Project[1] Number: 765497

Project Acronym: THERACAT

Project title: Bio-orthogonal catalysis for cancer therapy

Periodic Technical Report

Part B

Period covered by the report: from 01/03/2018 to 29/02/2020

Periodic report: 1st

[1] The term 'project' used in this template equates to an 'action' in certain other Horizon 2020 documentation

1. Explanation of the work carried out by the beneficiaries and Overview of the progress

Executive summary of the work carried out and of the progress

The **THERACAT** Training Network has overall progressed as planned and according to the structure of the Annex 1 to the Grant Agreement during this first period (Months 1-24). The project started on 01/03/2018 and the network was fully set up in this period, recruiting 13 Early Stage Researchers (ESRs) and conducting training and research activities described in Annex 1. The main deviation was the delay in the incorporation of ESR3, recruited by TEVA at month 19, due to the complex bureaucratic procedures of such a big pharma company and to the fact that it was the first time that a PhD student working in an industry enrolled in a PhD program at TAU. Despite the delay, ESR3 was promptly and successfully integrated in the Network after his incorporation. The other two modifications performed after the approval of the Amendment requested to the EC were the modification of TEVA's supervisor to be Dr. Avramovitch (former supervisor left the company), and the relocation of ESR6 from IBEC to TUE due to the new double appointment of the supervisor and coordinator of the network, Dr. Albertazzi, between IBEC and TUE.

In May 2018, the kick-off meeting was held at IBEC (Barcelona, SPAIN) with the attendance of all supervisors, during which all Network Boards and Assessment Commissions (ACs) were set up. Management tools were also implemented, such as the THERACAT website (https://theracat.eu/), which is used as the main tool for external and internal communication, as well as the signature of the Consortium Agreement.

During the first year, the initial dissemination and communication actions of the network took place, with the appearance of news providing information on the project in different beneficiaries' websites and the presentation of the project approach in some international visits and communications of the supervisors. In parallel, recruitment was performed individually by each beneficiary following common guidelines established at the kick-off meeting (call for applicants, evaluation phase, interview and contract). Of the final 13 contracted ESRs, 7 are women (54%) and 6 men (46%), reaching a gender balanced distribution of ESRs. All ESRs started their contracts and research project before month 12, with the exception of ESR3 (as mentioned above) and ESR8 (delay of 10 days), and have a good basis of work so far. Moreover, all ESRs enrolled in a Doctoral Programme except ESR9 (not pursuing a PhD due to BiogelX internal regulations).

During months 12-24, ESRs continued developing their research activities and receiving local and network training activities, according to their Personal Career Development Plans. The research activities gave rise, already, to the first poster and oral presentations in conferences, and presentations in seminars (5). Moreover, the first five scientific deliverables were completed and a significant part of the work corresponding to the scientific deliverables for the next year is already developed as planned. ESRs have also been very active in outreach activities for students at different levels.

The Ethics deliverables were also submitted at the beginning of the second year, complying with the ethics requirements received by the REA, as well as the Progress Report summarising all work performed since the beginning of the project until Month 13.

Regarding Meetings and Training Events, 2 Network Meetings, the Mid-Term Check Meeting with the Project Officer and 3 Training Events were organized during months 12-24. Network Meetings were always coupled with the corresponding Training Events (at months 12 and 24) to minimise expenses and facilitate organisational tasks.

Continuing with training activities, during months 12-24 three ESRs started their secondments but had to finish before the planned end date due to the COVID-19 outbreak and associated restrictions by the corresponding local/national authorities. Finally, the Assessment Commissions monitored the progress and concerns of the ESRs every 6 months (M12-Meeting 1, M18-Virtual Assessment, M24-Meeting 2), observing that the individual projects are being properly implemented and that ESRs are satisfied with the training received.

Finally, Training Event 4 that will be held at TAU (Tel Aviv, ISRAEL) and that was originally scheduled for month 30 (August 2020), is starting to be planned. However, final dates will not be defined until the current confinement measures finish and safe and unrestricted mobility of all researchers (ESRs and supervisors) can be guaranteed.

List of Deliverables and Milestones achieved during the first two years of the project

DELIVERABLES (MGT)

D2.1. Network meeting minutes (kick-off) (IBEC)
D2.2. Consortium Agreement (IBEC)
D2.3. Supervisory Board of the Network (IBEC)
D8.1. Website completion (EDI)
D7.1. Personal Career Development Plans (IBEC)
D2.4. Network Meetings minutes (Meeting 1) (IBEC)
D2.5. Recruitment completion (TUE)
D7.2. Training events minutes (Training event 1) (TAU)
D7.3. ESRs periodic short reports and AC recommendations (M12) (IBEC)
D8.2. Periodic report on dissemination and participation in outreach activities (M12) (EDI)
D2.6. Progress Report (IBEC)
D7.4. Training events minutes (Training event 2) (TAU)
D7.5. ESRs periodic short reports and AC recommendations (M18) (IBEC)
D2.7. Network Meetings minutes (Meeting 2) (IBEC)
D7.6. Training events minutes (Training event 3) (TAU)
D7.8. Updated Personal Career Development Plans (M24) (IBEC)
D8.3. Periodic report on dissemination and participation in outreach activities (M24) (EDI)

DELIVERABLES (RTD)

D1.1. A – Requirement No. 1 (IBEC)
D1.2. NEC – Requirement No. 2 (IBEC)
D1.3. HCT – Requirement No. 3 (IBEC)
D3.1. Novel metal complexes for bio-orthogonal catalysis (GRO)
D4.1. Library of anticancer prodrugs (EDI)
D5.1. Library of targeted catalysts carriers (TUE)
D6.1. Set of mCherry-labeled orthotopic models of cancer in mice (TAU)
D6.2. Results of the biocompatibility tests for the catalysts (TAU)

MILESTONES

MS1. Guidelines for recruitment and assessment of ESRs, PCDPs, strategy for dealing with scientific misconduct (IBEC)
MS2. Assessment Commissions (IBEC)
MS3. Intranet and extranet website (EDI)
MS4. ESRs Recruitment and PCDPs (IBEC)
MS5. ESR local doctoral studies (IBEC)
MS6. Project Check (IBEC)
MS7. Synthesis of the first prodye (IBEC)
MS8. Synthesis of the first catalyst (GRO)
MS9. Synthesis of the first prodrug (EDI)
MS11. Establishments of the protocol for in vivo cancer imaging (TAG)
MS12. First catalyst supported on a nanoparticle (TUE)
MS13. Establishment of an mCherry orthotopic cancer model (TAU)
MS14. Midterm project assessment (IBEC)
MS15. Midterm dissemination and outreach activities assessment (TAU)

1.1 Objectives

The global objective of the THERACAT Network is to train a new generation of researchers on the innovative topic of bio-orthogonal catalysis for cancer therapy. With the aim of establishing new and effective approaches for cancer therapies, the THERACAT strategy is based on nano- and micro-particles bearing a catalytic unit that are delivered to the tumour site, and subsequently non-active prodrugs are administered to the patient. The prodrugs are non-toxic and therefore generate limited side effects. Only at the tumour site the catalytic particles convert the prodrugs into active anticancer compounds that generate a local and strong effect, as single catalytic species can uncage a large number of drugs.

To achieve the global objective of the project, specific objectives have been defined. Each specific objective is addressed within a work package, each containing a set of tasks. The specific objectives addressed by the project and a summary of the work carried out during this first reporting period are:

1. Ensure that the project meets all Ethics requirements (WP1)
    - The ethical issues identified by the REA were properly addressed: animal experimentation (D1.1), involvement of third countries (D1.2) and origin of human cells (D1.3)
2. Implement an efficient management and coordination of the project (WP2)
    - A gender-balanced recruitment of ESRs was achieved: 54% women – 46% men (D2.5)
    - Consortium Agreement was signed (D2.2), the different boards of the Network were organised and their functions set (D2.3), Month 13 Progress Report was submitted (D2.6)
    - 4 Meetings were organised: Kick-off (D2.1), Network Meeting 1 (D2.4), Mid-term check Meeting with the Project Officer and Network Meeting 2 (D2.7)
3. Synthesize and characterize bio-orthogonal (nano)catalysts (WP3)
    - Ruthenium and Iridium catalysts for catalytic uncaging of prodrugs were prepared and evaluated in catalysis; micellar Pd(II) systems were prepared and evaluated in catalysis (D3.1)
    - Redox responsive shell crosslinked ABA tri block copolymers were synthesized and characterized using spectroscopic techniques
    - Single chain polymeric nanoparticles loaded with Pd(II) catalysts were synthesized and characterised using spectroscopic techniques. The ability of these SCPNs to uncage pro-dyes was studied in water, PBS and DMEM medium
4. Design novel catalytically-activable prodrugs and prodyes (WP4)
    - A library of prodrugs that can be activated by bioorthogonal palladium or gold catalysis, and two PROTAC precursors that can be assembled by bioorthogonal CuAAC reactions, was developed and is accessible for the consortium (D4.1)
    - The prodye propargyl-quinone-cyanine-7, was designed and synthesized for future *in vivo* imaging studies
    - The building of a microscopy setup for the imaging of catalysts and catalytic events at the single molecule level was achieved

5. Develop effective strategies for the selective delivery of catalysts in the cancer site (WP5)
    - A library of amphiphilic polymers bearing BTA grafts, dodecyl groups and Jeffamine@1000 were prepared that may be used as nanocarriers for targeted delivery of catalysts (D5.1)
    - Ruthenium-based catalysts were conjugated with sulfonamide anchors displaying high affinity for the active site of the hCAIX. The corresponding hCAIX Ru-cofactors have been fully characterized and are ready for metathesis assays on model substrates (caged drugs and caged fluorophores) on cell cultures and in solution
    - A platform for the fluorescent imaging of catalytic nanoparticles and their properties in an in-vivo-like 3D environment was established and used to study catalysts diffusion in the extracellular space
    - 2D and 3D cell culture conditions were developed in Biogelx hydrogels to allow assessment of the delivery and effectiveness of THERACAT biorthogonal catalytic systems in a relevant cellular microenvironment mimicking breast cancer
6. Validate the antitumor strategy in vivo using imaging and animal models (WP6)
    - Several clinically relevant breast cancer models that can be used for an accurate pre-clinical evaluation of therapeutics were established (D6.1)
    - Biocompatibility of the micellar polymeric carriers synthesized and characterized by ESR2-TAU was successfully evaluated (D6.2)
    - An in-vivo cancer imaging method based on click-to-release reaction was developed
7. Provide state of the art scientific and complementary skills training and guidance to the ESRs to develop their career (WP7)
    - 3 Training Events were organised (D7.2, D7.4, D7.6) and the first secondments were implemented
    - All ESRs prepared (D7.1) and updated (D7.8) their PCDP
    - 3 Assessment Commission sessions were performed (D7.3, D7.5, D7.7)
    - All ESRs are enrolled in a Doctoral Programme except ESR9 (MS5)
8. Promote the efficient awareness of scientists and general public of THERACAT, its training potential, and results (WP8)
    - The THERACAT webpage was launched and is continuously updated (D8.1)
    - ESRs presented their work in international conferences and seminars/workshops (D8.2, D8.3)
    - ESRs participated in communication and outreach activities (D8.2, D8.3)

A more detailed description of the work carried out is given in section 1.2 below (description of the work performed per work package). For those results already reported in a submitted deliverable, we provide only a short summary and refer to the respective deliverable reports for further details.

1.2 Explanation of the work carried per WP

1.2.1 Work Package 1: Ethics requirements

Objectives: To ensure compliance with the "ethics requirements" set out in this work package.

Work carried out during 1st reporting period:

During the project negotiation, several ethical issues were identified within the "Ethics Summary Report". In particular, several aspects required specific attention: (1) Authorisations to conduct animal experiments (it is confirmed that relevant authorisations, ethical approvals and personal licenses to work with animals have been already obtained); (2) Involvement of third countries (it is confirmed that ethical standards and guidelines of H2020 are rigorously applied regardless of the country in which the research is carried out); (3) Origin of human cells/tissues (it is confirmed that the samples of human origin to be used in the project are cells that are commercially available).

Further details are given in the reports of the submitted deliverables D1.1, D1.2 and D1.3.

1.2.2 Work package 2: Management and coordination

Objectives: To ensure a smooth management of the Network, including administrative coordination, contractual and financial management, and meetings organisation.

Work carried out during 1st reporting period:

Task 2.1. Gender-balanced ESRs recruitment

Good practices and guidelines for an open, transparent, international, competitive and based on an equal opportunity policy manner recruitment were discussed and approved at the kick-off meeting by the Supervisory Board and Recruitment Committee, being available to all partners in the THERACAT intranet (see D2.1, D2.5, D2.6). The main points included in the Recruitment Plan were:

- The Recruitment Committee (members & responsibilities)
- Eligibility criteria for ESRs
- Recruitment procedure:
    - Job description
    - Measures to ensure gender balance within the recruitment procedure
    - Advertising the positions
    - Good practices for candidate selection
    - Steps to be performed for the successful candidates
- Templates for: (1) Job description; (2) Interview Assessment; (3) Minutes of the selection process

In addition, it was agreed that the recruitment would be performed individually by each beneficiary and that the person responsible for organising and implementing the recruitment at each institution would liaise closely with his/her respective Human Resources teams, so that internal rules and regulations are adhered to, whilst ensuring that the rules of this specific project are met as well.

As a minimal provision agreed between all beneficiaries at the kick-off meeting, all positions were advertised on Euraxess, on the project's website (https://theracat.eu/jobs/), and on each beneficiary's own website. In addition, some beneficiaries advertised their vacancies on specialised, national-specific or broader job search websites such as Biocat, Academic Transfer and LinkedIn, among others. The consortium received 467 applications in total for the 13 positions that have been filled. On average, THERACAT received 35.9 applications for one position and, in total, beneficiaries shortlisted 64 candidates for interviews, which represents an average of 4.9 candidates per position.

After the recruitment process, 13 ESRs were selected: 7 are women (54%) and 6 men (46%), thus reaching a gender balanced recruitment that exceeded the objective set in Annex 1 of achieving at least 40% recruitment of women. Importantly, during the recruitment and in the implementation of the project, THERACAT beneficiaries took and will continue taking all measures to implement the principles set out in the "European Charter for Researchers" and the "Code of Conduct for Recruitment" of researchers and ensure that the THERACAT researchers are aware of them.

More details on the selected ESRs are given in the THERACAT webpage (https://theracat.eu/fellows/). All selected candidates met the eligibility criteria. They were, at the time of recruitment, in the first four years of their research careers and had not been awarded a doctoral degree. They had not resided or carried out their main activity (work, studies, etc.) in the country of their host organisation for more than 12 months in the 3 years immediately before the recruitment date.

Family allowance costs were only claimed for ESR8 since she fulfils the corresponding eligibility requirement. All candidates already obtained the corresponding visas and Researcher Declarations (RD) were filled in by all beneficiaries at the Funding & Tenders Portal.

Notably, the main deviation within the first year of the ITN was the delay in the recruitment of ESR3, to be incorporated at TEVA, due to bureaucratic difficulties; ESR3 finally started his contract at Month 19 (more details in section 5).

Task 2.2. Coordination of the ETN, including scientific and financial management and reporting to EC

The Coordinator (IBEC), supported by the Project Manager from IBEC, is responsible for supervising the day-to-day management of the project, both from the scientific and financial perspectives, in accordance to the EC guidelines and regulations. The management structures of the THERACAT Network are completed by the Supervisory Board (D2.3), the Recruitment Committee, the Training Committee, the IP and Innovation Committee and the Fellows Committee. These management structures (i) made management functions clear and verifiable, (ii) facilitated and managed the interaction between the different groups in the consortium and the integration of different backgrounds from academic and industrial environments, (iii) ensured the maintenance of research integrity, and (iv) guaranteed the highest quality in the recruitment, research and training programmes, as well as in the assessment of the scientific outcomes of the project in terms of IP and innovation. A Consortium Agreement (D2.2) was signed at the beginning of the project to specify the management structure and the relationship among the partners, the decision-making procedures, and the rights and obligations of the partners concerning liability, access rights, dispute resolution and intellectual property.

The management of the network is also supported by an effective internal communication strategy, which main instrument is the intranet section of the THERACAT webpage, with

access restricted to the members of the Supervisory Board and ESRs. In the intranet, a detailed schedule of activities is continuously updated together with a repository of official documents (deliverables, meeting/training events minutes and presentations, reports, guidelines and templates, among others). This is complemented by oral presentations and discussions at the Network Meetings, and by bilateral and multilateral exchanges of information by e-mail, phone or through videoconferences (more than 300 e-mail interactions and several phone and videocalls).

Several guidelines have been prepared and distributed to all partners (MS1), including the Recruitment Plan, guidelines to prepare Personal Career Development Plans of ESRs and the Strategy for dealing with scientific misconduct. The latter is used to prevent any potential misuse of research and scientific misconduct in the framework of the project (see D2.6).

Regarding the financial management, during the kick-off meeting IBEC explained that the funding mechanism is based on unit costs (1 unit = 1 month of eligible ESR), and detailed both the costs for recruited ESRs and institutional costs.

- Costs for recruited ESRs:
  - A1. Living allowance: Monthly salary for the fellow before any deductions. Monthly rate (3110€) is affected by country correction coefficient. We cannot pay less to the candidates (we can pay more). Progressive salary and/or 13-14 month pay regime is accepted.
  - A2. Mobility allowance: For all recruited fellows (600€ month) to cover private costs, not professional costs (e.g. secondments). This cost is usually taxed but depends on national taxation rules.
  - A3. Family allowance: For recruited fellows who have family at the time of recruitment (i.e. being officially married equivalent (or equivalent) and/or having children; 500€ month). Evidences are needed. Family allowance units need to be returned to EC in case fellows do not fulfill this requirement.
- Institutional costs:
  - B1. Research, training and networking costs (1800€ per unit cost): consumables, courses, conferences, secondments, visa, tuition fees, etc.
  - B2. Management and overheads: IBEC as coordinator retains the largest share to address the management activities. Distribution included in the Consortium Agreement.

Each beneficiary explained the salary conditions to the corresponding ESR(s) at the end of the recruitment, defining the taxes applied following the national rules as well as the possible differences (due to coefficient country factor) with other ESRs within the Network. Doing this at the very early stage avoided future problems or misunderstandings.

