AGREEMENT AND JOINT THESIS SUPERVISION

BETWEEN

University of Rouen Normandy, a public institution of a scientific, cultural and professional nature, having its registered office at 1 rue Thomas Becket 76821 Mont Saint Aignan cedex, FRANCE, SIRET 197 619 042 00017, APE 8542Z Hereinafter referred to as "**URN**" **(Institution of Origin),**
represented by M. **Laurent YON** in his capacity as **President**

and

The **Community of Universities and Establishments Normandy University,** a national public institution of a scientific, cultural and professional nature, whose registered office is located at Esplanade de la Paix – CS 14032 – 14032 Caen Cedex 5, France, SIRET 130 021 330 00019, APE 8542Z, hereinafter referred to as "NORMANDIE UNIVERSITE",
represented by M. **Ronan CONGAR,** in his capacity as **President**

and

Università degli Studi di Messina, an Italian public institution with its registered office at Piazza Pugliatti 1, 98121, Messina, ITALY, Tax Code 80004070837
Hereinafter referred to as " **UNIME** " **(Host Institution)**,
represented by Prof. **Giovanna Spatari** in his capacity as **Rector**

wherein the aforementioned parties are jointly referred to as "the Parties" and/or by "the Institutions" and individually by "the Party" and/or by "the Institution";

Regarding **URN** (France) :

- Decree of 26 August 2022 amending the Decree of 25 May 2016 setting the national framework for training and the procedures leading to the award of a national doctoral degree;

Considering **UNIME** (Italy)

- Article 4 of Italian Law No. 210 of 03/07/1998, which provides for the autonomy of universities in the establishment of doctoral courses within the framework of the governmental, educational and scientific autonomy of universities, as amended by Law No. 240/2010;
- the regulation of the Italian Ministry of Education, University and Research regarding doctoral programs, published with D.M. n. 226/2021;
- the Regulations of the University of Messina regarding the research doctoral course published with the Rector's Decree no. 834/2022;
- the report of the Doctoral Councils of Professors held on October 9, 2024 (prot. n. 128195, registration dated October 10, 2024) which approves the stipulation of a co-supervision agreement with the University of Rouen for the attribution of the double qualification at the end of the doctoral course.

Considering

- Framework Agreement on the Co-Supervision of these Universities between the Conference of University Presidents (CPU) and the Conference of Rectors of Italian Universities (CRUI), dated 13 February 1998;
- That the parties have signed a Research Project entitled "THERAGLIO" undertook, among other things, to activate a co-supervision and thesis collaboration agreement for the realization of a doctoral thesis entitled: "*New theranostic agents for the controlled release of temozolomide in glioblastoma*"; hereinafter the "**PROGRAM**", as described in Appendix 1

- As a result, the Parties met to conclude the Convention.

It was agreed as follows:

Article 1 - OBJECT - CO-SUPERVISION OF THE THESIS

The purpose of this agreement, hereinafter referred to as the "**Convention**", is to determine the conditions for the conduct of the international co-supervision of the thesis.

The partner institutions agree to jointly assume responsibility for the supervision of the doctoral student's doctoral research project. The two Institutions, driven by the desire to promote the exchange and mobility of doctoral students among themselves and thus to strengthen scientific and academic cooperation, decide by mutual agreement, within the framework of the legislation in force in their respective countries, to use the procedure of joint thesis supervision in favor of the doctoral student:

- First and last name of the PhD student: Deniz ONAL
- Address: 92130 ISSY (France)
- Baccalaureate: Science
- Master's degree : Biomedical Engineering

Article 2 – REGISTRATION - DURATION

The doctoral student must be enrolled in both institutions as of the 2024/2025 academic year. She must pay the registration fee to the **URN** (Home Institution). She is exempt from tuition fees at **UNIME** related to the COA (Contributo Onnicomprensivo Annuale). She will pay the annual amount of the regional tax for the right to education at **UNIME**.

The doctoral student must enrol each year in both institutions. He is required to respect the rules and practices of each establishment. It will benefit from the collective structures of the two establishments.

She will be required to provide an annual registration certificate for both parties.

The validity of the thesis prepared by the PhD student is automatically recognized by the Parties and this by virtue of the principle of reciprocity.

The Agreement is established for a period **of three (3) years**, starting from the academic year 2024-2025, upon signature by the Parties. Its validity is subject to the administrative registration of the PhD student in the two Institutions each year.

This period may be extended, by way of derogation, subject to an authorization to enrol in a derogatory year, on the reasoned opinion of the thesis directors, in accordance with the rules in force in each INSTITUTION, and according to the internal regulations of the doctoral school to which the doctoral school is attached.

Any extension or modification of the Agreement shall be the subject of a written amendment signed by the Parties.

