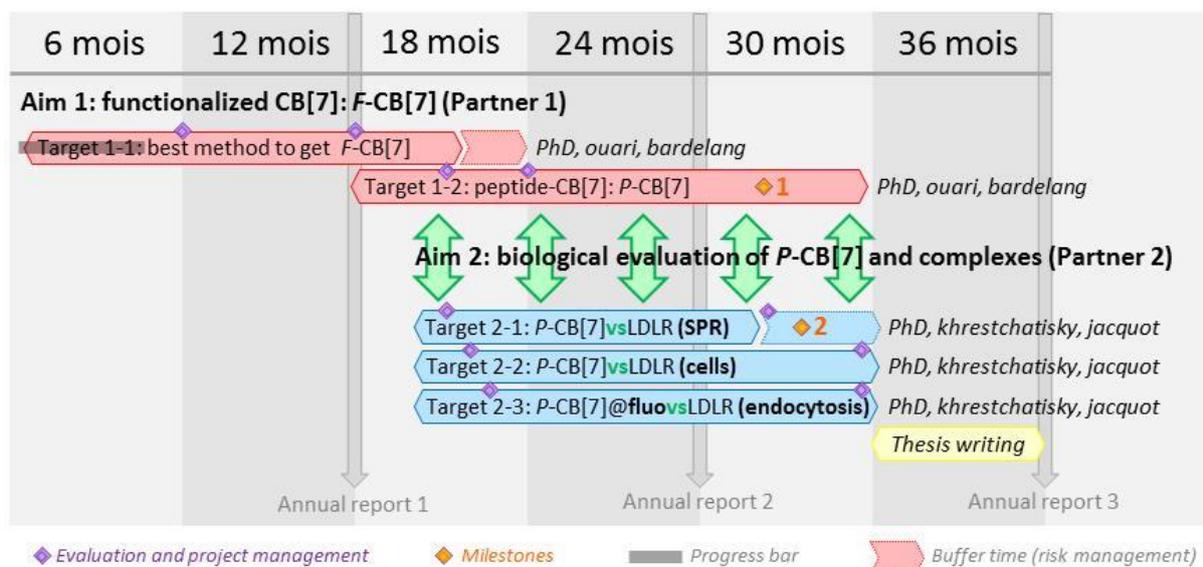


In the event that the 1st coupling approach does not work, the 2nd coupling approach (guest+anion metathesis) will be performed to get the conjugated-CB[7]. Once set, the experimental conditions will be adapted for the peptide conjugation before releasing of the dicationic guest *to ensure availability of the CB[7] cavity*. Finally, as CB[7]-(OH)₂ and CB[7]-(OH)₃ are also obtained in good quantities (several grams), multi-peptide conjugation will be assessed to get polyfunctional compounds in order to improve the binding affinity toward LDLR.

In parallel, after the first conjugate molecule will have been obtained, the binding properties of the *peptide*-CB[7] conjugate will be checked to ensure that the conjugation did not alter significantly the recognition toward LDLR. Surface Plasmon Resonance (SPR) studies will be performed by Partner 2 to quantitate the binding of the *peptide*-CB[7] conjugate toward the immobilized extracellular domain of the LDLR. Reference peptide displacement assays will also be performed on cell lines expressing human LDLR. Finally, the cavity of CB[7] has been reported to encapsulate efficiently several types of fluorophores thus enabling tracking of the *peptide*-CB[7]@fluo conjugate by fluorescence microscopy as a mean to monitor the cellular endocytosis and trafficking of the conjugate in live cells.

1.3 WORK PLAN

Gantt Chart.



The 1st part of the project will be devoted to setting the best method (yield, purification, scale) for obtaining functionalized-CB[7] (*F*-CB[7], Target 1-1) before applying it to the conjugation of a model LDLR targeting-peptide (Target 1-2) (Partner 1). This task should take time as we seek for finding the best method starting from CB[7]-(OH)₁ in terms of yield and efficiency. Then the *peptide*-CB[7] conjugate will be 1st checked for its binding affinity for the LDLR in SPR and cell-based assays and 2nd for its ability to incorporate model drugs with known binding affinity toward CB[7] and model fluorophores with good affinity toward CB[7] for fluorescence imaging. This work should enable to set CB[7] as a highly promising platform with targeting properties.