IBEC explained the rules regarding the financial aspects of the project. Each beneficiary is aware that records of recruitment of the fellows and the fulfilment of eligibility criteria must be kept for 5 years after last payment of the project. IBEC also informed about the project payments: pre-financing 80% (IBEC already transferred the budget received, 75%, proportional to budget agreed in the Consortium Agreement, and 5% was kept by the Commission as guarantee fund); interim payment (up to 10%); payment of the balance.

Concerning reporting and communication with the EC, IBEC monitors all requirements and informs the involved beneficiaries in advance. Apart from revising alignment of deliverables, milestones, secondments and Training Events/Meetings in every Network Meeting, Coordinator reminds the need to prepare a deliverable and/or accomplish a milestone to the corresponding leader at least 2 months before the deadline; the same applies to secondments

(reminded 3 months in advance). In case that any deviation is identified, Coordinator informs the Project Officer by means of a Formal Notification.

Task 2.3. Network meetings organisation

Network Meetings are used as the main tool to monitor the progress of the Network as a whole. To this end, yearly Network Meetings have been organised in which each partner reported in front of the other partners on the recruitment of fellows, research conducted, and training actions performed for a one-year period. The following Network Meetings have been done during Months 1-24:

- Kick-off meeting (1 day) was held at IBEC (Barcelona, SPAIN) – May 2018 (Month 3, D2.1), with the attendance of all supervisors, the Coordinator and the Project Manager. During the meeting, several aspects were reviewed and/or agreed, such as (1) the scope of the project (research and training), (2) presentation of beneficiaries, (3) relevant management information (boards, meetings, THERACAT website), (4) recruitment aspects, (5) training actions to be launched, (6) dissemination and communication actions, (7) financial and reporting information, (8) deliverables and milestones alignment, among others.
- Network Meeting 1 (1 day) was held at TUE (Eindhoven, THE NETHERLANDS) – March 2019 (Month 13, D2.4), coupled with Training Event 1 (4 days), with the attendance of all ESRs, supervisors (or representatives), the Coordinator and the Project Manager. In the Meeting, each PI presented his/her institution and each ESR introduced him/herself. The Project Manager presented an overview of the project management issues and management actions performed so far, addressing aspects related to recruitment, training, network boards, deliverables, milestones, dissemination and communication, and financial reporting. Moreover, the meetings and training events for the second year were scheduled, and the Fellows Committee was set up. The whole consortium aligned towards the implementation of the secondments and the plan of deliverables and milestones for the second year. Finally, the first meeting of the Assessment Commissions with the corresponding ESRs took place (D7.3).
- Mid-Term Check Meeting with the Project Officer Ms. Cristina Gómez Abad (1 day) was held at EDI (Edinburgh, UK) – June 2019 (Month 16, associated Progress Report D2.6) with the attendance of all ESRs, supervisors or representatives of all beneficiaries and partner organisations, the Coordinator, and the Project Manager. The meeting started with a brief presentation of each participant and then the Project Officer performed a presentation on the monitoring of project implementation, reporting and purpose of the MTC Meeting. Afterwards the Project Manager presented the Coordinator's report summarising all actions performed so far. Finally, each ESR presented herself/himself and the restricted session between the Project Officer and the fellows took place, followed by the final feedback given from the Project Officer to the whole consortium.
- Network Meeting 2 (1 day) was held at EDI (Edinburgh, UK) – February 2020 (Month 24, D2.7), coupled with Training Event 3 (4 days), with the attendance of all ESRs, supervisors (or representatives) and the Coordinator (Project Manager could not attend it as she was hospitalized due to an accident she had the day before the meeting). During the Meeting, the Coordinator presented a review of all management actions performed so far, carefully revising/planning future activities to be conducted within the third year (meetings, training events, secondments, alignment of deliverables and milestones, midterm report). Then, each ESR presented the work

performed so far and progress of his/her individual research project, which was discussed with the Network members, and finally the Assessment Commissions met with the corresponding ESRs (D7.7).

The organisation of the Meetings has been responsibility of the host institution supported by the Coordinator and the Project Manager. The dates and organizers of the Network Meetings 1 and 2 were chosen to coincide with the Training Events described in Section 1.2.7 to minimise travel expenses and organisation tasks.

Minutes of all Meetings were prepared by the Project Manager (IBEC) and sent to the beneficiaries with fifteen days for approval.

1.2.3 Work package 3: Catalysts synthesis

Objectives: 1. To synthesize metal complexes for bio-orthogonal catalysis; 2. To synthesize and formulate nano/micro particles loaded with catalysts; 3. To characterize (nano)catalysts structure and activity.

Work carried out during 1st reporting period:

Task 3.1. Synthesis of palladium and ruthenium complexes for dye/drug uncaging

ESR1-GRO: The aim of this project is developing transition metal complexes (with a particular focus on Ruthenium and Iridium) of pyridine and polypyridine ligands for catalytic uncaging of antitumor drugs in cancer cells. Metal complexes of polypyridyl ligands such as phenanthroline, terpyridine, TPA and N4Py are of interest because of their broad catalytic scope and, as recently shown in our group, are efficiently taken up by cancer cells. We want to investigate the activity of these complexes in uncaging of prodrugs, first in model reactions and then in vitro. When required, the complexes will be incorporated in delivery vehicles such as single chain polymer nanoparticles, micelles and lipidic nanoparticles developed in WP3.

Initially, we chose to focus on the N4Py ligand. It is a pentadentate ligand which consist of four pyridyl group that are connected to a central nitrogen atom. In our laboratory were already present four N4Py complexes: $[(N4Py)Fe(CH_3CN)](ClO_4)$, $[(N4Py)Mn(CH_3CN)](ClO_4)x2H_2O$, $[(N4Py)Cu(CH_3CN)](ClO_4)$ x H_2O, $[(N4Py)Fe(CH_3CN)](ClO_4)x2H_2O$, and we synthesized two Ruthenium(II) complexes. The synthesis of the Palladium-N4Py catalyst was unsuccessful.

After the synthesis of different prodrugs (propargyl-Floxuridine, allyl-Floxuridine, benzyl-Floxuridine, 1-propargyl-5FU, 3-propargyl-5FU, 1,3-dipropargyl-5FU) and prodyes (alloc-Rhodamine110), we tested all the N4Py complexes present in our library, unfortunately without any positive result. Once the poor activity of N4Py-metal complexes in uncaging reaction was established, we decided to re-adjust the project.

We chose to split the project in two different parts:

- SUB-PROJECT #1: Here, we chose to focus on development of novel complexes for deallylation/depropargylaation reactions based on complexes that show activity in Tsuji-Trost reactions.
- SUB-PROJECT #2: This entails a new approach that could enable us to exploit the Ruthenium catalyst already synthesized.

SUB-PROJECT #1. We started by analyzing complexes used by the Meggers group (Fig. 1). They found that higher electron density at the ruthenium center is crucial for elevated activity in the uncaging of allocprotected amines. Because there is no facile way to further increase the electron density at the ruthenium by introducing new substituents at the quinoline-2-carboxylato ligand, they designed a bidentate ligand that has greater electron-donating capacity (second generation catalysts Fig. 1 C). The best performances were obtained with the use of catalyst Ru9**.**

We chose to test Ruthenium complexed with the simplest polypyridine ligand [cpRu(dpy)PPh_3], that Meggers et al. found to have a poor reactivity in deallylation reactions, and simultaneously the corresponding para-OMe complex [cpRu(*para*-OMedpy)PPh_3], hoping that the increasing of electron density on metallic center could also increase the catalytic activity.

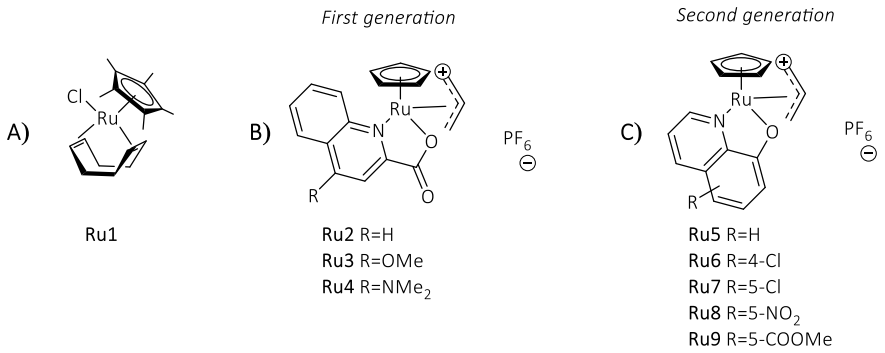

Fig. 1. Meggers catalysts

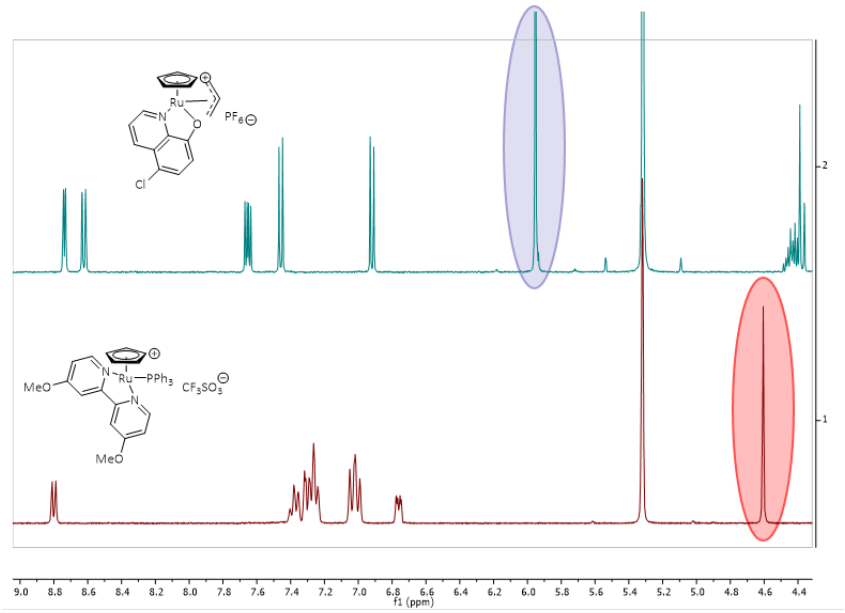

Fig. 2. Comparison between ^{1}H-NMR of Meeggers complex Ru7 and [cpRu(*para*-OMedpy)PPh_3]

Unfortunately, both the complexes were inefficient in uncaging alloc-Rhodamine110. Comparing the ^{1}H-NMR spectra of catalyst used as positive control (Meggers complex Ru7) and the ^{1}H-NMR spectra of [cpRu(*para*-OMedpy)PPh_3] (Fig. 2), and in particular taking a look at the signals deriving from cyclopentadienyl moiety (the ones circled in the figure), we can say that the electron density on the metal center in the two complexes is not similar. In the

complex [cpRu(*para*-OMedpy)PPh_3] the electron density on Ruthenium is too low, and probably for this reason is not able to catalyzed deallylations and depropargylations.
A collaboration with TU/Eindhoven and IBEC is anticipated for better understanding the intracellular position of the catalysts with super resolution microscopy. The aim of this project is better understanding Meggers second-generation catalyst behavior in cellular environment, but, for doing that, we are going to use a new to literature catalyst, that we can name Ru10 (Fig. 3 A) and the corresponding Cy5 conjugated (Fig. 3 B).

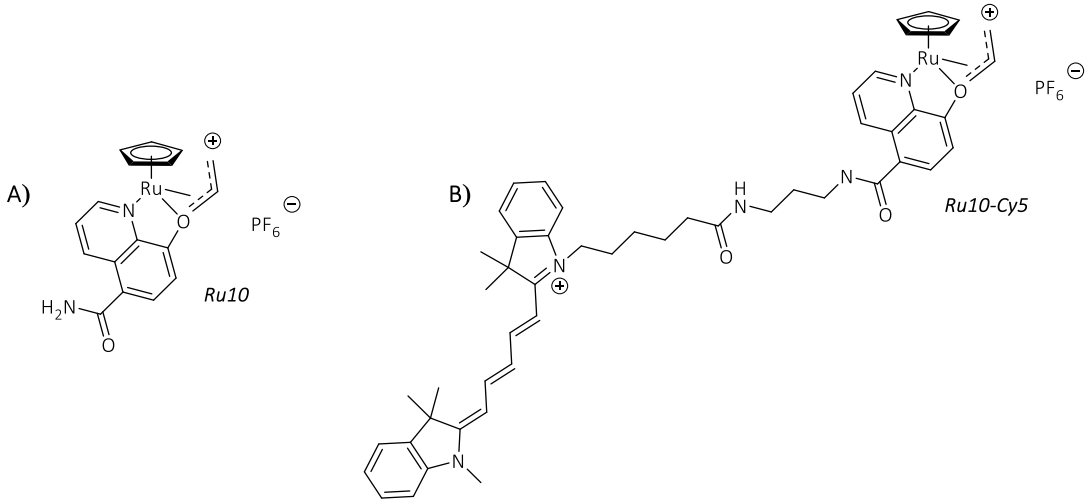

Fig. 3. A) Ru10 B) Ru10-Cy5

We chose to use Ru10 instead of Ru9 because the synthesis of the fluorescent equivalent is easier in presence of an amide instead of an ester. The activity of Ru9 and Ru10 should be very similar, this because the electron withdrawing capability of esters and amides are quite similar, as the values of Hammet costants show (σp-CONH2=0.36 σp-COOMe=0.38), thus also electron densities on metallic centers should be analogous.

SUB-PROJECT #2. [Ru(N4Py)(CH_3CN)] shows photocatalytic activity. We would like to protect the biological active group of a drug with a group that could be easily converted, in physiological conditions and under light irradiation, into a good leaving group that will abandon spontaneously the molecule, restoring the active drug. We call this approach Photo-to-Release.
Inspired by the work of Wissinger et al., we designed the reaction pathway (Fig. 4). We chose to focus on a cyclic amine because a lot of drugs present an amine as active site, and if the amine is endocyclic is not possible to protect it directly as an azide.

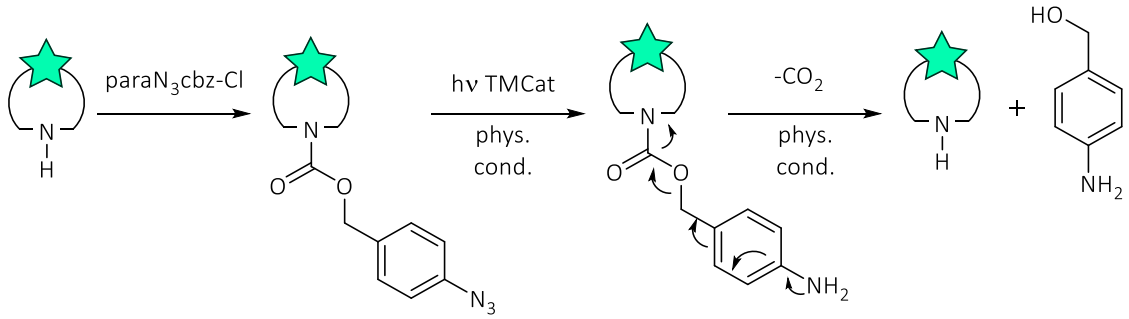

Fig. 4. Photo-to-Release approach on aminocyclic substrates

The biologically active amino group occurs will be protected with *para*-N_3Cbz. The azide will be reduced in physiological conditions by the action of transition metal catalyst and light irradiation obtaining the corresponding amine. The group obtained is a self-immolative group, that will leave spontaneously the molecule, restoring the active species. This kind of approach can be applied also to some drugs that present the active amine in para position in a benzene ring highly conjugated (Fig. 5). In fact, highly conjugated substrates protected as azide can undergo spontaneous uncaging *in vivo*. The use of *para*-N_3Cbz breaks the conjugation (due to the presence of the $–CH_2$ moiety), and we can speculate that this can help to avoid the self-uncaging in vivo.

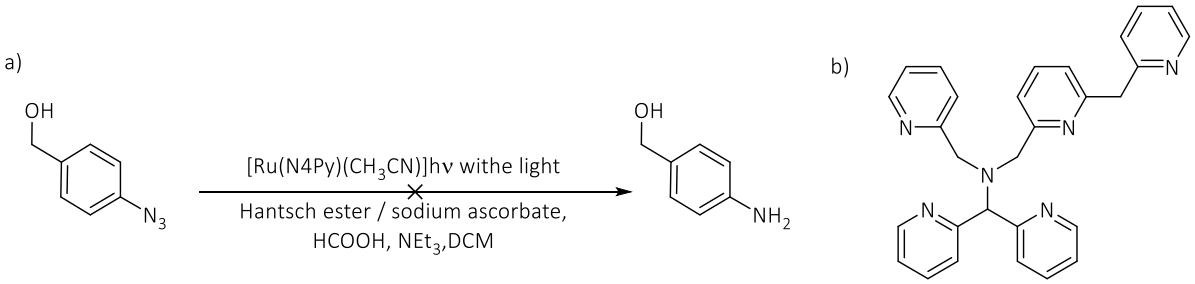

Fig. 5. a) Reduction of azides with [Ru(N4Py)(CH_3CN)]; b) structure of N5Py

First of all we tested whether this approach could be feasible with [Ru(N4Py)(CH_3CN)] as catalyst, using published procedures for azide reduction with $Ru(bpy)_3$. (Fig.6).

Unfortunately, we detected only a poor activity of N4Py complex in these reactions. Therefore, we designed a new N4Py derived ligand, named N5Py. We still need to complexate this ligand to Ruthenium and test it in light promoted azide reductions.

We tested our Photo-to-Release approach using the commercial catalyst $Ru(bpy)_3$. First, following modifications of literature proceedings, we synthesized the *para*-N_3Cbz protective group Then we protected 5FU with *para*-N_3Cbz. We carried out the uncaging reaction on this substrate (conc. 300 µM, solvent: water, catalyst: $Ru(bpy)_3$ conc. 100 µM, white light, sodium ascorbate 3000 µM), and we detected, with UPLC-MS, the presence of some uncaged product. Unfortunately, the control reaction without catalyst showed some uncaged product too. This is because 5FU protected as carbamate has some tendency to undergo self-uncaging. So, we decided to synthesize a model starting material, with same procedure as cited above, from pyrrolidine, and test it in Photo-to-Release approach (Fig. 6). The progress of the reaction is shown in Fig. 7.