Article 3 – RESEARCH PERIODS

The alternating periods of work in each INSTITUTION are divided by the thesis directors according to the scientific requirements and the conditions of preparation of the thesis and decided by mutual agreement, as follows:

	Periods
➢ **Establishment** At **the URN (COBRA** UMR 6014)	*First academic year (2024-2025) = 12 months, from December 1, 2024 to November 30, 2025* *Third academic year (2026-2027) = 6 months, from December 1, 2026 to May 31, 2027*
➢ At **UNIME**	*Second academic year (2025-2026) = 12 months from December 1, 2025 to November 30, 2026* *Third academic year (2026-2027) = 6 months from June 01, 2027 to November 30, 2027*

This calendar is subject to modification by means of an amendment at the request of the thesis directors. Over the entire duration of the thesis, the period spent in each institution may not be less than twelve (12) months.

Article 4 - FINANCING

The doctoral student will be recruited by URN for 18 months with funding comes from the Normandy region and for 18 months with funding from the University of Messina.

Article 5 – SUPERVISION OF THE DOCTORAL STUDENT

In each of the institutions concerned, the doctoral student will carry out the research work under the

supervision and responsibility of the following thesis directors:

- At **the URN** (Home Institution):

  Name: Mrs. Géraldine GOUHIER, University Professor, geraldine.gouhier@univ-rouen.fr, COBRA Laboratory.

- At **UNIME** (host institution) :

  Name: Mr. Antonino MAZZAGLIA, antonino.mazzaglia@unime.it, antonino.mazzaglia@cnr.it, in the department: Scienze Chimiche, Biologiche, Farmaceutiche e Ambientali, **ChiBioFarAm** (*Chemical, Biological, Pharmaceutical and Environmental Sciences),* Director of Research at the Consiglio Nazionale della Ricerca, **CNR-** ISMN (National Research Council); - Member of the Faculty Council of the Doctoral Course in Scienze *Chimiche*.

The thesis directors undertake to fully exercise, jointly with the doctoral student, the responsibilities assigned to them by the regulations in force and the academic traditions of their respective countries. They also undertake to ensure the application of the provisions of this agreement.

In the event of a change in the direction of the thesis, the procedure followed will be that of the institution concerned. This change must be communicated to the other institution.

Article 6 – ATTACHMENT TO THE DOCTORAL SCHOOL

For **the URN** and **NORMANDIE UNIVERSITE**, the PhD student is attached to the Normandy Doctoral School **of Chemistry ED NC** (508) enrolled in a doctorate in (Chemistry), and integrated within the laboratory / research unit (UR) Organic and Bioorganic Chemistry (UMR 6014), hereinafter referred to as "**COBRA**"

For **UNIME**, the PhD student is enrolled in the Scienze Chimiche doctoral course, cycle 40° and integrated within (laboratory CNR ISMN URT Messina c/o Department ChiBioFarAm (polo Papardo) Viale F. Stagno d'Alcontres 98166 Messina/ research unit of Prof. Antonino Mazzaglia).

Article 7 – TRAINING ACTIVITIES

The training followed by the doctoral student during the thesis will be recognized according to the regulatory provisions of each institution. The doctoral student will follow the activities of the doctoral programs during his stay at **UNIME** and **URN**, he will undertake to follow them and will be subject to the internal regulations of the academic schools of the institutions.

Article 8 – INSURANCE COVERAGE

The doctoral student undertakes to take out social security coverage for the duration of his thesis, covering him both in France and abroad.

The PhD student certifies that he or she is covered for the duration of his or her thesis by a civil liability insurance in the organization of his or her choice, which guarantees him or her for all accidents of which he or she may be a victim or for which he or she may be personally liable.

During its stay in the host country **(UNIME/Italy)**, UNIME guarantees civil liability insurance and accident insurance **within the limits established by the UNIME insurance policy**. Upon arrival in Italy, the URN doctoral student must have appropriate health coverage that also covers repatriation in the event of accident or illness, as well as insurance coverage against bodily injury and civil liability in the performance of their duties within the framework of the doctoral research project in co-supervision of the thesis.

The civil liability of the Parties may not be engaged

Article 9 – ADMISSION TO THE SOLE FINAL EXAMINATION BOARD

According to Italian rules, in order to obtain admission to the single final examination, the thesis will be evaluated by two external examiners appointed by the Council of Italian Professors who do not participate in the final examination. If the evaluation of the examiners and the Italian Council of Professors is positive and they do not request changes to the thesis, the final exam can be taken up to 60 days before the end of the doctoral course. If the evaluation of the examiners and the Italian Council of Professors is positive, but they request changes to the thesis, the final exam could be postponed for up to six months.

Article 10 – DEFENSE OF A DOCTORAL THESIS (PUBLIC DEBATE)

The deadline for submission of the final thesis will be up to 15 days before the end of the PhD course.

Authorization to defend the thesis is requested from each INSTITUTION according to the procedures and deadlines as well as the legal and regulatory provisions in force.

In the event of any particularities of the conditions of defense specific to the partner country (potentially incompatible with French legislation), the clauses should be specified.

External rapporteurs will be jointly appointed by the Parties. The evaluation reports and the authorization of the defense will be drawn up in English.

The date and place of the defense are set by mutual agreement between the two ESTABLISHMENTS and indicated in the application for authorization to defend.