1.4 SUPERVISORS AND RESEARCH GROUPS DESCRIPTION

The work will be supervised by Drs. Olivier Ouari and David Bardelang (ICR, AMU-CNRS, Marseille) and Dr. Michel khrestchatisky (NICN, AMU-CNRS, Marseille) for the synthetic and biological parts of the work respectively. Olivier Ouari (team leader) has a large experience in macrocyclic compounds, their synthesis and their supramolecular chemistry with nitroxide free radicals that are also used for several other purposes (batteries, spin probing, spin labeling and as polarizing agents for Dynamic Nuclear Polarization). Pr. Michel khrestchatisky (UMR-7259 director) is an expert of neurobiology and neurophysiopathologies. Michel Khrestchatisky will supervise the biological and biophysical evaluation of the *peptide*-CB[7] conjugate using cell biology and surface plasmon resonance approaches. In particular one of the potential interests of the *peptide*-CB[7] conjugate is the transport of drugs across the blood brain barrier and his group with Vect-Horus has developed several models of *in vitro* BBB models that could prove useful to assess the transport properties of the *peptide*-CB[7] conjugate. His actual lab is well equipped for the proposed approaches, encompassing 4 teams of experts in the fields of neuroscience, molecular and cellular biology, imaging etc. In the near future (2018) the laboratory directed by Michel Khrestchatisky will encompass 11 scientific groups within the *Institut de Neurophysiopathologie* that is part of the large NeuroTimone project.

This project is part of a larger program toward the use of CB[*n*] and functionalized CB[*n*] in paramagnetic systems, functional systems and molecular machines. One national (ANR) funding has been requested about CB[*n*] functionalization and team allowances are devoted to support further this line of research toward a new generation of nano-machine based on smart CB[*n*] materials.

2. 3I DIMENSIONS AND OTHER ASPECTS OF THE PROJECT

2.1 INTERDISCIPLINARY DIMENSION

Drs. O. Ouari and D. Bardelang will be in charge of directing the doctoral work toward obtaining the functionalized and targeting compounds. This work will largely involve synthetic organic chemistry and routine characterization studies but as the number of targeted compounds is limited, and preliminary results have been obtained (*F*-CB[5] and *F*-CB[6]), the feasibility of the project over 3 years is good. On the other hand, conjugation might perturb the recognition of the target peptide toward LDLR. In this event, several linkers/spacers may be used to optimize the affinity of the targeting peptide for the LDLR. Finally, polyfunctional versions starting from CB[7]-(OH)₂ and CB[7]-(OH)₃ will also be synthesized. Because the essence of the work is turned toward the biological recognition of LDLR used as an endocytic receptor for transport of small (Å) and large (nanoscale) objects, this topic is situated at the interface of chemistry and biology but not only. If BBB crossing is possible while transporting several model drugs via the grafted CB[7], biomedical evaluations, especially in neurosciences could rapidly emerge paving the way for pharmaceutical applications. SPR binding measurements of the *peptide*-CB[7] conjugate will be performed on immobilized LDLR. Then, if promising, the *peptide*-CB[7] conjugates will be tested in cell lines expressing the LDLR before fluorescence imaging of endocytosis using encapsulated fluorescent probes (red or NIR probes). As such, the two laboratories are complementary, in that organic chemistry will provide with new objects, highly relevant to biology which will, in turn, stimulate chemical and supramolecular input toward tailored receptors for advanced drug transportation. Beyond a bidirectional exchange of skills and data toward the success of this work, each discipline will be strengthened by the contribution of the relevant partner.