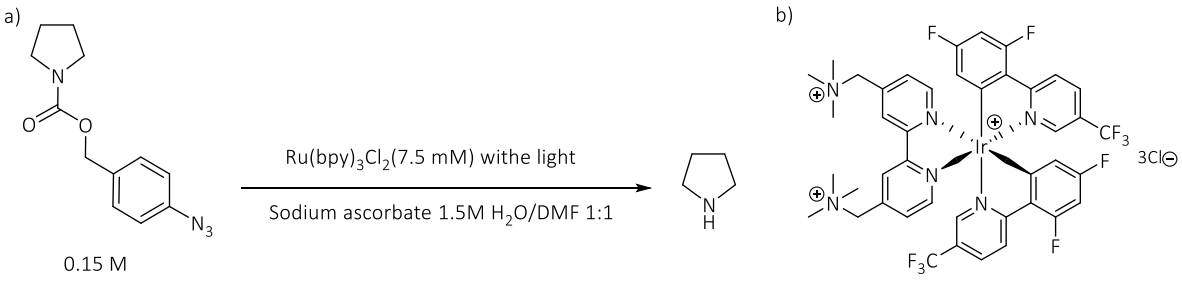

Fig. 6. a) Photo-to-Release approach on protected pyrrolidine; b) Iridium water-soluble photocatalyst

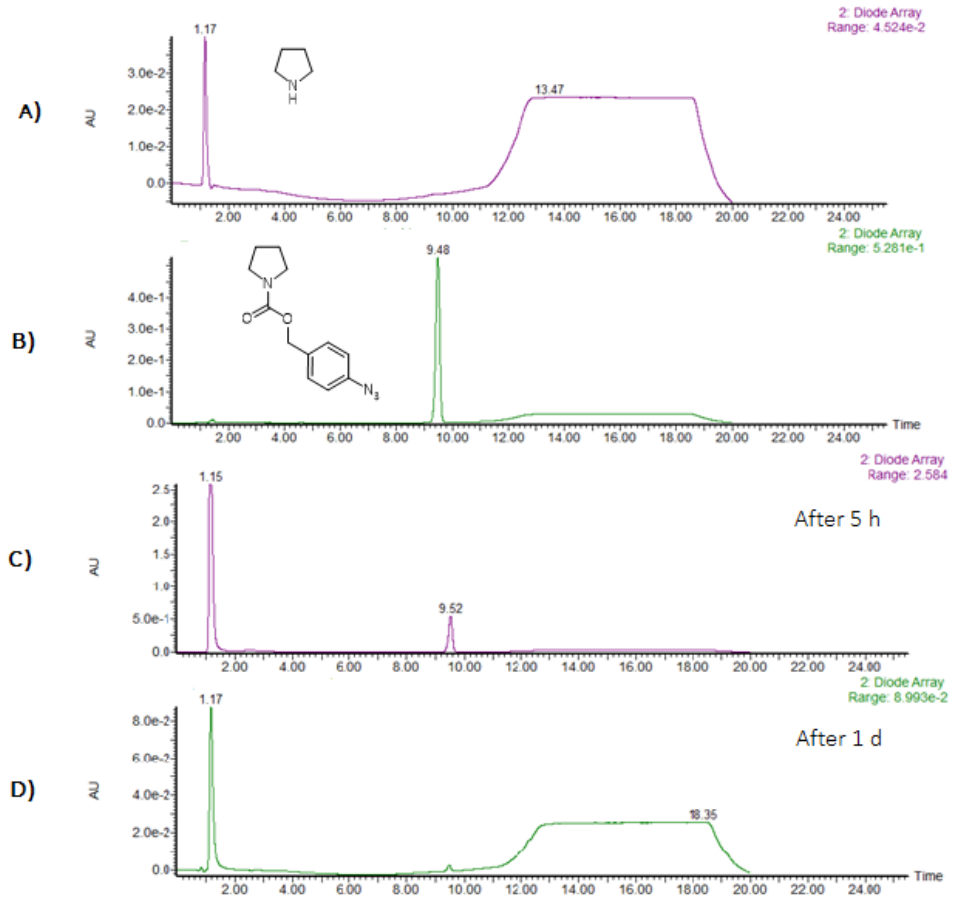

Fig. 7. UPLC traces of A) pyrrolidine B) pyrrolidine protected with *para*-N_3Cbz C) reaction mixture after 5 h D) reaction mixture after 1 d

Therefore, $Ru(bpy)_3$ seems not to work in very diluted environment, so we chose to test another catalyst, based on Iridium. In our group was designed an Iridium water-soluble photocatalyst (Fig.6b), and we tested it in the Photo-to-Release approach (substrate: 300 μM; catalyst: 100 μM; sodium ascorbare: 3000 μM). In this case we used blue led instead of white light.

The reaction goes to complete conversion in less than 15 h, and control reactions demonstrated that both catalyst and light irradiation are necessary for obtaining the product. At the moment we can talk only about conversion and not about yield, but we hope to gain this information as soon as possible.

ESR2-TAU: Polymeric amphiphiles can self-assemble to create hydrophobic domains by forming micellar structures, which can be utilized for encapsulation of a variety of small molecules. With the right modifications such hydrophobic pockets can also facilitate in carrying out organic reactions in an aqueous environment. Thus, we synthesized monofunctional PEG-dendron hybrids and covalently attached them to bipyridine and phenanthroline ligands for coordinating the Pd(II) ions. The amphiphilic polymers were used to prepare catalytic polymeric micelles.

The PEG chains formed the hydrophilic shells of the micelles while the hydrophobic segment was based on dendritic structures. The high structural precision and modularity of the

dendrons allow tuning the amphiphilicity of the polymers and their micellar stability. To attach the coordinating ligands to our amphiphiles we attached a boc-protected lysine group between the hydrophobic and hydrophilic blocks at the interface of the two domains to be formed in the micellar structures. The boc group was removed at the last step of the synthesis and the obtained free amine group was linked to the acid functionalized ligands (bipyridine and phenanthroline) to yield the structures shown below in Fig. 8.

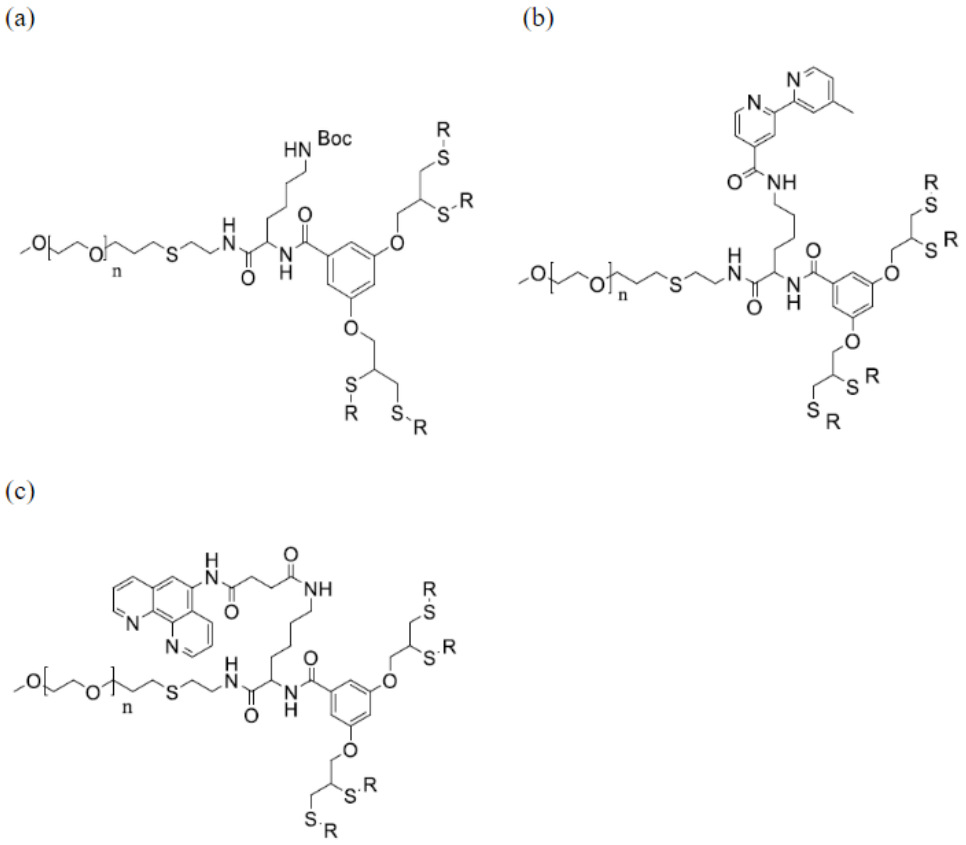

Fig. 8. Chemical structures of polymer amphiphiles (a) $mPEG_{5kDa}$-Lysine-(Boc)-D-$(Octyl)_4$, (b) $mPEG_{5kDa}$-Lysine-(Bipyridine)-D-$(Octyl)_4$ and (c) $mPEG_{5kDa}$-Lysine -(phenanthroline) -D-$(Octyl)_4$ to be used as catalytic micelles. R=C_8H_{15}.

We complexed the coordinating ligands in these amphiphiles with Pd(II) ions using $Pd(OAc)_2$ salt, and used the metal-loaded micelles to conduct the catalytic experiments in PBS. We observed that the attached ligand was not helping with rate of depropargylation as we hoped to but the metal loaded micelles on their own could essentially catalyze the reaction. This could be plausible due to the presence of 1,2-dimercapto ethers present as part of the dendrites in the amphiphile itself is sufficiently active enough to conduct depropargylation as seen in Fig. 9.

Additionally, we observed precipitation of the substrate due to its hydrophobicity in control experiments. We started the control experiment with 400 μM of the substrate and ended up with only around 250 μM at the end of it showing that precipitation of substrate was a big issue and this could also be the cause of the yield being around 60%. Based on these results, subsequent experiments will be carried out at lower substrate concentrations and the hydrophobicity of the micelles will be tuned to study the effect of the hydrophobicity of the amphiphile on the self-assembly of the micelles (size, critical micelle concentration) and their catalytic activity in the depropargylation model reaction.

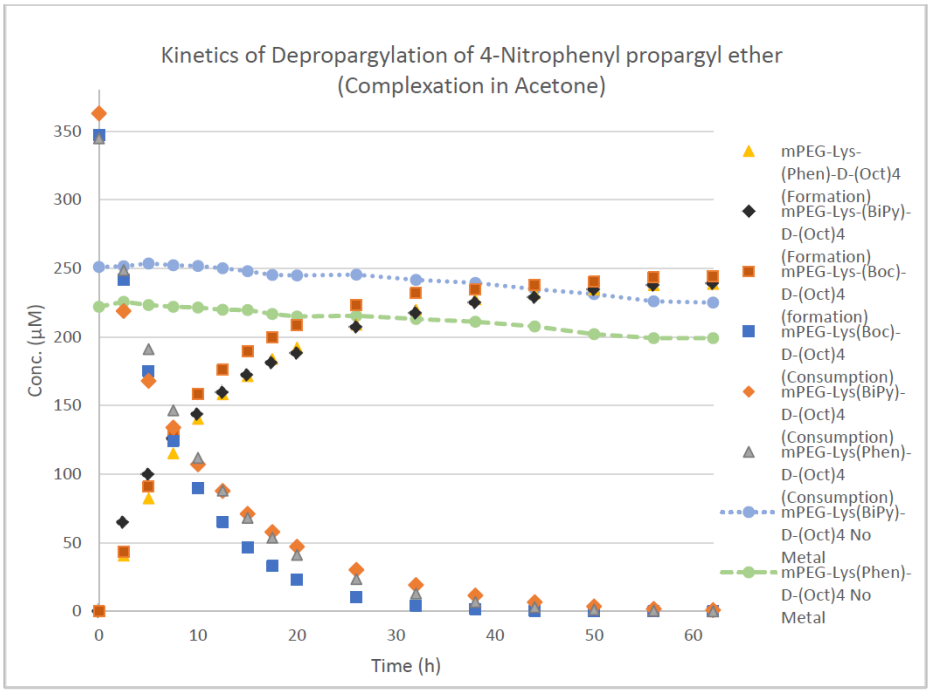

Fig. 9. Reaction kinetics of depropargylation of 4-Nitrophenyl propargyl ether by catalytic micelles complexed with $Pd(OAc)_2$.

We labelled two of our polymers, $mPEG_{5kDa}$-Lys-(Dye)-D-$(Hexyl)_4$ & $mPEG_{5kDa}$-Lys-(Dye)-D-$(Dodecyl)_4$, with fluorescent dyes Cy5 and Cy3. Then we made polymer solutions containing10% Cy5-labelled polymers, 10% Cy3-labelled polymers and 80% non-labelled polymers for both sets of polymers. This mixture of polymers was selected because they form micelles that can undergo FRET (fluorescence resonance energy transfer) where the Cy3 acts as the FRET donor and the Cy5 dye acts as the FRET acceptor. We made these solutions to conduct FACS, Confocal microscopy experiments in collaboration with ESR13 (TAU) to understand how the polymers entered the cells as micelles or as individual polymers depending on whether we observe FRET due to micelles retaining their self-assembled state inside the cells or do we observe only individual polymers. The structure of the labelled polymers are shown in Fig. 10 below.

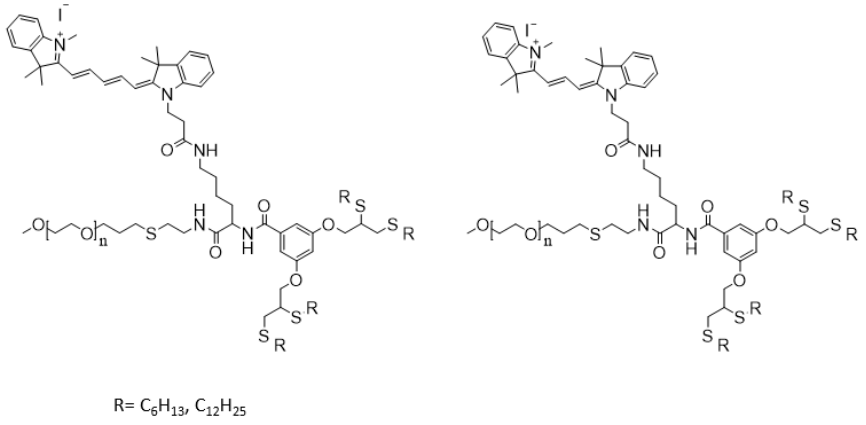

Fig. 10. PEG-dendron hybrids labelled with fluorescent dyes (a) Cy5 and (b) Cy3.

Further details on the work performed in this task are given in the report of the submitted deliverable D3.1.

Task 3.2. Formulations of catalytic polymeric nanoparticles, micelles and vesicles

ESR2-TAU: From our previous experiments, we observed that our PEG-dendron based micelles are capable of conducting depropargylation reactions in an aqueous environment such as PBS. We observed that the micelles without any coordinating ligand were sufficient enough to carry out the uncaging of 4-nitropropargyl ether. As explained above, this could be due to the presence of 1,2-dimercaptoethers. So we synthesized amphiphiles with different degrees of hydrophobicity without the lysine moieties and conducted depropagylation reaction using the 4-nitrpropargyl ether substrate we used for our previous experiments. The structures of the synthesized substrates can be seen below in Fig. 11.

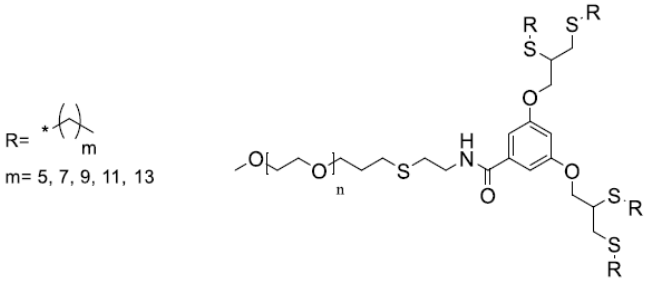

Fig. 11. Chemical structure of mPEG-D-$(R)_4$.

In total, we synthesized a series of five amphiphiles with dendrons with four end-groups based on: 1-hexanethiol, 1-octanethiol, 1-decanethiol, 1-dodecanethiol or 1-tetradecanethiol and conducted the same type of catalysis experiment. We used all the five amphiphiles to coordinate with $Pd(OAc)_2$ and conducted the catalysis experiment for around 80 hours and the starting amount of the substrate used was reduced to 160 µM instead of the 400 µM used previously to avoid precipitation of the substrate.