The thesis defense is unique and will take place at the URN.

The financial arrangements for covering travel related to the thesis defense (travel, accommodation) of the members of the jury (thesis directors, personalities of the INSTITUTIONS and external personalities) or of the doctoral student, will be determined jointly between the UR 6014 of the URN in France and the UNIME in Italy.

The constitution of the defense jury obeys the regulations in force in the country where the defense takes place, without prejudice to the regulations specific to each institution.
The defense jury is composed of six members, including the two thesis directors who do not evaluate the candidate. In accordance with the regulations in force, the members of the jury are composed of a balanced number of women and men appointed by the two establishments and include members and external personalities appointed by each establishment. At least half of the members of the thesis defense must be university professors or equivalent.

At the end of the defense, the president draws up a report which is countersigned by the members of the jury present and by the president on behalf of the members by videoconference, indicating the mention "*present by videoconference in accordance with the delegations of signature*".

For the URN, on an exceptional basis, the Doctoral Student, and the members of the jury, in whole or in part, may participate in the defense by means of videoconference or electronic communication allowing their identification and their effective participation in a collegial deliberation and satisfying technical characteristics guaranteeing the continuous and simultaneous transmission of the debates.

The videoconference defense must comply with the conditions required for any thesis defense as well as the relevant rules in force in each Institution.

Article 11 – LANGUAGE OF THE THESIS

The doctoral thesis and research findings must be written in English.
A summary of the doctoral thesis must be provided in English and French.
The thesis will be defended in English.

Article 12 – GRADUATION

The degree of doctor will be awarded simultaneously in each of the two universities (double degree). The doctorate diploma will indicate the speciality or discipline, the title of the thesis or the title of the main works, the mention of the co-supervision, the names and titles of the members of the jury and the date of the defence.

NORMANDIE UNIVERSITE and **the URN** (name of the French institutions) will award the diploma of "Doctor of Chemistry", in accordance with the French regulations in force.

UNIME (name of the Italian institution) will award the diploma of Dottore di ricerca (Ph.D) in cycle 40 "Scienze Chimiche", in accordance with the Italian regulations in force.

Article 13 – INTELLECTUAL PROPERTY RIGHTS

Intellectual property will be governed in accordance with the respective policies of each of the partner institutions.

Therefore, the protection of the research results resulting from the doctoral research project, as well as any issues related to their deposit, availability, publication and exploitation must be discussed and approved by the partner institutions and comply with the national and institutional regulations in force, by respecting the provisions of the research project that the doctoral student undertakes to respect.

A separate specific agreement will be drawn up between the Parties to deal with intellectual property, the financing and confidentiality of the thesis.

Article 14 – PROCESSING OF PERSONAL DATA

The processing of personal data related to this Agreement is carried out by the University in accordance with the European General Regulation No. 679/2016 on the Protection of Personal Data and the Code on the Protection of Personal Data, Legislative Decree No. 196/2003 and subsequent amendments and additions.

Article 15 – FILING, COPYING AND PUBLICATION OF THE DOCTORAL THESIS

Details regarding the deposit copy, author information and printing of the doctoral thesis are defined in the applicable regulations of each higher education institution.
The publication of the doctoral thesis is guaranteed by the two higher education institutions in accordance with their applicable regulations, by respecting the provisions relating to research projects that the doctoral student undertakes to respect.

Article 16 – ENTRY INTO FORCE AND VALIDITY OF THE CONTRACT

This Agreement shall take effect from the date of its signature by the authorized representative of each Contracting Institution and shall expire at the conclusion of the final examination procedure.

The doctoral program lasts three years and starts at December 1st 2024 and it will end on November 30, 2027.

Article 17 – VALIDITY OF THE AGREEMENT AND DISPUTE RESOLUTION

The provisions of this agreement may not conflict with the provisions of the Rules of Procedure of the two institutions. Disputes should be resolved amicably through friendly bilateral consultations.
Amendments to this Agreement shall not be effective unless in writing and signed by the authorized representatives of the Contracting Parties.

Article 18 – DEMATERIALIZATION OF THE SIGNATURE OF THE AGREEMENT

Parties may sign the Convention in electronic form by exchanging documents in PDF format digitally signed or equivalent. It is expressly agreed that the document thus signed shall have the value of an original between the Parties and shall be enforceable between them.

Signatures

(Done digitally, including one copy for each Party)

University of Rouen Normandy **Università degli Studi di Messina**

Date: Notice and signature of the President University of Rouen Normandy (URN) **Mr. Laurent YON** Vincent RICHARD Vice-Président Université de Rouen Normandie	Date: Notice and signature of the Rector Università degli Studi di Messina (UNIME) **Prof.ssa Giovanna SPATARI**
Date: December 10th, 2024 Notice and signature of the President Normandy University (NU) **Mr. Ronan CONGAR** Pour le Président et par délégation, Le Vice-Président en charge de la Formation doctorale et Directeur du Collège des Ecoles Doctorales de Normandie Université François DAUPHIN	

Visas

The thesis director

Mrs. Géraldine GOUHIER Dr.