2.2 INTERSECTORAL DIMENSION:

The private company Vect-Horus has shown a strong interest in the prospects of our functionalization strategy toward functional CB[*n*] macrocycles. Dr. Guillaume Jacquot (Vect-Horus, PharmD, PhD - Preclinical Project Manager) will follow the work toward the establishment of a robust strategy enabling the grafting of proprietary LDLR-targeting peptides on CB[*n*] for use as a tailored drug carrier with anticipated biodistribution and safety advantages. This breakthrough technology combines both an innovative chemistry approach together with the highly efficient LDLR-targeting technology developed by Vect-Horus. These new *peptide*-CB[*n*] conjugates will open new avenues in the field of targeted drug delivery for therapeutic and imaging applications, especially for those drugs or imaging agents that would benefit from transportation of unmodified drugs with recognized activity (avoiding chemical ligation). The PhD candidate will be able to present his findings to the company and possibly contribute to a future collaboration toward developing another application of functionalized CB[*n*] compounds for biological compounds. Beside the academic viewpoint, a project about CB[*n*] encapsulation of free radicals has been funded by the regional antenna of the French administration (Region PACA, project "Masked Spins") in 2012 and 2013 whose results highly supported the setup of the reaction enabling getting CB[*n*]-OH compounds. So Region PACA supported our research and we just applied in 2016 for another project implying CB[*n*]. The essence of the present proposal matches well with *one* of the priority axes developed by Region PACA: Domaines d'Activités Stratégiques (DAS) – Strategic Activity Domains. The SRI-3S policy (Smart Specialisation Strategy) has been integrated in the "Teaching, Research and Innovation Regional Scheme" (Schéma Régional d'Enseignement Supérieur, de Recherche et d'Innovation (SRESRI)) by defining 5 Strategic Activity Domains. This proposal matches well with the Strategic Activity Domain: "Health, Food and Well-Being" and Vect-Horus is a clearly identified regional actor stimulating and improving innovation around Aix-Marseille University and the Marseille bay. In particular, skills related to neurosciences have been pointed to be highly relevant, skills largely represented by Partner 2. In summary, this PhD proposes to investigate a chemistry/biology interface with contacts

in industry for an applied perspective of (i) our research in the two laboratories concerned with the project and (ii) a continuous interest from a local biotechnology company in our work.

2.3 INTERNATIONAL DIMENSION:

Because the essence of the project is focused on setting a new advanced drug carrier that combine (i) versatility for transported compounds and (ii) targeting to tissues of interest, there are no doubts about the impact of the present study. Indeed, current limitations of cyclodextrins (world market evaluated > \$600 million) can be surpassed by the advantages offered by CB[7]. As such, economical perspectives could merge from the results obtained and patents can be deposited. Communications at international conferences and workshops are planned for example at the occasion of the annual “International Symposium on Macrocyclic and Supramolecular Chemistry (ISMSC)”, the biannual “International Conference on Cucurbiturils (ICCB)” or the RSC / SCI Medicinal Chemistry Symposium, or the International Symposium on Advances in Synthetic and Medicinal Chemistry.

3. RECENT PUBLICATIONS

Partner 1 (chemistry):

EPR studies of the binding properties, guest dynamics and the inner space dimensions of a water-soluble resorcinarene capsule, M. M. Ayhan, G. Casano, H. Karoui, A. Rockenbauer, V. Monnier, M. Hardy, P. Tordo, D. Bardelang, O. Ouari,* *Chem.-Eur. J.*, **2015**, *21*, 16404–16410. (frontispiece).

Comprehensive synthesis of monohydroxy-cucurbit[n]urils (n = 5, 6, 7, 8): high purity and high conversions, M. M. Ayhan, H. Karoui, M. Hardy, A. Rockenbauer, L. Charles, R. Rosas, K. Udachin, P. Tordo, D. Bardelang,* O. Ouari,* *J. Am. Chem. Soc.*, **2015**, *137*, 10238–10245.

High binding yet accelerated guest rotation within a cucurbit[7]uril complex. Toward paramagnetic gyroscopes and rolling nanomachines, G. Casano, F. Poulhès, T. K. Tran, M. M. Ayhan, H. Karoui, D. Siri, A. Gaudel-Siri, A. Rockenbauer, G. Jeschke, D. Bardelang,* P. Tordo,* O. Ouari,* *Nanoscale*, **2015**, *7*, 12143–12150.

Spin exchange monitoring of the strong positive homotropic allosteric binding of a tetraradical by a synthetic receptor in water, D. Bardelang,* G. Casano, F. Poulhès, H. Karoui, J. Filippini, A. Rockenbauer, R. Rosas, V. Monnier, D. Siri, A. Gaudel-Siri, O. Ouari,* P. Tordo,* *J. Am. Chem. Soc.*, **2014**, *136*, 17570–17577.

Partner 2 (biology):

Optimization and in Vivo Validation of Peptide Vectors Targeting the LDL Receptor, G. Jacquot, P. Lecorche, J.D. Malcor, M. Laurencin, M. Smirnova, K. Varini, C. Malicet, F. Gassiot, A. Faucon, M. David, N. Gaudin, M. Masse, G. Ferracci, V. Dive, S. Cisternino, M. Khrestchatisky, *Mol. Pharmaceutics*, **2016**, *13*, 4094–4105.