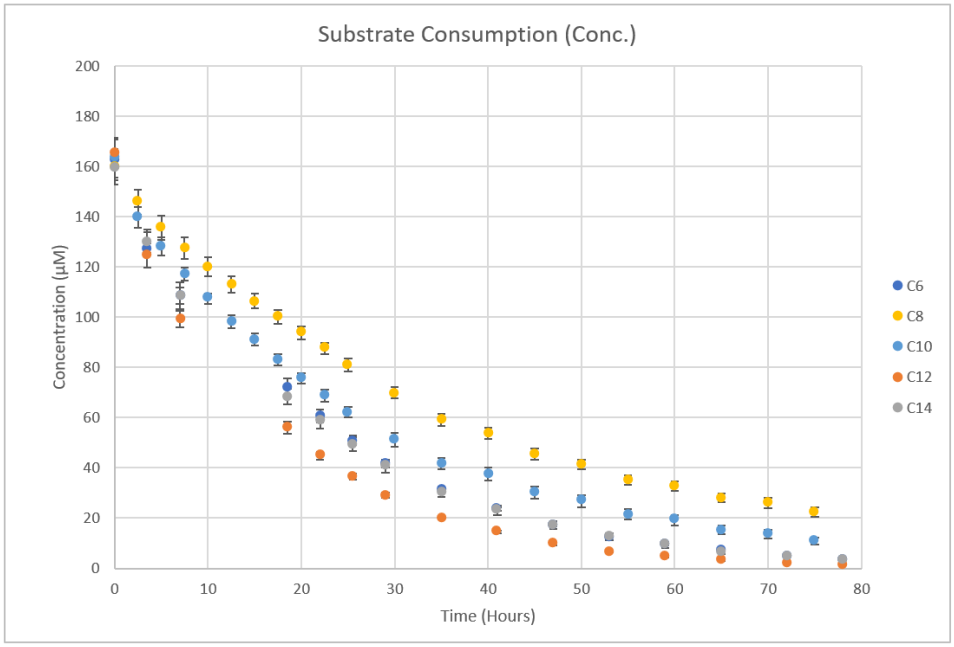

Fig. 12. Consumption of 4-nitropropargyl ether for depropargylation using different catalytic micelles

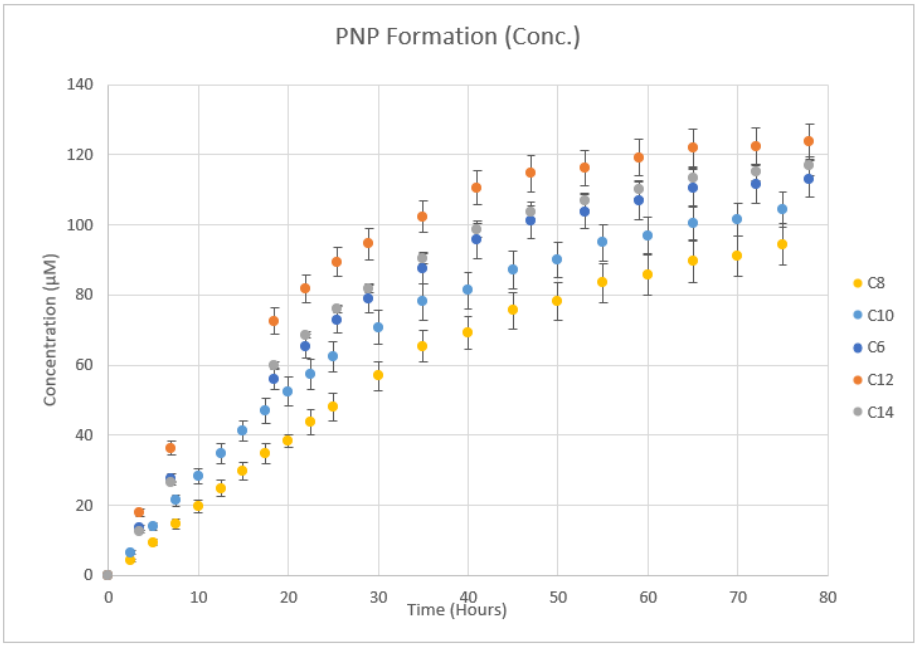

Fig. 13. Formation 4-nitrophenol during depropargylation reaction using different catalytic micelles.

We observed that the micelles made of mPEG$_{5kDa}$-D-(Dodecyl)$_4$ worked the best but the difference between the micelles with respect to their rate of depropargylation is not extremely large as observed in Figs. 12 and 13. Further experiments looking at the relative concentrations of the metal ions and micelles are currently on-going. The results obtained so far indicate that even although the micelles made of mPEG$_{5kDa}$-D-(Tetradecyl)$_4$ are more hydrophobic, it does not necessarily correlate to higher catalytic activity as mPEG$_{5kDa}$-D-(Dodecyl)$_4$ seems to have the optimum level of hydrophobicity required to conduct depropargylation faster than the others.

ESR3-TEVA: Model formulations were initially designed with star block copolymers but due to their tendency to form gels and limited availability, hydrophilic-hydrophobic-hydrophilic triblock copolymers were chosen to be the starting material. Furthermore, triblock ABA copolymer with PEO: PPO: PEO with different ratios of the PEO to PPO are commercially available and known to have an interesting efflux pump inhibitory activity for p-glycoprotein.

Crosslinking micelles not only gives an extra layer of control over the drug delivery system by preventing premature diffusion of drug in the capillary but also an ability to make it stimuli- responsive which adds another layer of specificity. Even though crosslinking micelles has been previously reported to make micelles more stable, attempts to understand how specific changes in cross-linking architecture and their ability to selectively release drugs in redox environment have yet to be established. To elucidate this, polymers with different architectures that are redox responsive were designed for synthesis.

Pluronic 123 polymer was used to initially test this theory. Synthesis has been done. Currently on hold due to COVID-19 pandemic.

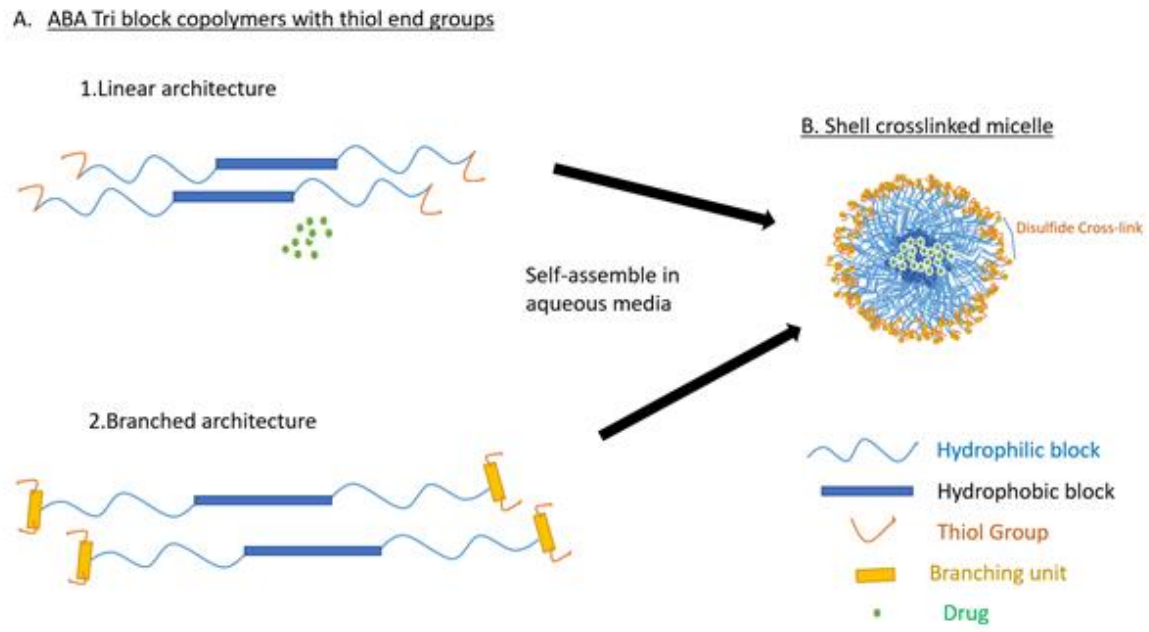

Fig. 14. Schematic representation: (A) polymers self-assembled along with the drug in aqueous media to form micelles with drug trapped inside the micellar core. (1) mono-functional crosslinkers, (2) di-functionalized crosslinkers. (B) Micelles are then crosslinked by oxygenated to from to disulfide crosslinks which increases in the stability at lower CMC and against plasma proteins.

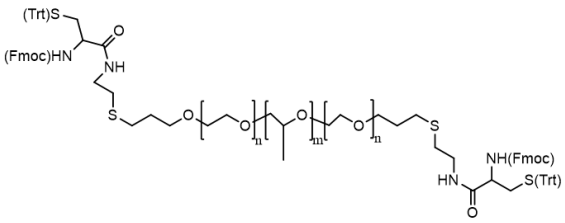

Fig. 15. Structure of protected precursor of redox responsive polymer with a single thiol on each chain end.

ESR4-TUE: Single chain polymeric nanoparticles are amphiphilic polymers that can form structured nanoparticles in solution due to the optimal balance of hydrophobic and hydrophilic groups in the polymer backbone. With the aim of developing SCPNs that can function efficiently in cells to perform prodrug activation in cancer therapy, amphiphilic polyacrylamide polymers bearing palladium binding ligands, BTA, dodecyl units and jeffamine were developed by post functionalization approach. Two polymers P1 and P2 were synthesized by functionalizing poly(pentafluorophenyl acrylate) polymer with different pendant groups with the desired ratio in a single polymer chain. Both polymers were equipped with bipyridine ligands that can bind to Pd(II) to catalyse depropargylation reactions. Polymer P1 has BTA units that can form self-assembled stacks, which will help in forming a structured interior when the polymer collapses to form nanoparticles in solution. Polymer P2 was designed without BTA units to determine the role of a structured hydrophobic cavity in catalysis. These amphiphilic polymers are soluble in water and can be formulated to SCPNs by following a reported protocol. The amphiphilic polymers were dissolved in water by sonication for 45 minutes followed by heating at 80ºC for 45 minutes and was allowed to cool and equilibrate overnight to form SCPNs.

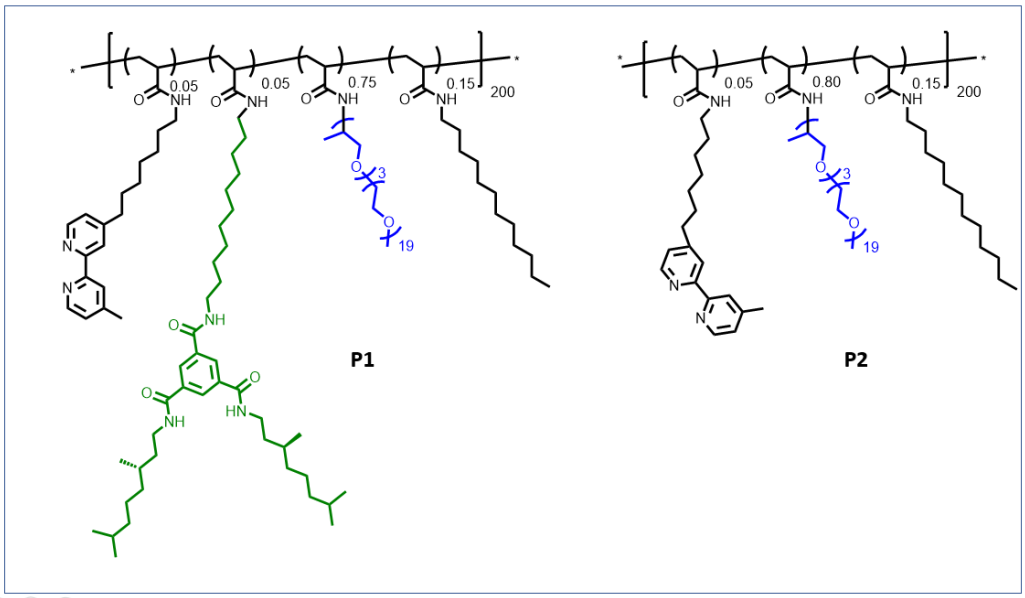

Fig. 16. Structure of amphiphilic polymers

Task 3.3. Studying of (nano)catalysts structure using spectroscopy, SAXS, fluorescence spectroscopy, light scattering

ESR3-TEVA: Dynamic light scattering (DLS) was used to measure the size of the micelles, Gel permeation chromatography (GPC) to measure the dispersity (Đ) and 1H NMR to elucidate the structure of the polymers post synthesis.

ESR4-TUE: SCPNs formed from amphiphilic polymers equipped with bipyridine ligands were characterized using spectroscopic and scattering techniques. The BTAs self assemble into helical supramolecular structures and it results in the hydrophobic collapse of amphiphilic polymer into nanoparticles in solution. The well-defined BTA stacks formed inside SCPNs was confirmed using circular dichroism spectroscopy. A negative CD effect was observed confirming the left handed helical assemblies formed by BTA grafts. The size of the SCPNs formed was determined using dynamic light scattering technique and the majority of particles had a size around 8-10 nm. After the formulation of SCPNs, they were complexed with palladium and their ability to catalyze depropargylation reactions was tested in water, PBS and DMEM medium on various propargyl protected substrates. The formation of products from pro-dyes was monitored using UV and fluorescence spectroscopy.

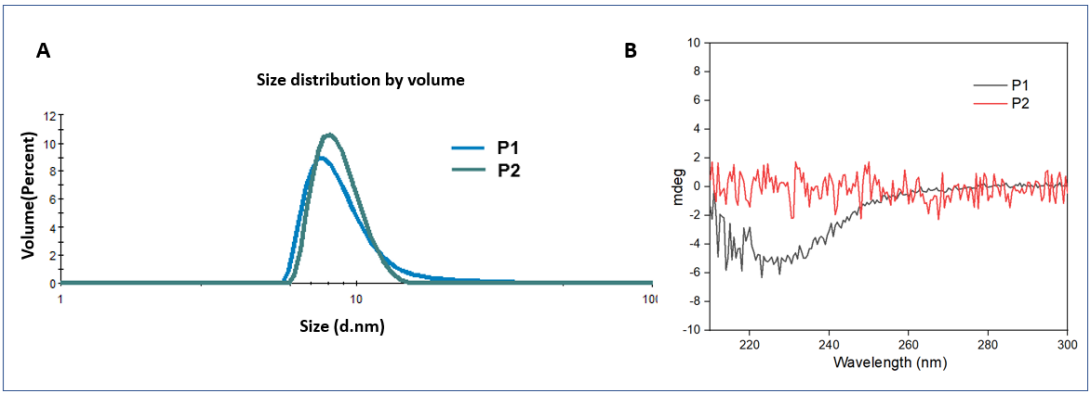

Fig. 17. A) Size of SCPNs from DLS B) CD spectra of SCPNs formed

1.2.4 Work package 4: Prodrugs design and synthesis

Objectives: 1. To synthesize prodrugs; 2. To synthesize prodyes; 3. To understand prodrug/dyes activation kinetics, stability and turnover rates.

Work carried out during 1st reporting period:

Task 4.1. Synthesis of a library of anti-cancer drugs (e.g. selumetinib and panobinostat) protected with propargyl/allyl groups

ESR5-EDI: The collection of prodrugs synthesized during this task is shown in Fig. 18. Palladium/gold-activatable prodrugs **1-7** were synthesized as previously described by EDI (30 to 500 mg available), while two new prodrugs were also prepared: **8** and **9**, which had not been published.

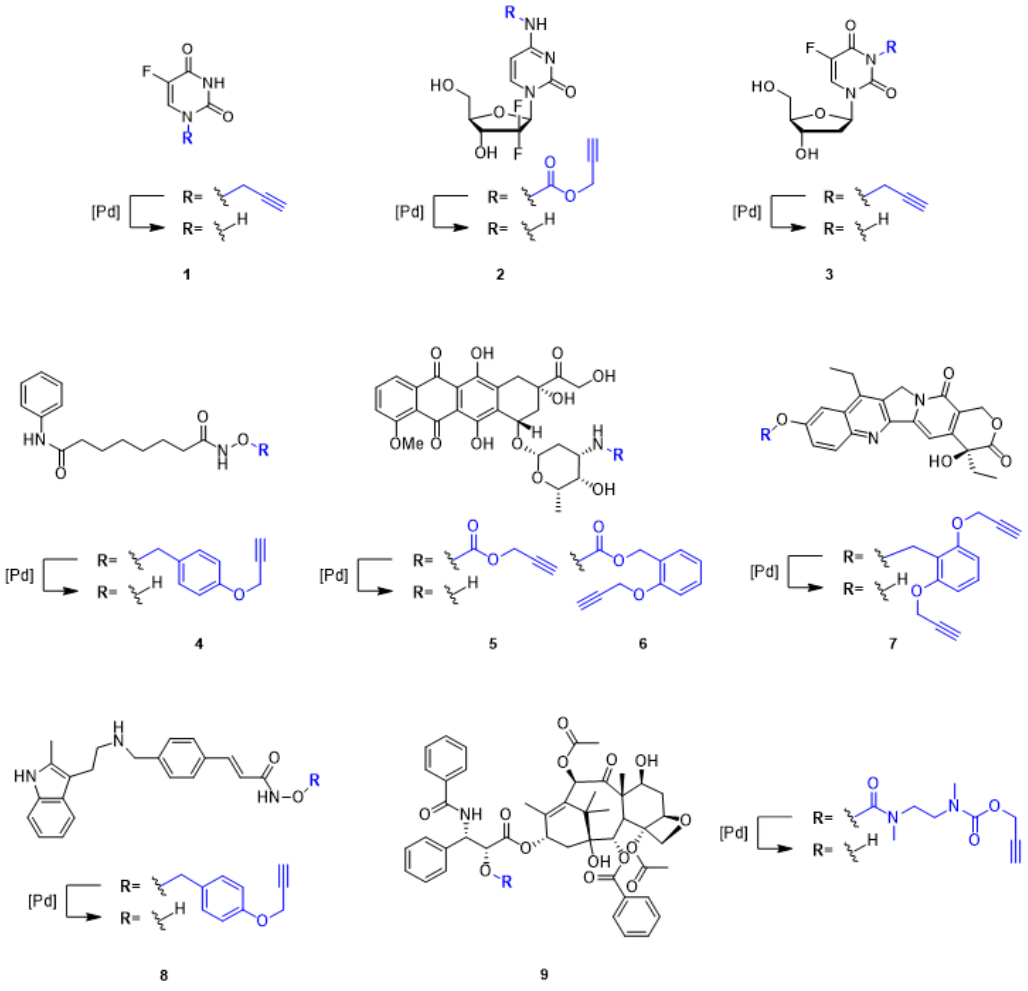

Fig. 18. Structure of the bioorthogonally-activated prodrugs synthesized in task 4.1.

Besides the synthesis of prodrugs **1-9**, the main body of research work of ESR5 have consisted on developing a new design for PROTACs (proteolysis targeting chimeras), an emerging class of cancer therapeutics with PK problems due to their large size and systemic toxicity. The novel design aims to improve PK properties by administering PROTAC precursors as two half-size components that can be assembled by a bioorthogonal copper-based catalytic device to generate the therapeutic PROTAC at the desired site. The new strategy required the synthesis of two precursor molecules functionalized with adequate

bioorthogonal tags that can be assembled into a triazole group by a Copper-catalyzed alkyne-azide cycloaddition (CuAAC) reaction (see Fig. 19): an alkyne-tagged cereblon ligand (thalidomide ligand of E3 ligase to promote ubiquitination) and azide-functionalized dasatinib (a ligand of the kinases BCR-ABL and SRC, which are highly overexpressed in many cancers including chronic myeloid leukaemia and breast cancer, respectively).