The Co-Thesis Director

Mr. Antonino Mazzaglia

The Director of The NC Doctoral School

Mr. Thomas LECOURT

Doctoral Course Coordinator Scienze Chimiche

Mrs. Concetta DE STEFANO

The Directorof the COBRA Laboratory

Mr Pierre-Yves RENARD

Professeur Pierre-Yves RENARD
Directeur du Laboratoire COBRA
UMR CNRS 6014

The doctoral student for admission

Mrs. Deniz ONAL

URN Ref: 012893

APPENDIX 1 - SCIENTIFIC PROJECT

Abstract

This research project aims to propose novel theranostic nanoparticles to improve the treatment of glioblastoma, visualize its effect on the tumor and study the activity of the treatment over time. This approach involves the use of multiple entities associated by supramolecular interactions. First, a ferromagnetic nanoparticle encapsulated by cyclodextrin-based vectoring agents functionalized by polyethylene glycol chains. This nanomaterial will generate an MRI contrast and therefore a follow-up over time. The chemotherapy part is represented by temozolomide functionalized by an adamantane chemical anchor by a hydrazone bond that can be cleaved under the action of an acid stimulus and therefore more specific to cancer cells known to be more acidic than normal cells. In addition, a fluorescent probe will be co-trapped to monitor the cellular uptake of magnetic nanoentities. The targeting of the nanobiomaterial for glioblastoma will be evaluated by antibody decoration. This project requires close collaboration between the materials chemistry, organic chemistry, biology and biomedical imaging teams.

Objectives

This research project aims to propose novel theranostic nanoparticles to improve the treatment of glioblastoma, visualize its effect on the tumor and study the activity of the treatment over time. This approach involves the use of two entities associated by supramolecular interactions. First, a ferromagnetic nanoparticle encapsulated by cyclodextrin-based vectoring agents functionalized by polyethylene glycol chains (MNP@CD-PEG, Italian Group). This nanomaterial will generate an MRI contrast and therefore follow the fate of the NPMs. The therapeutic part is represented by temozolomide (TMZ) functionalized by an adamantane chemical anchor (ADA) by a cleavable hydrazone bond (HYD) under the action of an acidic stimulus and therefore specific to cancer cells (French Group). The host-guest inclusion complex (cyclodextrin-adamantane) (MNP@CD-PEG:ADA-PEG-HYD-TMZ) formed will be soluble in the aqueous phase. In addition, the magnetic nanoparticles will be doped with fluorescent markers (fluoroscein-isothiocyanate FITC) connected to ADA for monitoring cellular uptake *in vitro* to obtain MNP@CD-PEG:ADA-PEG-FITC). Targeting on glioblastoma cells will be evaluated with a decoration with transferrin antibody (TfR Ab). The activity of this theranostic agent will be studied *in vitro*, then *in vivo*, in particular its evolution during treatment on mice and its elimination over time (Partner France, ISTCT Laboratory, Caen).

State of the art

Glioblastoma (GBM) is a high-grade brain tumor with about 2000 new cases per year in France. Treatment combining surgery, radiotherapy (RT) and temozolomide chemotherapy (TMZ) improves survival rates, but does not prevent tumour recurrence. Temozolomide is an imidazotetrazine and the standard chemotherapy for glioblastoma. It is a water-insoluble prodrug and DNA methylating agent that is much more potent and effective than traditional drugs because it can penetrate the blood-brain barrier, leading to apoptosis. A recent Phase 3 clinical study included survival data from approximately 160 patients treated with RT alone or in combination with RT TMZ was reported and the results showed no benefit to the use of TMZ for these patients (Tesileanu et al, Clin. Cancer. 2022, DOI org/10.1158/1078-0432.CCR-21-4283). These results highlight that while TMZ is very effective *in vitro* in radiosensitizing cells to RT, its use *in vivo* is more complex. Indeed, a particularity of TMZ is the solubility in water of this molecule. This requires a more complex dosage form with the need for solubilization of DMSO, which reduces its bioavailability. In addition, only a small proportion of systemic TMZ can reach the brain. Finally, a characteristic of GBM compared to other gliomas is the presence of a very pronounced hypoxic component. One

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of the effects of hypoxia explaining its influence on drugs would be an increase in the expression of efflux pumps, which leads to the need for an almost continuous delivery of an active substance. Hypoxia is also associated with the acidification of the tumor bed.

The use of anti-transferrin antibody (TfR-Ab) improves delivery across the blood-brain barrier (BBB) and selectivity in GBM tumor cells. TfR-Ab selectively targets the transferrin receptor, which is highly distributed in the BBB and in GBM cells (Ramalho et al., Pharmaceutics 2022, DOI 10.3390/pharmaceutics14020279). Monoclonal antibodies against TfR have been extensively explored for targeted drug delivery in many applications, and various clones have been reported (Monsalve et al., Nanomedicine 2015, DOI 10.2217/nnm.15.29). The OX26 antibody caught our attention because Ashrafzadeh et al. (Monsalve et al., Nanomedicine 2015, DOI 10.2217/nnm.15.29) recently showed that liposomes adapted to OX26 were efficiently internalized in GBM cells. *In vivo biodistribution* in Wistar rats with intracranial tumors confirmed that drug accumulation by the adapted liposomes was higher than by the unmodified liposomes, resulting in a significant result in terms of inhibition of tumor growth and prolongation of animal survival.