Chemical optimization of new ligands of the low-density lipoprotein receptor as potential vectors for central nervous system targeting, J.D. Malcor, N. Payrot, M. David, A. Faucon, K. Abouzid, G. Jacquot, N. Floquet, F. Debarbieux, G. Rougon, J. Martinez, M. Khrestchatisky, P. Vlieghe, V. Lisowski, *J. Med. Chem.*, **2012**, *55*, 2227–2241.

Setting-up an in vitro model of rat blood-brain barrier (BBB): a focus on BBB impermeability and receptor-mediated transport, Y. Molino, F. Jabes, E. Lacassagne, N. Gaudin, M. Khrestchatisky, *J. Vis. Exp.*, **2014**, e51278.

Use of LDLR-targeting peptide-vectors for in vitro and in vivo cargo transport across the blood-brain barrier, Y. Molino, M. David, K. Varini, F. Jabès, N. Gaudin, A. Fortoul, K. Bakloul, M. Masse, A. Bernard, L. Drobecq, P. Lécorché, J. Tamsamani, G. Jacquot, M. Khrestchatisky, *FASEB J*, in press.

4. EXPECTED PROFILE OF THE CANDIDATE

The PhD candidate will have to be familiar with the rules and methods of the modern synthetic organic chemistry. NMR spectroscopy, mass spectrometry, IR and UV-vis spectroscopies will be necessary to be mastered as they will be routinely applied for the characterization of the newly prepared compounds. A background or biology will be highly appreciated to meet with the requirements of the 2nd part of the project. Thus knowledges of binding determination of biological targets will be even better but are not compulsory for the project as the necessary skills will be gained during the 3-year period of the PhD thesis. Similarly, skills or preliminary knowledges about macrocyclic chemistry, such as CDs or CB[n] chemistry will be appreciated but are not strictly necessary to start the PhD. A culture of supramolecular chemistry (basis, main forces and recognition motifs, families of macrocycles) will also be another positive point for the candidate.

The PhD candidate will be attached to the two doctoral schools:

- 1) ED 250 – Sciences chimiques (Doctoral School 250: Chemical Sciences).
- 2) ED 62 - Sciences de la vie et de la santé (Doctoral School 62: Life and Health Sciences).

The PhD candidate will thus develop a two-fold background. First, as an organic chemist (syntheses and characterization), and second as a biochemist (molecular recognition, peptide receptors, neurosciences, fluorescence imaging), aware and devoted to bring new solutions to long-lasting biological problems. Such opportunity seems ideally suited for a future carrier in the pharmaceutical industry or for researches at the chemistry/biology/health interface.

The Doctoral School in Chemistry (ED 250) requires candidates having a high ranking at the Master degree (mention Bien in the French system or equivalent).

5. SUPERVISORS' PROFILES

The work will be supervised by Dr. Olivier Ouari (ICR AMU-CNRS, Marseille) who has published >75 articles in peer-reviewed journals, some in high-profile media, 2 book chapters, and 6 patents (2 under licence). He has been the supervisor of 4 PhD thesis and all of the young researchers have joined a private company or a postdoctoral position. He is currently supervising two PhD students (starting date: 01/10/2014 and 1/11/2015, ending date: 15/09/2017 and 10/2018). Olivier Ouari has been a research fellow at UCL (Belgium, 1999-2000), at UNED (Madrid, 2002-2003), and UTD (Dallas, TX, 2003-2005) before joining AMU in October 2005 as an associate professor. Since 2011, he is a team leader at the Institute of Free Radical Chemistry. He has been part of the national evaluating CNRS committee (2012-2016) and he is regularly invited for seminars, PhD and HDR committees and international conferences.

Michel KHRESTCHATISKY, DR1 at the CNRS, PhD in cellular and molecular biology, specialized in neurobiology, 4 years of research at the University of California Los Angeles (UCLA, USA) and 10 year experience as group leader at INSERM, Paris. Director during the last 14 years of the NICN-UMR7259 neurobiology laboratory supported by the CNRS and Aix-Marseille University, and leader of the BBB and Neuroinflammation group. Michel Khrestchatisky supervised 12 doctoral thesis, published over 110 peer-reviewed articles in international scientific journals, a dozen book chapters and is co-inventor in 5 families of patents with more than 20 patents delivered worldwide and 30 patent applications pending. He currently supervises 2 PhD students, one in the context of a European program (09/2015 to 08/2018) and another in co-supervision within an CIFRE contract (01-2015 to 12/2017).