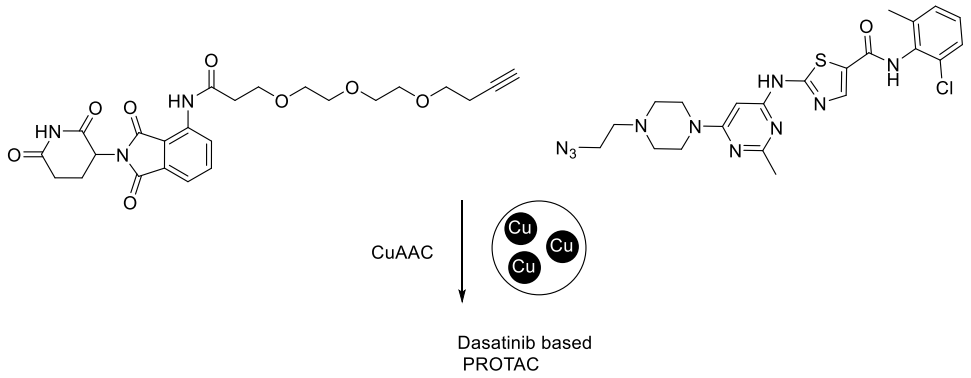

Fig. 19. Alkyne-tagged thalidomide and azide-functionalized dasatinib (PROTAC precursors) synthesized in task 4.1. Once assembled, the PROTAC will bring together E3 ligases into the proximity of dasatinib's targets, thus inducing protein degradation and treating the disease locally.

These smaller precursor molecules obey the Lipinski rule of 5 rules and thus give a PK profile that is akin to drug-like molecules. A biocompatible device containing Cu^{+} or Cu-nanoparticles will be used to catalyse an azide-alkyne cycloaddition reaction. When this catalytic device is combined with the hemi-PROTAC partners, this should result in the PROTAC being generated *in situ* at the tumour site, limiting targeted protein degradation to the tumour site. This will hopefully avoid any toxicity concerns regarding systemic protein degradation.

A PROTAC series based on the BCR/ABL and SRC kinase inhibitor, dasatinib, is being investigated as a proof of concept to determine if the new design is active for protein degradation. Initially the series, **eSKC101-3**, was synthesised *ex situ* in order to validate the most potent compound (Fig. 20).

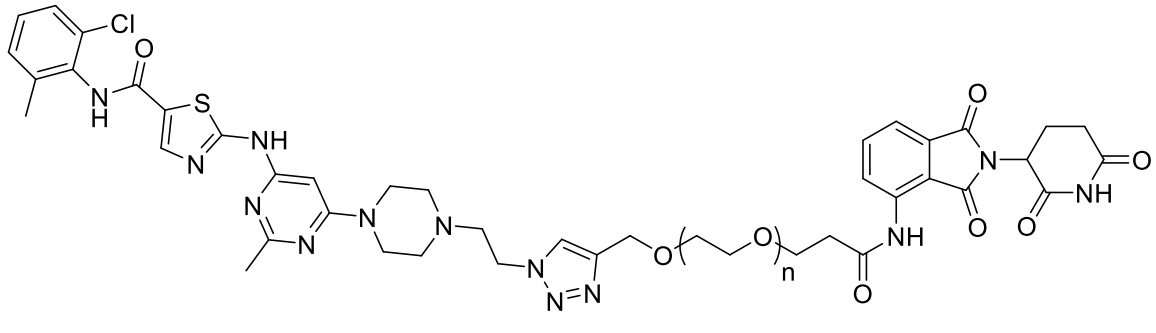

Fig. 20. Dasatinib PROTAC series.

eSKC101-3 were screened for protein degradation in breast cancer cells MDA-MB-231 and MDA-MB-436 cell lines. Each cell line were treated with eSKC101-3 for 24 h at a dose range (30−3000 nM) and compared with untreated cells (0.1% v/v DMSO), dasatinib and dasatinib azido treatment (3000 nM) for protein degradation of ABL and SRC proteins (both being dasatinib's targets). As can be seen eSKC101-3 achieve a clear decrease dose dependent decrease in ABL levels in both cell lines. A trend can be seen; as the length of linker increases the protein degradation decreases. A presto blue assay was performed to determine the antiproliferation effect of eSKC101-3 in MDA-MB-231. eSKC101 was shown to have an IC50 value of 557 nM, 414 nM for eSKC102 and 232 nM for eSCK103 in this cell line.

	Percentage of ABL protein				
	MDA-MB-231		MDA-MB-436		
	eSKC101	eSKC102	eSKC101	eSKC102	eSKC103
3000 nM	63	0	0	26	71
1000 nM	0	0	10	37	82
300 nM	7	0	12	32	84
100 nM	6	24	22	53	83
30 nM	18	74	31	75	76
DMSO	100	100	100	100	100
DAS (3000 nM)	86	66	149	182	80
DAS N_3 (3000 nM)	68	147	149	243	35

Table 1. Percentage of ABL protein levels after treatment with different concentration of compounds. The percentage of immunoblots' ABL band intensities at concentration indicated normalised to DMSO control for compound indicated in cell lines MDA-MB-231 and MDA-MD-436.

While these results are promising and show that the assembled PROTAC can induce protein, there are ongoing efforts being made to discover a SRC targeting PROTAC. A second series has been developed based on the SRC selective inhibitor, eCF506, **eSKC301-3** (Fig. 21). Similarly, the series has been synthesised *ex situ* to confirm SRC degradation. Unfortunately, the impact of COVID-19 interrupted the experiments before the results could be obtained.

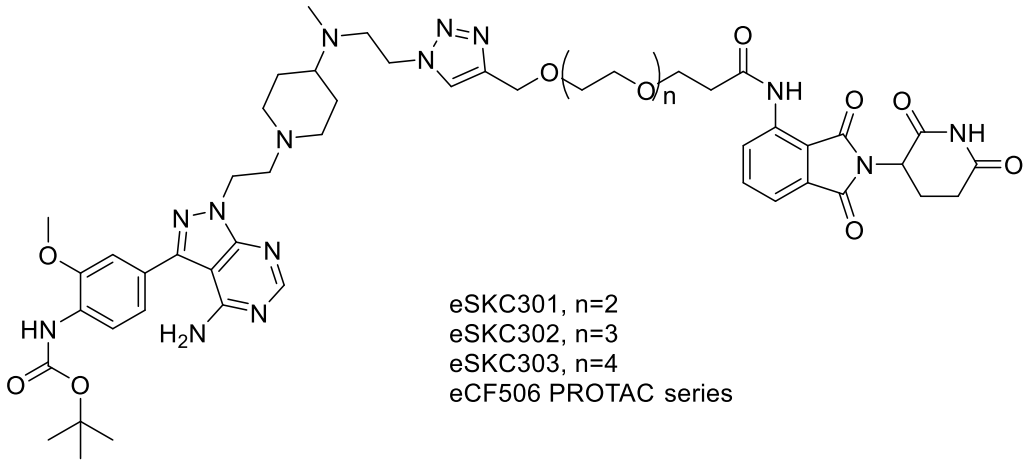

Fig. 21. eCF506 PROTAC series.

There is ongoing research being conducted to improve our current design of bioorthogonally assembled PROTAC, by incorporating additional features of bioorthogonal prodrugs. Our

group has extensive experience using metal catalyst to activate benign prodrugs at tumour sites through depropargylation. The current design we are working on prevents protein degradation occurring throughout an organism, but it must be recognised that the warhead is still capable of inhibiting the target protein throughout an organism. This represents a drawback of the current design which could be avoided by using a prodrug version of the warhead. Thus, an investigation into incorporating an eCF506 prodrug that can be activated through depropargylation and be converted into a PROTAC is being conducted.

A derivative of compound eCF506, **eSKC201**, has been synthesised and shown to be equipotent (Fig. 22). Crystal structures of eCF506 bound to SRC have revealed that the dimethylamino moiety is protonated under physiological conditions and interacts strongly with amino acids in present in SRC, contributing to its binding affinity to SRC. Therefore, **eSKC202** has been synthesised to confirm this observation. The conversion of the amino group into a carbamate is expected to result in a considerable reduction in potency, which can then be converted to its active form in the presence of a Cu catalyst. However, the impact of COVID-19 has prevented the confirmation of **eSKC202** as a prodrug of eCF506.

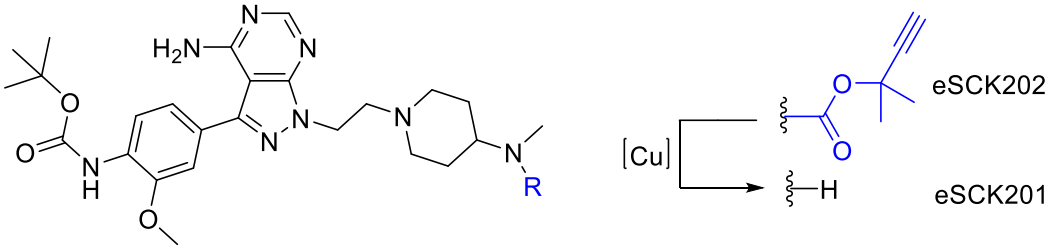

Fig. 22. Derivative of eCF506, eSCK201, and eCF596 prodrug, eSCK202.

If **eSKC202** is confirmed to be prodrug then an azido derivative shall be synthesised that enables the *in situ* activation of the warhead and conversion to an active PROTAC.

Further details on the work performed in this task are given in the report of the submitted deliverable D4.1.

Task 4.2. Synthesis of fluorescent dyes such (rhodamines, cyanines) protected with propargyl/allyl groups

ESR4-TUE: Propargyl protected fluorescent substrates 1-4 were synthesized to study the catalytic activity of SCPNs in vitro. Fluorescent substrates serve as the model compounds of prodrugs. Substrates with variable hydrophobicity help to study the trend of catalytic activity with respect to the hydrophobicity. Higher the hydrophobicity greater is the tendency of substrates to move into the hydrophobic pocket of SCPNs. Inside the hydrophobic pocket, these substrates get converted to hydrophilic products, which will move out of the hydrophobic cavity and help the catalysis to go on. Fluorescent model compounds help to study the performance of catalysts using spectroscopic and microscopic methods. The catalytic activity of the SCPNs can be monitored using fluorescence spectroscopy, while the turnover rates, stability, and heterogeneity at a single catalyst level can be studied using single-molecule fluorescence microscopy.

The catalytic activity of SCPNs was initially tested on 2,4-dinitrophenyl propargyl ether (DNPPE) 1, which on depropargylation yields 2, 4-dinitrophenol (DNP) and the reaction was monitored using UV-Vis spectroscopy at a wavelength of 400 nm. N-Proc-caged Coumarin 2 was synthesized next and was used as the fluorogenic substrate where catalysis was

monitored using fluorescence spectroscopy. The uncaged product has an excitation maximum at 370 nm and emission maximum at 440 nm when inside the SCPNs. The uncaging of 1 and 2 was successful in water and PBS but was not successful in the DMEM medium. This can be due to the low hydrophobicity which decreases the substrate accumulation in the presence of competing elements present in the DMEM medium. Propargyl protected benzothiazole derivative 3 was also synthesized however uncaging was not successful even in water. This led to the synthesis of N-proc Rhodamine 110 4 which on depropargylation yields fluorescent Rhodamine 110. The uncaging was successful in water, PBS and DMEM medium. It was found to be the best candidate owing to its higher hydrophobicity. Therefore, N-proc Rhodamine 110 4 can be used to study the performance of the catalysts in complex biological media by fluorescence spectroscopy and single-molecule fluorescence microscopy.

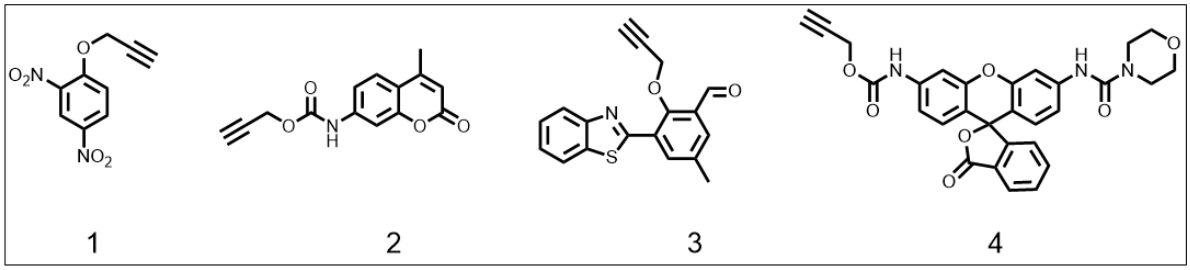

Fig. 23. Structure of pro-dyes

Task 4.3. Spectroscopic (bulk) and microscopic evaluation (single molecule) study of catalysis

ESR5-EDI: Work is expected to begin shortly on task 4.3 with the library of prodrugs and prodyes synthesised within the consortium.

ESR6-TUE: The understanding of catalysis at the single molecule level is crucial for the achievement of THERACAT goals for two main reasons: i) many catalytic nanoparticles synthesized in this consortium are intrinsically heterogeneous and therefore need to be studied individually and not in bulk to correct assess their physicochemical properties; ii) the ability to study catalysis at the single molecule level complement the bulk measurements and provides new hints on how to improve the catalysts design. To achieve this ESR6 has designed and built a single molecule spectral imaging setup. This custom-made microscope can detect at the same time the presence and the position of a single molecule with nanometric precision (spatial resolution) and at the same time its fluorescence emission spectra (spectral resolution). This allows to identify the position of individual catalysts and to study their spectroscopic features one by one. This setup is obtained by inserting in a standard super-resolution configuration a grating as a diffraction element at a short distance from the camera. This results in the separation of the zero order incoming light and the creation of a stripe due to the diffraction of the different colors of the fluorescence (first order). A typical image is presented below and shows the particles localization (spherical spots on the left) and the corresponding spectra (stripes on the right). The localization is fitted with a 2D gaussian profile to find the centroid and the spectra peak identified with an automated software. By the reconstruction of many events and many spectra an histogram can be recorded showing the spectroscopic features of two different nanoparticles.

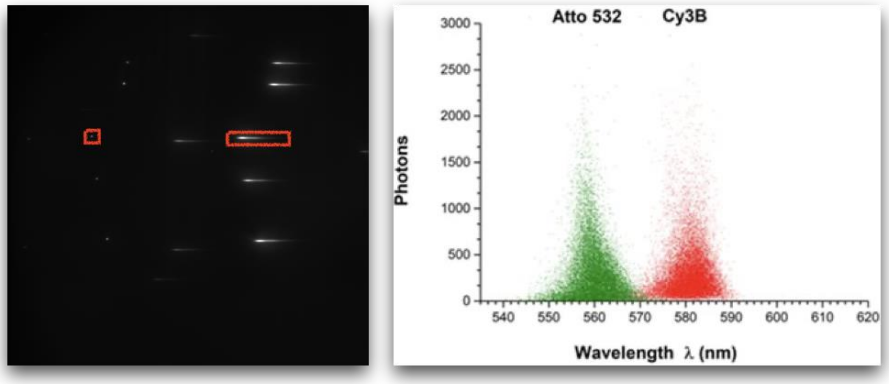

Fig. 24. (a) Typical raw data of sPAINT with the dye localization on the left and the corresponding spectra on the right (one pair highlighted in red). (b) Example of two dyes (atto532 and Cy3b) spectrally resolved. Every dot in the scatterplot corresponds to a single localization.

Furthermore, ESR6 applied this spatial and spectral resolved technique on SCPNs (labeled with covalently attached Nile red), which were synthesized for imaging by ESR8. Three different images are illustrated below. By measuring a single particle distance from the left part (spatial domain) to the right part (spectral domain) we can see - as a proof of concept, what the catalyst can "feel" inside the hydrophobic interior - a clear spectra shift of the nile red on different nanoparticles (nile red emission shifts depending on the nano environment that it belongs), something that can be denoted only by this type of measure (single particle level). Related to this, four different individual spectral histograms from four different nanoparticles, were plotted to show the proof of concept and the potential use of the spectrally resolved methodology on the characterization of the catalytic materials.

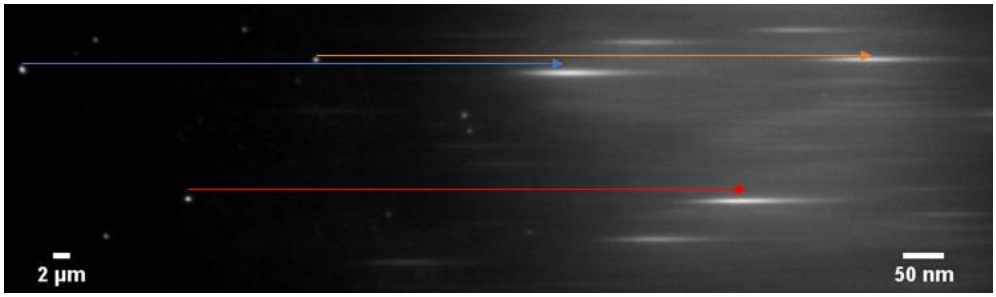

Fig. 25. Imaging of SCPN with sPAINT.

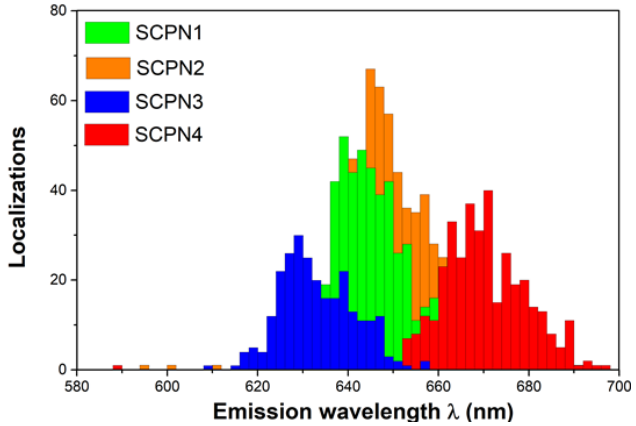

Fig. 26. Spectra of individual SCPN measured with sPAINT. The difference between the particles highlights the sample heterogeneity.

1.2.5 Work package 5: In vitro delivery and imaging

Objectives: 1. To synthesize a library of cell-targeted catalysts carriers; 2. To understand cell-material interactions with optical microscopy; 3. To screen the best-performing catalysts in *in vitro* models.

Work carried out during 1st reporting period:

Task 5.1. Synthesis catalysts carriers bearing targeting ligands

ESR8-TUE: SCPNs can create an adaptive reaction compartment to shield the catalyst from aqueous environment that allows for the catalytic reactions in the hydrophobic domain, serving as carriers to transport catalysts to the tumour site and to activate prodrugs in situ. To study SCPNs as catalyst carriers, we designed a modelling system by incorporating Nile Red into the polymer backbone to mimic the hydrophobicity of catalytic units and meanwhile study the stability of SCPNs in complex media.