Prof. Géraldine Gouhier from the University of Rouen Normandy (COBRA laboratory, UMR 6014 CNRS) has expertise in the chemical functionalization of cyclodextrins and the formation of host-guest inclusion complexes. Beta-cyclodextrins (DCs) are naturally occurring cyclic oligosaccharides composed of 7 glucose units connected by alpha-(1,4) bonds derived from the enzymatic breakdown of starch. They are widely used for complex molecules of interest, including anti-cancer molecules, improving their water solubility, bioavailability, and stability. TMZ is a water-insoluble prodrug and decomposes at a pH greater than 8. TMZ cannot be permanently embedded in the hydrophobic cavity of a CD. It was necessary to find a new way to functionalize the TMZ with an adamantane chemical anchor (ADA) known to form stable inclusion complexes with the beta-CD cavity (thesis defended in 2021). Under these conditions, the CD:ADA-TMZ complex becomes soluble in water. The complexes improve the solubility of the aqueous phase and bioavailability. Prof. Gouhier's team also synthesized CD-Gd-based T1 contrast agents and obtained inclusion complexes and a theranostic probe (Gouhier et al, RSC Adv 2021, DOI 10.1039/D1RA04084G, Gouhier et al, Patent WO 2019/213756, PCT/CA2019/050603). We have recently demonstrated that the structural variation of these supramolecular associations, after the release of chemotherapy, would hinder a variation in the signal. The *in vitro* and *in vivo* effects on glioblastoma of host-guest complexes (CD:ADA-TMZ, CD-Gd:ADA-TMZ) are ongoing (collaborations with Dr. H. Castel, INSERM U1245 and Dr. S. Valide, ISTCT, Cyceron, RIN Tremplin Neuroncochimie 2021).

This interdisciplinary collaboration is also possible thanks to the participation of Dr. Antonino Mazzaglia at the CNR in Italy, whose expertise is the use of cyclodextrins to vectorize chemotherapy (Mazzaglia et al, Biomacromol. 2015, DOI org/10.1021/acs.biomac.5b01082, Zagami et al, Biomacromol. 2019, DOI org/10.1021/acs.biomac.9b00306). In 2022, he published new magnetic nanoparticles of iron oxide (Fe3O4MNP) coated with amphiphilic cyclodextrins via polyethylene glycol chains (CD-PEG) used as a tool for separating biological material by magnetization (Mazzaglia et al., J Colloid Interface Sci 2022, DOI org/10.1016/j.jcis.2022.01.051). The resulting MNP@CD-PEGs are about 30-50 nm in size. Inclusion complexes with a pentapeptide conjugated adamantane guest (ADA) via a PEG spacer arm were used. Thus, this nanomagnetic assembly was based on the strong affinity between the ADA group and the CD cavity, and the interactions between the PEG arms of the same lengths as the linker, and the substituents on the macrocycle crown (MNP@CD-PEG:ADA-PEG-peptide). Dr . Mazzaglia's team will be in charge of the co-assembly of the molecular components synthesized by Gouhier's group and the decoration of TfR-Ab on magnetic nanoparticles.

Therefore, this project is a continuation of the interdisciplinary research begun with the RIN Springboard Neuroncochemistry obtained in 2021. Thanks to our new skills and this international collaboration, innovative theranostic formulations will be developed to improve the effectiveness of cancer treatments.

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Scientific Program – Methodology

Task 1. French part (PhD, 18 months): Synthesis and physicochemical characterizations of precursors 2 and 3

The objective of this project is to synthesize theranostic probes. The therapeutic agent will be temozolomide (TMZ) and the diagnostic tool will consist of iron nanoparticles (MNP), a T2 MRI contrast agent. To assemble these two properties and obtain a theranostic tool, MNPs of cyclodextrin nanoassemblies MNP@CD-PEG **1**, already published by Dr. Mazzaglia, were selected as a vector (Figure 2). To vectorize the drug, a supramolecular association will be used via the formation of a host-guest complex between the cavity of MNP@CD-PEG **1** and adamantane (ADA) immobilized at TMZ. The final water-soluble vector will increase solubility, BBB crossover, and absorption into tumor tissue by enhancing the therapeutic activity of TMZ. As the CD is decorated with PEG ligands on both sides, the bond between the active agent (TMZ) and the chemical anchor (ADA) will be a PEG with the same number of ethylene glycol units forming the new ADA-PEG-TMZ **2** guest (Figure 1). Finally, in order to control the release of TMZ on cancer cells, a cleavable hydrazone link (HYD) will be inserted on the spacer arm forming the ADA-PEG-HYD-TMZ **3** guest (Figure 1). Indeed, since the pH varies from 7.3 to 7.5 in the blood to 5.5 to 6.5 in tumor cells, it is possible to control the release of the drug specifically in cancer cells by introducing such a cleavable binding agent at the pH.