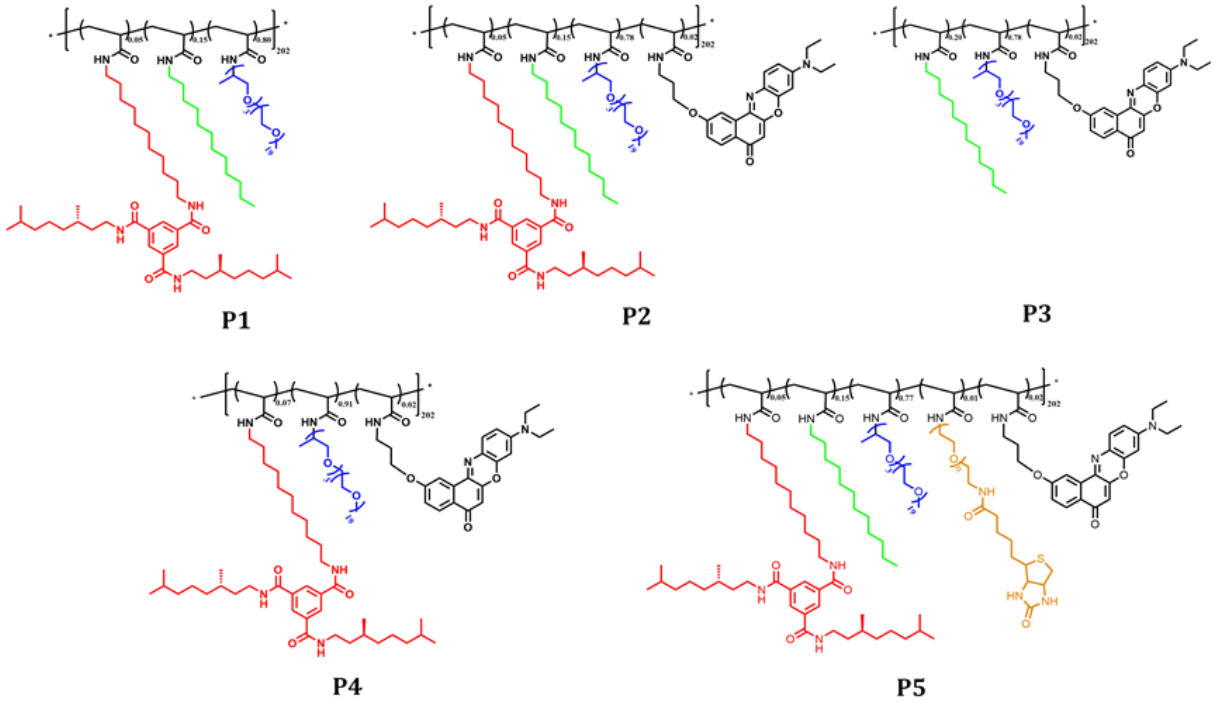

Fig. 27. Structure of polymers as catalyst carriers P1-P5

A set of amphiphilic polymers designed as catalyst carriers were synthesized (Fig. 27). P1 contains 5% benzene-1,3,5-tricarboxamide (BTA) grafts, 15% dodecyl groups and 80% Jeffamine@1000. P2-P5 all bear Nile Red as the side chain to mimic the hydrophobicity of catalytic units. P3 and P4 are either with BTA grafts or with dodecyl grafts acting as the hydrophobic part. P5 was synthesized with additional biotin to dock the particles on a glass-streptavidin substrate and therefore to study the catalytic activities of SCPNs at a single molecule level using single molecule fluorescence microscopy.

After the successful preparation of polymers P1- P5, we tested whether these polymers were able to form SCPNs in water. By performing dynamic light scattering (DLS) experiments, we obtained the hydrodynamic diameter (Dh) of polymers P1- P5 in water with a standard

protocol of sample preparation. The DLS results (Fig. 28) show that all the polymers formed nanoparticles in water with hydrodynamic diameter smaller than 12 nm.

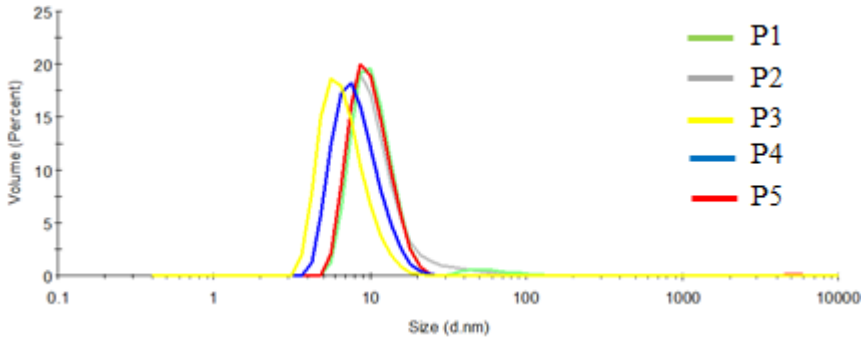

Fig. 28. P1-P5 volume distribution of the hydrodynamic diameter

To further understand the stability of these nanoparticles in complex media, fluorescence spectra of Nile Red covalently attached to the polymer were monitored. According to our previous study, P2 can form more compact and globular single-chain polymeric nanoparticles compared to other catalysts carriers that we prepared in task 5.1, so we are firstly interested in studying the stability of SCPNs formed by P2 in complex media. After forming SCPNs, we injected water, PBS buffer, DMEM buffer and bovine serum albumin solution into SCPNs to reach the final concentration of SCPNs at 2.5mg/3mL. The fluorescence intensity of SCPNs around 620 nm (Fig. 29) did not change significantly in complex media compared to that of SCPNs in water, which indicates that SCPNs in PBS buffer, DMEM buffer and bovine serum albumin solution are stable. The stability of other catalysts carriers which we synthesized in task 5.1 is still under investigation.

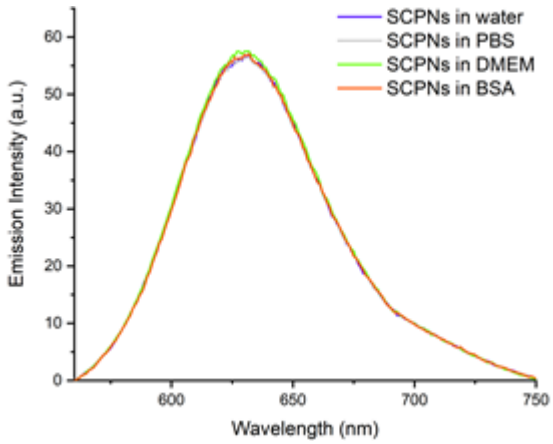

Fig. 29. Fluorescence measurement of SCPNs in complex media

Further details on the work performed in this task are given in the report of the submitted deliverable D5.1.

ESR10-BAS: Based on careful scrutiny of literature, various anchors were selected for anchoring and accumulating the Ru-cofactors on the surface of cancer cells overexpressing to hCAIX. These include: i) derivatives of fluorinated benzenesulfonamide (**1a-f**), ii) benzenesulfonamide (**2a-d**) and iii) azolamide (**3a-c**) (Fig. 30). As spacers between the cofactor/fluorophore and the sulphonamide anchor, we introduced various polar and low-polarity chains (aliphatic, aromatic and PEG-based). To evaluate the binding specificity to

hCAIX, we additionally synthesized sulphonamide-bearing derivatives equipped with a fluorescent dye (SRB) in place of the Ru-catalyst (Hov-II).

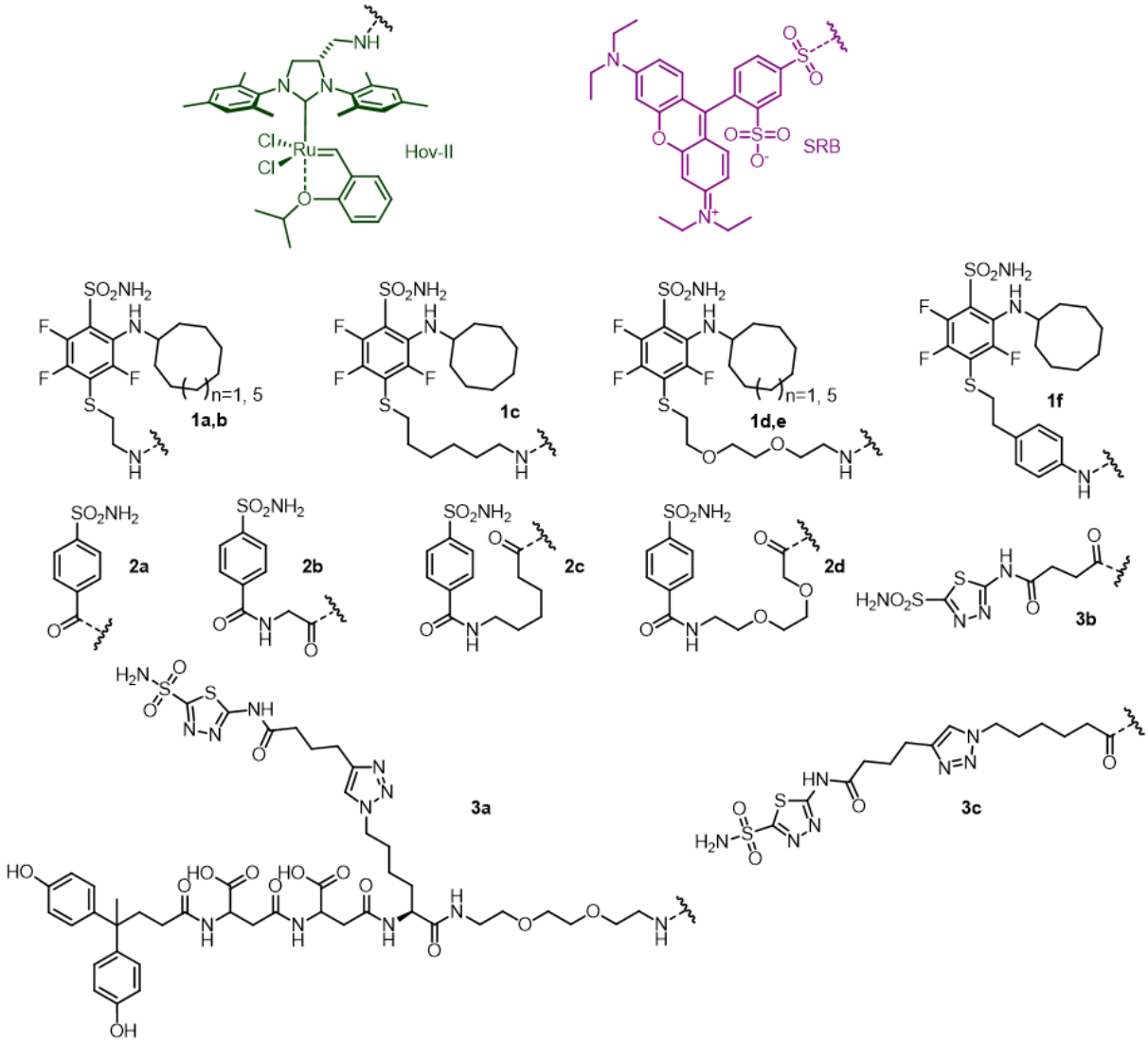

Fig. 30. Ru-cofactors and fluorescent dye bearing sulfonamide anchors to target hCAIX on the surface of cancer cells.

Task 5.2. Fluorescence and super resolution optical imaging of carriers' interactions with cancer cells

ESR7-IBEC: Understanding the interactions of catalytic nanoparticles with the biological system is crucial to design devices able to overcome the in vivo barriers towards cancer therapy. In this regard one of the key obstacles that a catalytic nanoparticle has to face is the diffusion through the thick and complex extracellular matrix (ECM). It is important to understand the structural features that allow the nanodevices to navigate through the ECM without being trapped and therefore to reach the cancer cells. ESR7 designed an ECM-on-a-chip model to study particle diffusion. The device is made by a fabricated microfluidic chip that can be filled with an artificial ECM (e.g. collagen or mixture of collagen with other fibrous proteins). The chip allows to perfuse the nanocatalysts of interest through a pump system and to image the behaviour through various types of fluorescence microscopies. For this specific scientific question ESR7 used Fluorescence Recovery after Photobleaching (FRAP).

FRAP allows to measure particle diffusion in a media evaluating both diffusion coefficient and the immobile fraction (the % of particles not able to move). The platform was first validated with dextran as standards and then two type of carriers have been tried: micelles from TAU and SCPNs from TUE. In Fig. 31 it can be seen a confocal image of the chip filled with fluorescent micelles (left, in black the pillars of the chip) and a typical FRAP recovery curve (right).

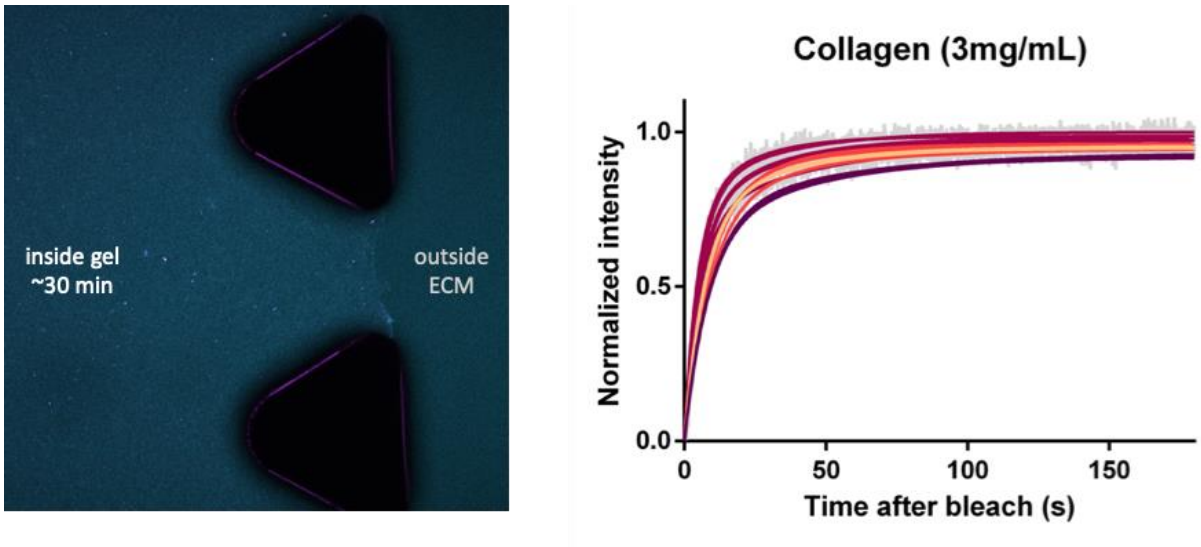

Fig. 31. ECM mimic FRAP imaging of micelles. Left: example of a field of view in the microfluidic chip. Right: recovery curves in collagen gels.

Task 5.3. Test the efficiency of prodrug conversion in 2D and 3D cancer models

ESR9-BGX: 3D in vitro cell culture platforms provide cells with an extracellular microenvironment, offering a better alternative to 2D cultures and replicating morphology, function and behaviour of cells in their natural matrix in vivo, thus bridging the gap between unrealistic in vitro 2D models and complex animal models. However, adoption of in vitro 3D systems in the drug discovery industry presents challenges in standardization and analysis. Biogelx hydrogels are synthetic, and therefore provide an in vitro 3D matrix that is simple, reproducible and can be tailored to mimic the requirements of any cell type. The stiffness of Biogelx hydrogels can be modulated and their biochemical composition allows the incorporation of biomimetic peptide sequences derived from key extracellular matrix proteins (e.g. the RGD biomimetic peptide sequence from fibronectin).

In this study, Biogelx 3D in vitro cancer models are provided to assess the delivery and effectiveness of the developed biorthogonal catalytic systems, including the efficiency of prodrug conversion at the cancer site. At present, ESR9's work has focused on optimising and characterising Biogelx's most suitable hydrogel for 3D tissue modelling in this project. This included customising parameters of unfunctionalized Biogelx hydrogels and fibronectin bioinspired hydrogels (Biogelx-RGD), and evidencing biocompatibility and correct morphology of MCF-7 cancer cells in 2D and 3D Biogelx in vitro platforms.

Hydrogel characterisation and identification of most suitable MCF7 culture conditions was done in 2D Biogelx in vitro platforms first. 0.8-1.1 kPa (low) and 3-4 kPa (medium stiffness) Biogelx-RGD hydrogels were the most appropriate to work with in these specific experiments. Evidence of cell viability, correct morphology and clustering of MCF7s initially seeded on 2D Biogelx-RGD hydrogels at early time points (3 days in culture) was provided at low to high cell densities (4 x 104, 1.6 x 105, 5 x 105 and 106 MCF7s/cm2) and low to

medium stiffness hydrogels (representative images shown in Fig. 32). The optimised 2D conditions provided us with insightful data to move into 3D in vitro experiments. MCF7s were encapsulated in 3D Biogelx-RGD bioinspired hydrogels at an analogous cell density. MCF7s seeded at low to high cell density (1.5 x 105, 5 x 105, 106 and 3.5 x 106 cells/ml) in medium stiffness Biogelx-RGD hydrogels remained viable in 3D culture for 7 days, as evidenced by the percentage reduction of the AlamarBlue reagent, an indicator of cell viability (Fig. 33A). MCF7s seeded at high cell density in low stiffness Biogelx-RGD hydrogels also remained viable in 3D culture for 7 days (Fig. 33B). Although non statistically significant, viability in medium stiffness 3D platforms seeded with a high cell density of cells was higher than in low stiffness 3D platforms.

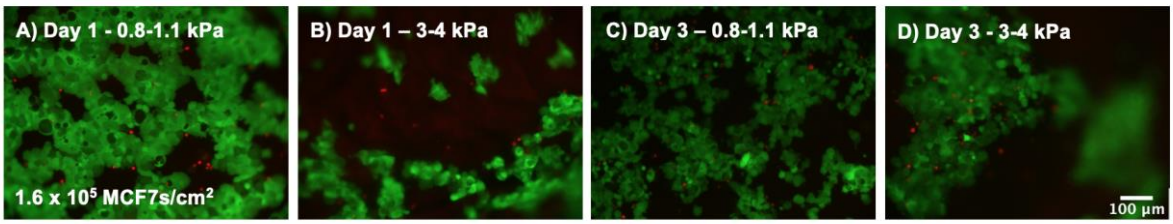

Fig. 32. Representative images of Live (green)/dead (red) stained MCF7s seeded at 1.6 x 105 cells/cm2 (medium cell density) on 0.8-1.1 kPa (low) and 3-4 kPa (medium stiffness) 2D Biogelx-RGD hydrogels. MCF7s were viable on 2D Biogelx-RGD in vitro platforms for 3 days: Day 1 (A and B) and Day 3 (C and D). Images displayed MCF7 correct morphology and clusters. Scale bar = 100 µm. N=1.

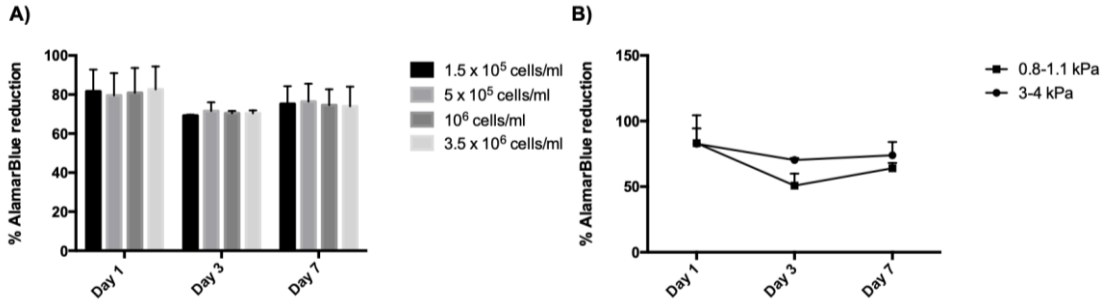

Fig. 33. Percentage of AlamarBlue reduction as an indicator of viability of MCF7s encapsulated in 0.8-1.1 kPa (low) and 3-4 kPa (medium stiffness) 3D Biogelx-RGD in vitro platforms. A) MCF7s seeded at low to high cell density (1.5 x 105, 5 x 105, 106 and 3.5 x 106 cells/ml) in 3-4 kPa Biogelx-RGD hydrogels remained viable in 3D culture for 7 days. B) MCF7s seeded at high cell density in 0.8-1.1 kPa Biogelx-RGD hydrogels remained viable in 3D culture for 7 days. Although non statistically significant, viability in 3-4 kPa 3D platforms seeded with a high cell density of cells was higher than in 0.8-1.1 kPa hydrogels. Mean + SEM; N=3.

Biogelx-RGD bioinspired hydrogels are biocompatible and could be used for the development of a 3D Biogelx-MCF7 breast cancer model for further assessment of THERACAT developed drugs. Based on noticeable variability in the presented data as well as evidence collected in the course of these experiments, the scope of future ESR9's experiments will be reduced to 5 x 105 to 106 MCF7s/ml encapsulated in 3-4 kPa Biogelx-RGD hydrogels. Live/dead and cytoskeleton imaging in 3D Biogelx-RGD in vitro models, as well as quantitative PCR experiments, will be performed next. These results will complement the present work in 3D in terms of MCF7s morphology, confluency, growth and functionality.

ESR10-BAS: Initial assays were carried out with the sulfonamide-bearing fluorophore **3a-SRB** in the presence of hypoxic and normoxic HeLa cells. The latter overexpress hCAIX and thus allows to accumulate **3a-SRB** on their surface, as evidenced by confocal microscopy (Fig. 34).

Next, we treated both the normoxic- and hypoxic HeLa cells with **2d-HovII** (50 mM). Following incubation, the cells were washed and subjected to ICP-MS analysis. In stark contrast to previous experiments carried out with a different Ru-cofactor used for deallylation, we could not observe any difference in [Ru] concentration for either hypoxic or normoxic cells. In both cases, high [Ru] concentrations were observed ($1.35 \cdot 10^9$ per hypoxic cell vs. $1.59 \cdot 10^9$ per normoxic cell). We hypothesize that the metathesis catalyst **2d-HovII** undergoes cross-metathesis with the double bonds present in the lipid bilayer constituting the mammalian cell membrane. To circumvent this challenge, we plan to treat the incubated cells with a reactive olefin to displace the lipid-entrapped **2d-HovII**. Unfortunately, we had to interrupt this work due to the COVID-19 confinement.

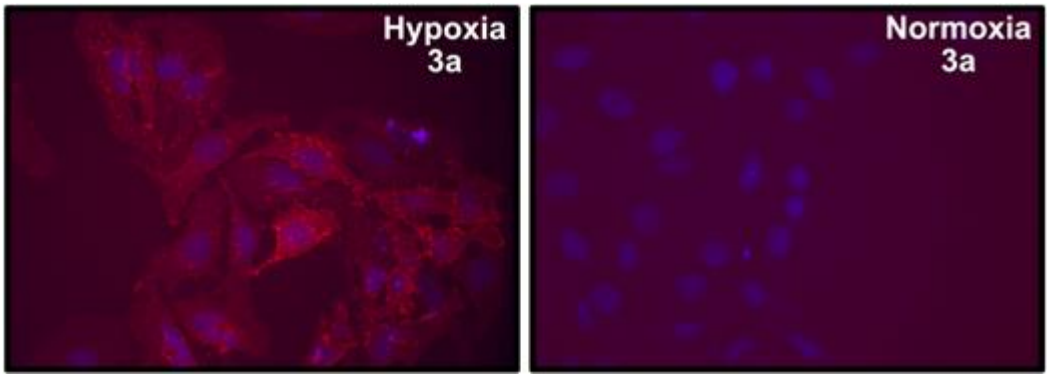

Fig. 34. Comparison of HeLA cells grown at 1% (hypoxia) and 21% oxygen (normoxia) (in PBS at pH 6.0). blue - DAPI, red - **3a-SRB**.

1.2.6 Work package 6: In vivo evaluation

Objectives: 1. To establish reliable in vivo cancer models; 2. To study toxicity and biocompatibility of the selected catalysts; 3. To image catalyst localization and efficacy in vivo; 4. To test anticancer efficacy in vivo.

Work carried out during 1st reporting period:

Task 6.1. Create mCherry fluorescent tumour in vivo models

ESR13-TAU: Patient-derived xenografts (PDX). Fresh human breast cancer tissues were obtained from The Chaim Sheba Medical Center (Tel HaShomer, Israel) in accordance with a protocol approved by the IRB committee (IRB). Tumor tissues were obtained during surgical resection, kept in cold PBS and processed within 40 min. In order to isolate tumor cells and generate cells monolayer, tumor specimens were dissected to 0.5 mm pieces, plated in 6 cm plates and cultured with 1 mL DMEM supplemented with 10% FBS, 100 U/mL Penicillin, 100 µg/mL Streptomycin and 2 mM L-glutamine. Following continuous media replacement, viable cancer cells remained attached to culture plates and kept growing in culture, while stroma and cell debris were washed. Cells were routinely tested for mycoplasma contamination with a mycoplasma detection kit (Biological Industries, Israel). All cells were grown at 37°C in 5% CO_2. Furthermore, fresh tumor tissues were used to generate patient-derived xenogtrafts by implanting small tissue fragments subcutaneously (sc) into SCID mice,

allowing them to grow and then excise them. Additionally, patient-derived samples were used to generate tumor spheres. Tumor spheres were grown in ultra-low attachment surface flasks and then implanted into SCID mice (Fig. 35A).

Generation of mCherry-infected human and murine breast cancer cell lines and mouse models. Human embryonic kidney 293T (HEK 293T) cells were cotransfected with pQC-mCherry and the compatible packaging plasmids (pMD.G.VSVG and pGag-pol.gpt). Forty-eight hours following transfection, the pQC-mCherry retroviral particles containing supernatant was collected. MDA-MB-231 and 4T1 were infected with the retroviral particles media, and 48 h following the infection, mCherry positive cells were selected by puromycin resistance. Afterwards, Nu/nu female mice were inoculated to the mammary fat pad with 1x10 6 MDA-MB-231 mCherry-labeled mammary adenocarcinoma cells. Balb/C female mice were inoculated to the mammary fat pad with 0.5x10 6 mCherry-labeled 4T1 mammary adenocarcinoma cells. Tumor growth was continuously followed by non-invasive intravital imaging systems (MaestroCri™, IVIS SpectrumCT) (Fig. 35B).

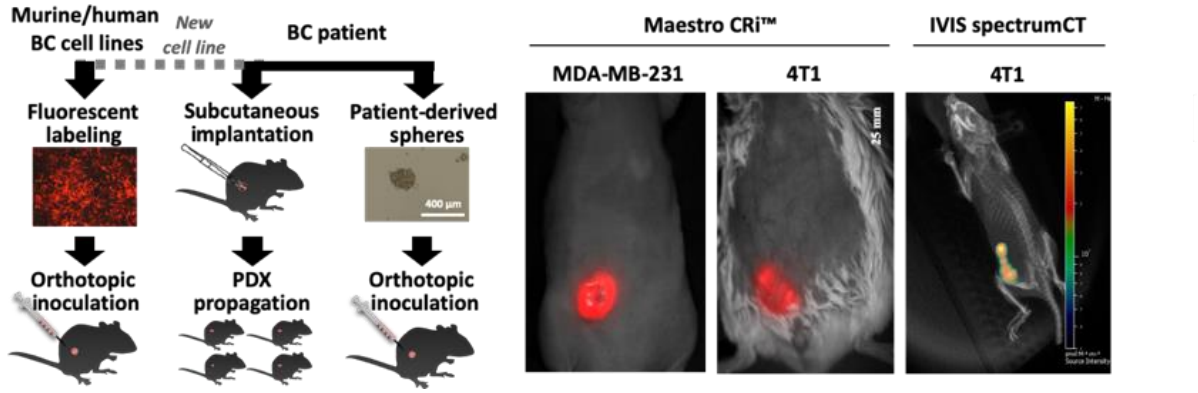

Fig. 35. Models of breast cancer (BC). (A) Human, murine and patient-derived xenograft (PDX) mouse models for BC. (B) Non-invasive imaging of mice bearing BC. Right panel: Fluorescence-imaging of mCherry-labeled MD-MB-231 and 4T1 mammary tumors, performed by Maestro CRi™; Left panel: 3D reconstruction combining fluorescence and µCT imaging of mice bearing mCherry-labeled 4T1 tumors, performed by IVIS SpectrumCT Imaging System.

Further details on the work performed in this task are given in the report of the submitted deliverable D6.1.

Task 6.2. In vivo administration of the catalysts and study of biocompatibility

ESR11-EDI: The initial steps have focused on preparing and selecting biocompatible nanoparticle catalysts (Au, Pd) and propargylated prodyes, which will be used for future *in vivo* imaging studies, during the 2nd secondment of ESR11-EDI at TAU planned in month 36. The detection and demonstration of the catalytic activity of transition-metal nanoparticles, which are able to cleave propargyl moieties, are currently being performed with the prodye propargyl-resorufin. This prodye, previously established from the Unciti-Broceta group at EDI, has been successfully used to indicate the presence of Au and Pd nanoparticles *in vitro*. Moreover, the cleavage of the propargyl moiety from the prodye is quick and visually detectable via the generation of bright pink fluorescence from resorufin, which is quantitatively measured like a standard Alamar Blue fluorescence assay.

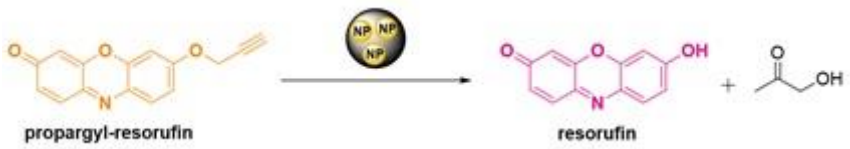

Fig. 36. Catalytic cleavage of propargyl-resorufin results into bright pink resorufin and 1-hydroxyacetone

In order to conduct the study of the catalytic activity of transition-metal nanoparticles *in vitro* and *in vivo*, dialysis membrane sachets were made. These organic biocompatible semi-permeable membrane sachets will function as a second protection layer for transition-metal nanoparticles, improving their catalytic activity and bio stability, by minimizing deactivation interactions with sulphur or amine bearing components present in living micro-environments. In addition, the sachets would enable surgical localized implantation of these catalyst loaded devices for *in vivo* studies.

The proof-of-concept for the nanoparticle loaded dialysis membrane sachet application has been successful, demonstrating the diffusion of the prodye (propargyl-resorufin) present in surrounding PBS-serum medium through the semi-permeable membrane pores into the sachets. Followed by the coordination of the prodye with either Au or Pd nanoparticles, resulted into cleavage of the propargyl moiety and subsequently released bright pink resorufin. The resorufin was detected after diffusion back into the surrounding medium, meanwhile the nanoparticles remain inside the sachets.

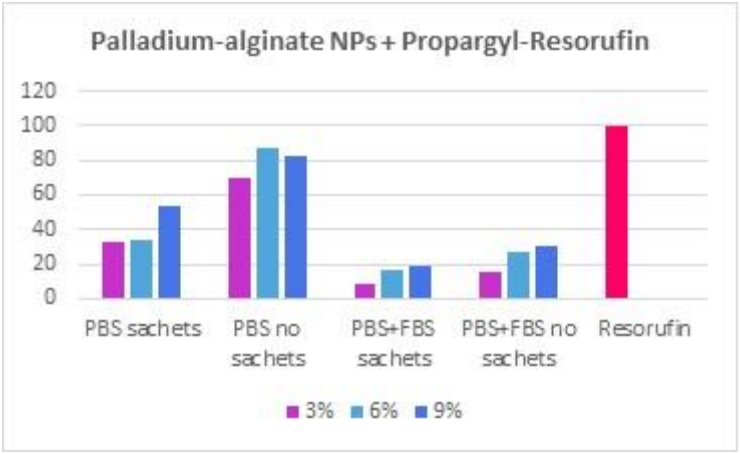

Fig. 37. Results of the catalytic cleavage of propargyl-resorufin by Pd-alginate nanoparticles at different concentrations. Experiments were performed with the Pd-alginate nanoparticles protected in dialysis membrane sachets or free (no sachets), and in the absence (PBS) and presence (PBS+FBS) of serum components.

The propargyl-resorufin prodye is quick and easy to prepare and therefore ideal to study catalytic activity of nanoparticles *in vitro*. However, its emission wavelength of 585-590nm makes it unsuitable for *in vivo* imaging studies. In order to gain better signal-to-noise ratio for *in vivo* fluorescence imaging, the use of near-infrared fluorophores is necessary. Therefore a propargylated derivative of quinone-cyanine-7 (QCy7), earlier published by TAU, has been developed. Future studies in collaboration with ESR13 at TAU will be performed to complete Task 6.2.

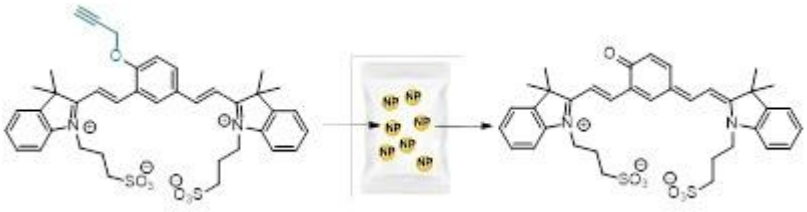

Fig. 38. Schematic presentation of the prodye propargyl-QCy7 (left) catalytically converted into QCy7 (right).

ESR12-TAG: To ensure a targeted tumor accumulation of the catalyst, two methods have been designed, so far, aiming for antibody conjugation of a catalyst. A non-internalizing antibody has been chosen (CC49, anti-TAG-72) and two different Pd-catalysts have been designed with the ability to catalyze depropargylation reactions. The first method consisted of the direct conjugation of the Pd-catalyst with the antibody and the second method consisted of two steps, where the conjugation is indirect using in vivo click chemistry between a trans-cyclooctene (TCO)-modified mAb and a tetrazine-catalyst construct. The development of these approaches was started during the secondment of the ESR12 in EDI but was cut short due to the COVID-19 outbreak, resulting in an unfinished project. Among the results that have been achieved before the COVID-19 outbreak, is the semi-synthesis of two different Pd-catalysts (Fig. 39), a successful TCO conjugation with the CC49 antibody, a successful synthesis of a fluorescent dye to serve as the substrate of the depropargylation of the catalyst (Fig. 40) and finally an unfinished tetrazine-Pd-catalyst construct (Fig. 41).

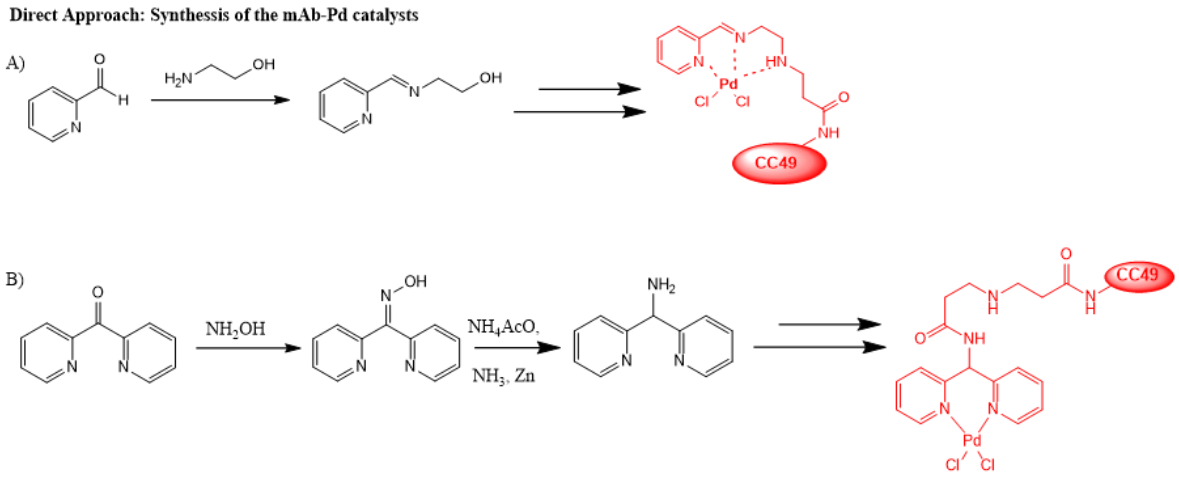

Fig. 39. Synthesis of the two Pd-catalysts, using the direct approach. (In black is depicted the work performed so far and in red the final desired construct)

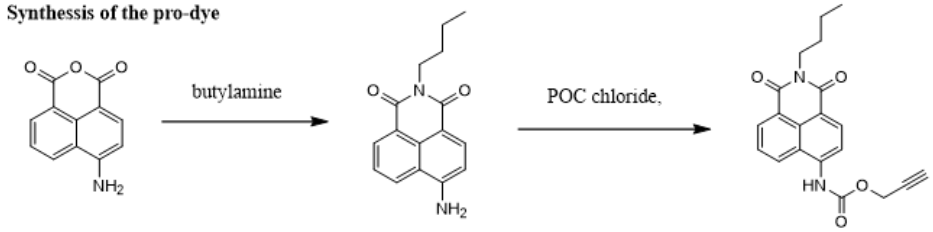

Fig. 40. Synthesis of the pro-dye.