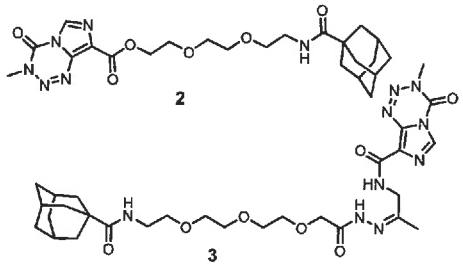

Figure 1. Guest Structures ADA-PEG-TMZ **2** and ADA-PEG-HYD-TMZ **3**

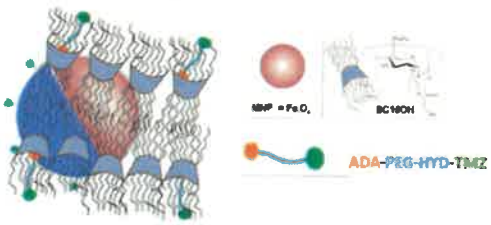

Figure 2. Theranostic probe MNP@CD-PEG **1** :ADA-PEG-HYD-TMZ **3**

The synthesis of the ADA-PEG-HYD-TMZ 3 guest will require two precursors, the functionalized TMZ **5** and the ADA-PEG-hydrazide **4** (Figure 3). The new compound **4** could be obtained from a commercial PEG aminoester carrying three units of ethylene glycol. After the protection step and amination reaction (Bricelj et al., Med Chem Lett 2021, DOI10.1021/acsmedchemlett.1C00368), adamantane carboxylic acid will be introduced, resulting in amide function (Liu et al., Nature, 2017, DOI 10.1038/nature23652).To introduce the hydrazine function, a methylester function is required (César et al., J Org Chem 2017 DOI: 10.1021/acs.joc.7B01486).

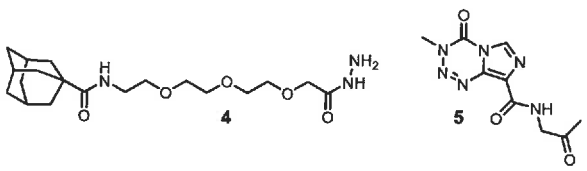

Figure 3. Structures of ADA-PEG-Hydrazide **4** and TMZ-ketone **5 Precursors**

To form the hydrazone bond, a ketone function must be introduced into the TMZ (Structure **5**, Figure 3). This functionalization has been implemented in the French laboratory (S. Sembo, PhD 2021, publication in progress). This reaction is based on an amidation reaction between the carboxylic

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acid of TMZ and 1-aminopropan-2-one catalyzed by a peptide coupling agent. Compound **5** was obtained without purification with 83% yield.
The coupling reaction between TMZ-ketone **5** and ADA-PEG-hydrazine **4**, a reaction never described, will be tested, for example, using a TBTU reagent or an acid catalyst.
In order to validate the effect of drug control release and to highlight the importance of hydrazide binding, a more stable binding agent will be synthesized. The ADA-PEG-TMZ **2** guest could be synthesized in two steps from the commercial amino-PEG (Diagram 1). In the same way, a fluorescent probe will be obtained by immobilizing the FTIC unit for *in vitro* studies.

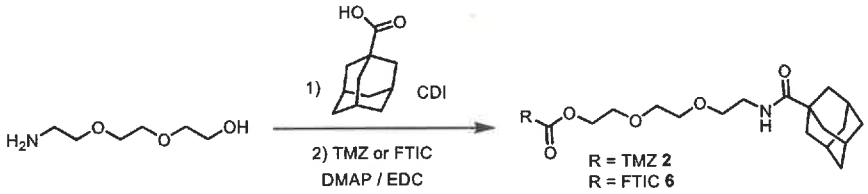

Figure 1. Proposal for a summary of the ADA-PEG-TMZ **2** and ADA-PEG-FTIC **6 invitees**

The first step will introduce the adamantan group of carboxylic acid by forming an amide bond. Then, the addition of TMZ carboxylic acid will lead to a more stable ester 2 and **6 function**. Guests **2, 3** and **6** will be analyzed by NMR and mass spectrometry. It should be noted that the sensitivity of TMZ up to pH 8 and in a strongly acidic environment is a constraint that must be overcome. The kinetic study of hydrolysis as a function of pH will be carried out at the Rouen laboratory by ^{1H} NMR. In addition, *in vivo* studies will validate the efficiency of the hydrazone's activatable function by comparison with an ester function (ISTCT Unit, Caen, see task 3). A stock will be built up for the second part of the project involving the incorporation of synthesized products into iron nanoparticles.

Task 2 Italian part (PhD, 18 months) Synthesis and physicochemical characterizations of complexes 1/2 and 1/3
The second part of this project is the synthesis of MNP@CD-PEG **1** according to a protocol published in 2022. To enhance the crossing of BBBs, the CD-PEG 1 nano-assemblies will be formed by an appropriate amount of non-ionic CD-PEG-OH mixed with CD-PEG-NH2 (the cationic amphiphilic CD analogue carrying the same PEG linker as CD-PEG-OH). The formation of two host-guest inclusion complexes between NMP@CD-PEG **1**, ADA-PEG-TMZ **2**, ADA-PEG-HYD-TMZ **3**, ADA-PEG-FITC **6** and TfR-Ab is planned. Preliminarily, the presence of inclusion complexes with **2, 3** and **6** will be confirmed by ROESY NMR on □-CD, CD-PEG-OH, CD-PEG-NH2 to confirm intermolecular interactions with the internal protons of the CD cavity. It will highlight the mechanism of binding with the cavity and the interaction between the PEG linkers. The supramolecular complexes manufactured in Italy will be analyzed by mass, TGA, XPS, DLS, TEM, AFM, FT-ATR-IR and magnetization (Mazzaglia et al., J Colloid Sci Interface 2022, DOI org/10.1016/j.jcis.2022.01.051) and steady-state and time-resolved fluorescence measurements. Using the T2 1 contrast agent, **the 1:2/3/6** complexes will be analyzed by relaxometry (Minispec mq 20) in Rouen with different host-guest ratios. An increase in r1 relaxivity is expected in the presence of inclusion complexes (Gouhier et al., Org. Biomol Chem 2017, DOI org/10.1039/C6OB02583H). In order to monitor the cellular uptake of magnetic nanoparticles, a FITC derivative 6 will also be co-trapped with an anti-cancer drug. Targeting on the GBM tumor will be achieved by the decoration of magnetic nanoparticles with an anti-transferrin antibody by reaction coupling with CD-PEG. The best conditions for complexation must be reached and the skills of both teams will be useful. Stability studies on biologically relevant media (H2O, 0.9% NaCl and PBS at pH 7:4) will be performed in solution and solid form after freeze-drying and storage at 4°C, 25°C and -20°C. Similarly, the kinetic study of TMZ release should be performed in the

presence of different pH (5.5-6-7). In addition, loading TMZ guests into NPMs must be optimal to reduce the volume of injection to mice and deliver the greatest amount of active drug to cancer sites.

Task 3 *In vitro* and *in vivo* studies ISTCT, Cyceron, Samuel Valide unit, funding obtained thanks to the Cancéropôle Grand Ouest 20 Keuros

The **1:2, 1:3, 1:6** complexes and precursors will be sent to the University of Caen for evaluation and comparison with free TMZ on two GBM cell lines. This project, submitted in its preliminary form, was selected by the Cancéropôle Grand Ouest in 2023 (€20K for biological tests) in collaboration with the three partners (Mazzaglia-Gouhier-Valide). Valide has extensive experience using these cell lines to study the effects of various therapies. The 2 human cell lines U251-MG and U87-MG will be used in parallel as they represent large molecular panels of GBM and also have a different metabolism and therefore small differences in terms of the acidic microenvironment. The team also has nanoparticle incorporation capabilities as recently demonstrated (Hélaine et al., Colloids Surf Biointerfaces 2022, DOI org/10.1016/j.colsurfb.2022.112732). As a first step, the cell penetration of the two **1:2/3/6** complexes will be evaluated by ICP-MS (COBRA Laboratory) by quantifying the iron in the cells and determine the time required for the complex to enter the tumor cells. Thanks to the presence of FITC, we will also monitor intracellular uptake using a 1:6 complex time-lapse microscope. We will measure the cytotoxic LC50 activity of **the 1:2** and **1:3** complexes at different concentrations by WST-1 assays and violet crystal in normoxia and hypoxia. Based on the results of the *in vitro studies*, a model will be selected. After verification of tumor growth by MRI imaging (Bruker 7T BioSpec, GIP Cyceron), the complexes will be injected i.v. into mice and then an MRI will be performed early (2 hours after injection) and at 24 hours to ensure that the nanoparticles are well integrated into the tumor by taking advantage of the EPR effect (Anfray et al, Biomaterials 2020, DOI 10.1016/j.biomaterials.2020.120249) in T2* imaging. Alternatively, the complex could also be monitored *in vivo* by fluorescence using the FITC fraction using the IVIS system (GIP Cyceron) and on brain sections at the end of the experiments using the fluorescence microscopes present on site. Following this brain distribution study, a therapeutic efficacy study will be conducted. A potential barrier would be too little penetration of complexes into the tumor. However, previous work at ISTCT has already shown significant accumulations of NP after intravenous injection. If so, an intratumoral implantation strategy could be deployed.

Conclusion-Perspectives

This funding will synergize multidisciplinary skills to obtain new theranostic probes to visualize the controlled release of TMZ in glioblastoma cells by T2 MRI. The activity of this new tool will first be studied *in vitro*, then *in vivo* to monitor its evolution during treatment on mice and its elimination over time. The comparison with the effect of TMZ alone and without a cleavable linker will allow us to validate the strategy. This project requires close collaboration between the teams of materials chemistry, organic chemistry, biology (specialized in cancer) and biomedical imaging.

Added value of collaboration

This new collaboration between the universities of Rouen and Messina will validate the *in vivo intracellular* feasibility of this project with a high valorization impact. The three teams have complementary skills. This partnership with this impressive Italian team expert in the fields of chemistry to biology will be an asset for the French team. This project is also an opportunity for the Italian group to broaden its field of research in MRI imaging.

Expected consequences

Our scientific approach aims to bring together multiple and complementary skills in biology, imaging, materials chemistry, organic and bio-organic chemistry. This synergy based on syntheses and analyses mastered by the partners will make it possible to carry out this project.

Our objective is to demonstrate a better efficacy of TMZ by improving its formulation and therefore its bioavailability and by gradually releasing it, thus limiting its too rapid elimination.

The program will make it possible to obtain new theranostic complexes, and to study their effects in vitro according to different parameters such as hypoxia and finally *in vivo effects*.

URN Ref: 012893

The combination of the theranostic agent MNP@CD-PEG:ADA-PEG-HYD-TMZ **1:3** and radiotherapy could bring real added value to the project as this RT/TMZ combination is the gold standard for the treatment of GBM.

Actions planned as part of the dissemination of Scientific, Technical and Industrial Culture (STIC) in addition to the actions of the Science Festival (1 page maximum): Scientific results and expected scientific productions

- Publications in journals with a high impact factor
- Papers and poster at the symposium
-Conference
-Website
- Answering an international call

Researchers are committed to disseminating technological advances during regional scientific days:
- Regional conferences (Norman Biomedical Research Day, Northwest Cancer Day, OncoNeurotox Network Day, Norman Biomedical Research Day, Norman Cancer League Day, etc.)
- Public days (Brain Awareness Week, Open Days, etc.)

Géraldine Gouhier is also a project manager at the Carnot i2C. It is the contact with companies and establishes new contracts on a daily basis to develop their research and innovation. Through this position, she was able to present the results to the pharmaceutical industries and imaging probe suppliers.

Thus, this joint thesis will give us the opportunity to respond to other calls such as the CRA or the CNIB.

Impact on Normandy

Oncology is now a priority sector in the Normandy Region, which in 2019 set up an Oncology CEI to develop new molecules and diagnostic methods to treat cancers. We will also work with the Findmed Carnot sector, a strategic research partner for French small and medium-sized companies, and the pharmavalley competitiveness cluster, Europe's leading pharmaceutical cluster supported by the Grand Ouest Cancinogen. Thanks to this industrial network and this unique research potential, the unification of our skills will allow us to acquire the European recognition that is essential for a European project consortium. In terms of excellence in scientific research and training, this project is perfectly in line with the strategic axes of the CBSB (Chemistry, Biology, Health and Well-being) regional cluster, in particular through the INC3M Normandy Institute of Chemistry and the 2 Labex SynOrg and Fer recently renewed in 2019 for 5 years. The Carnot i2C and the XL-CHEM University Research School, recently selected as part of the PIA3 EUR call for projects, will ensure future industrial partnerships and the training of future managers in the health sector. These higher value-added products and these new design methods require breakthrough innovations that will be possible thanks to this funding, which will bring international influence to Normandy's skills. The experts involved are distinguished by the excellence of their internationally recognized scientific work and by collaborative European research programs

In 2018, the global pharmaceutical market generated sales of €928 billion (+5% compared to 2017). The American market remains the largest today (45% of the world share) and France remains the

2nd largest European market behind Germany. The French pharmaceutical industry reinvests 10%/year of its turnover (€4.5 million) in the R&D of new therapeutic solutions. The global MRI medical imaging market is valued at $7 billion in 2017 with an estimate of $11.7 billion for 2025 (Bayer, GE Healthcare, Guerbet, Lantheus Medical Imaging, Bracco Diagnostics, etc.). There are 5 main suppliers of temozolomide (Temodar© or Temodal©): Merck Sl Pharme, Sun Pharma, Mayne Pharma, Schering-Plough, Cipla. In 2013, sales brought in $1 trillion for the year. The leader of the Normandy chemical industry at the national and even European level (2nd French region in pharmaceutical chemistry and 3rd for the production of medicines) makes it possible to establish legitimacy in this field. This innovative therapeutic approach via new active ingredient formulations (bioavailability, controlled and targeted release coupled with imaging) in the fields of brain cancer is innovative. The challenge of this approach can be overcome by pooling unique knowledge and know-how with the same objective, which is the health of the patient. The innovation will range from formulation and medical imaging (diagnostic and monitoring tools) via new molecular probes to *in vitro* and *in vivo* studies on small animals.

The results of this first study could be applied to other tumors for which chemoresistance remains a real problem, such as other hypoxic tumors such as bronchial cancers, breast cancers, colorectal cancers, etc. Thus, the leverage effect is considerable.

URN Ref: 012893