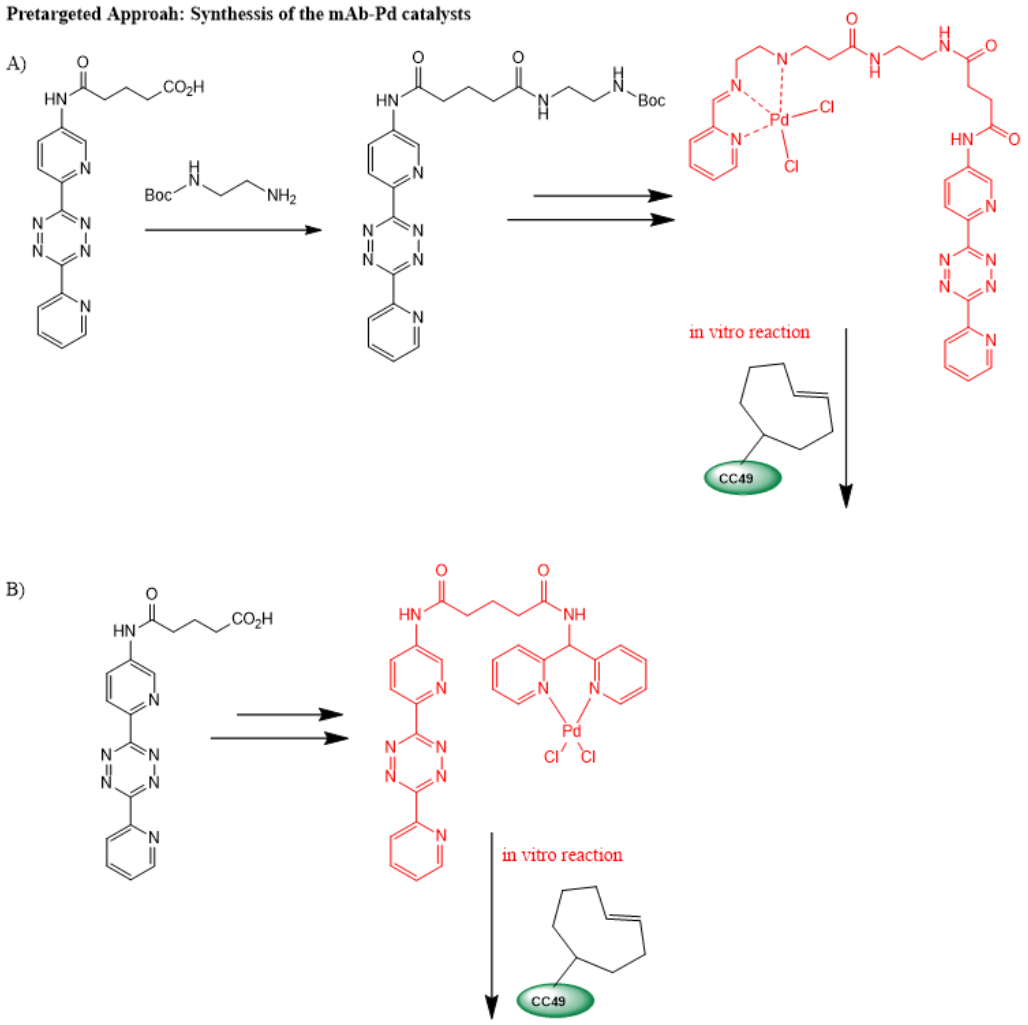

Fig. 41. Synthesis of the two Tetrazines-Pd-catalysts, using the pretargeted approach. (In black is depicted the work performed so far and in red red final desired construct and the final desired in vitro assay).

ESR13-TAU: Six different micellar polymeric carriers were provided by ESR2. Four non-degradable polymers (C6, C7, C8 and C12) and two degradable polymers (6x6Cd and 4x8Cd) were synthesized. All the micelles have similar hydrophilic blocks (5 kDa mPEG). The evaluation of their biocompatibility allowed as to choose the inert and most promising nanocarriers for further prodrug encapsulation and/or catalytic efficiency that will be evaluated in in vivo experiments.

We assessed the potential toxicity of each micelle type in terms of cellular proliferation using MTT assay (Protocol described in D6.2). None of the non-degradable micelles (C6, C7, C8 and C12) showed cytotoxicity up to 1 mg/mL (Fig. 42). However, the 4x8Cd showed some cytotoxic effects at concentrations higher than 0.01 mg/mL.

We also tested the potential ability of each type of micelle to lysate red blood cells (RBC) via RBC lysis assay (Protocol described in D6.2) in order to avoid any undesirable effect when going through *in vivo experiments*. None of the carriers induced the lysis of the red cells making them biocompatible for iv injections (Fig. 43).
The biocompatible carriers were selected for further prodrug and catalyst encapsulation and internalization assays in 2D plated and 3D spheroids cell cultures.

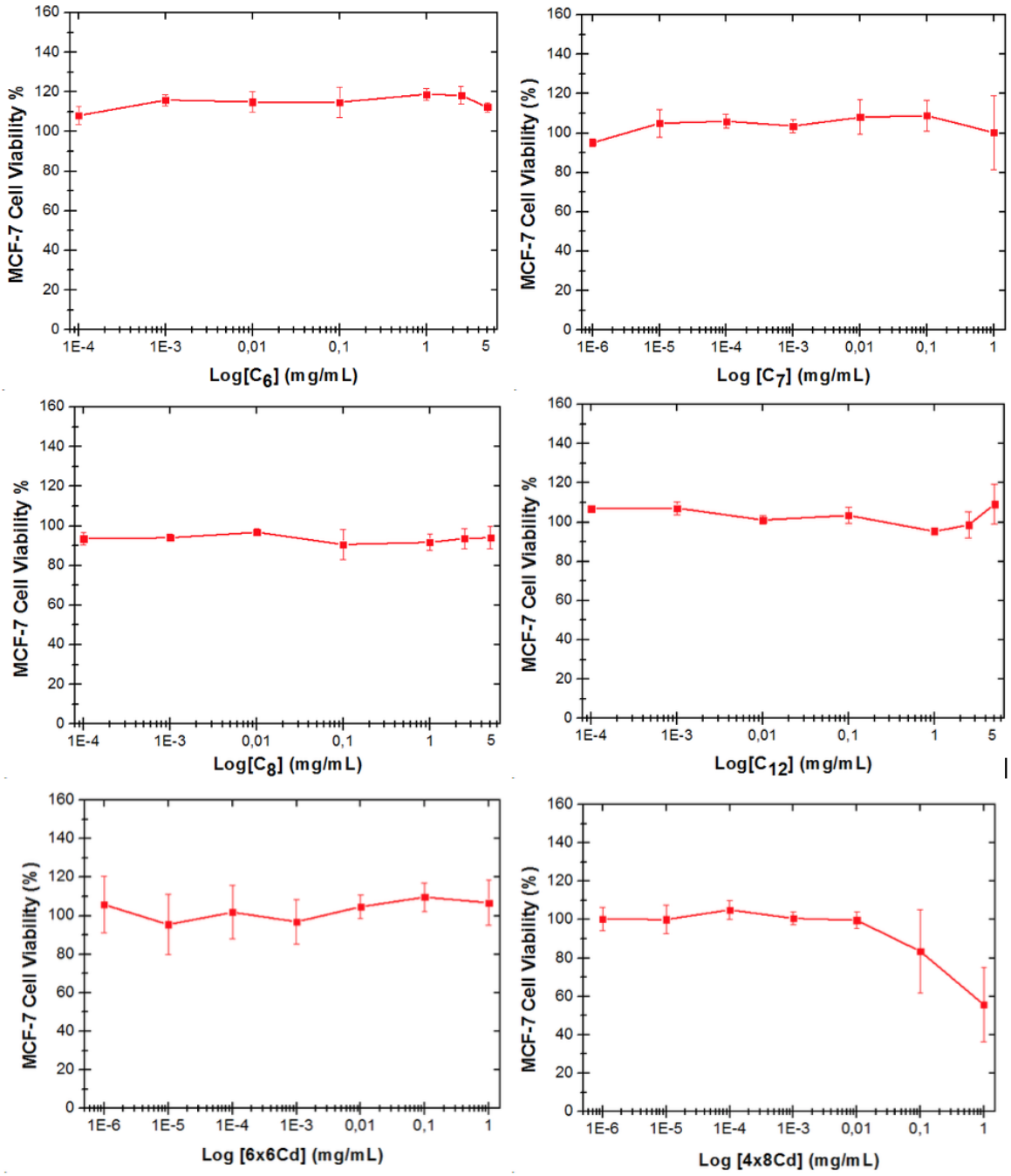

Fig. 42. Cell viability of MCF-7 cells assesed with MTT test after incubation with each micelle. None of the non-degradable micelles (C6, C7, C8 and C12) show cytotoxic effects up to 1 mg/mL whereas the 4x8 Cd starts to show cytotoxicity at concentrations higher than 0.01 mg/mL. Mean ± SD (N = 3 independent experiments).

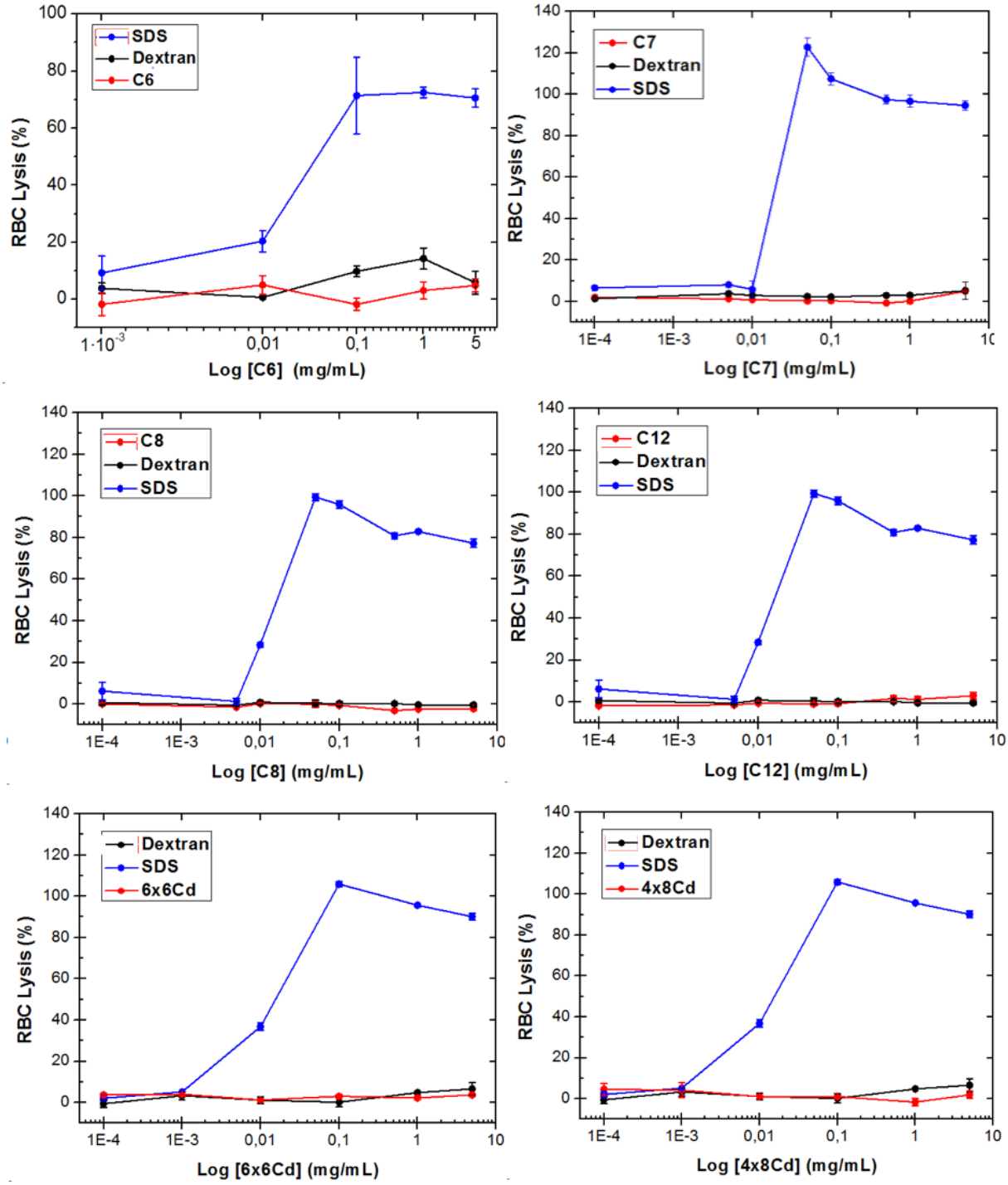

Fig. 43. RBC Lysis assay following 1 h incubation. None of the polymeric micelles (C6, C7, C8, C12, 6x6Cd and 4x8Cd) promoted RBC lysis at concentrations up to 5 mg/mL. Sodium dodecyl sulphate (SDS) was used as a positive control and dextran as a negative control. Mean ± SD (N = 3 independent experiments).

Further details on the work performed in this task are given in the report of the submitted deliverable D6.2.

Task 6.3. Use intravital optical and PET imaging to study catalyst localization and efficacy

ESR12-TAG: One major obstacle in in-vivo PET imaging of the catalyst/catalytic nanoparticles would be the potentially long circulation of the radiolabeled catalyst in the

blood stream, hindering the imaging of the tumor site. In addition, simply imaging a target-bound radiolabeled catalyst will not inform us whether the catalyst is extracellular or intracellular, which is relevant as this internalization may negatively affect the prodrug conversion efficiency. To address these aspects, we have developed an imaging method that centers on the chemically triggered removal of the radiolabel from the catalyst after a desired interval, employing the so called click-to-release reaction. In this approach, the radiolabelled catalyst is administered and allowed to circulate and bind the target. After a desired time, for example when sufficient target uptake has occurred, an Activator, a tetrazine derivative, is administered and this reacts with the TCO linker between the catalyst and the radiolabel (e.g. a radiolabeled chelate), resulting in cleavage of the radiolabel from the catalyst and its rapid excretion. The tetrazine can be designed such that it cannot efficiently extravasate. As a result only the still freely circulating catalyst in blood will be "unlabeled", boosting the target-blood ratio in imaging. Alternatively, the tetrazine can be designed such that it can extravasate but cannot internalize, resulting in that only the internalized catalyst fraction remains visible. Comparing images before and after tetrazine administration will report on the relative amount of internalized catalyst.

A protocol for this in-vivo cancer imaging method has been successfully developed in model systems, antibody-chelate conjugates, leading to robust in-vivo validation in mice. This includes the optimization of the TCO linker and the chelate-TCO-mAb conjugate, development of radiolabeling protocols (89Zr, 111In) of the constructs, development of optimal tetrazine-based Activators, demonstration of efficient tetrazine-triggered cleavage of radioactivity from antibodies in vitro and in mice, followed by protocol optimization in mice including tetrazine dose and timing. As far as the optimization in the timing and dosing of the tetrazine, we can safely conclude that the best timing is 24h after the administration and localization of the chelator-TCO-conjugate and the optimal dosing was found to be 1 mg/kg.

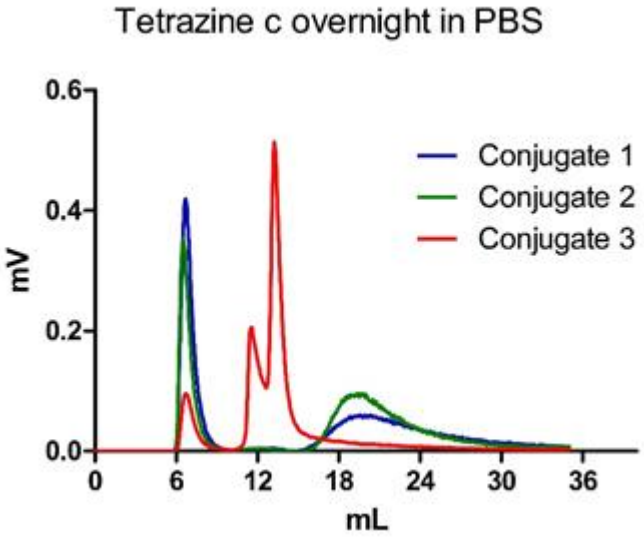

Fig. 44. Monitoring the click-to-release in PBS using SEC-FPLC, using three different types of zirconium-89 radiolabeled conjugates.

Task 6.4. Test in vivo efficacy against melanoma, breast and prostate cancer

This task deals with the final in vivo evaluation of the catalysts developed in THERACAT. According to the scheduled plan this task has just started, please find below the first preliminary results.

ESR11-EDI: To refer back to the nanoparticle loaded dialysis membrane sachet devices (Task 6.2), the idea was to further explore and develop a pre-*in vivo* screening method, performed in an advanced *in vitro* model that better represents the complexity of living environs. The design of the envisioned screening platform was being established in collaboration with Biogelx, during the first secondment of ESR11. A preliminary 3D-*in vitro* set-up has been created from Biogelx-RGD hydrogel matrix and biocompatible Au or Pd nanoparticles. Moreover, the uptake and diffusion of propargyl-resorufin through the hydrogel matrix, catalytic cleavage thereof and stability of the gel spheres and plaques were tested. The next steps for this pre-3D-*in vitro* model, would be exploration of the options to incorporate the NP loaded sachets into the model. Furthermore, a collaboration with ESR9-BGX, could be beneficial to explore the combination of this model with the MCF7 breast cancer cell line hydrogel. In addition, such a resulting combined 3D-*in vitro* model could lead to a desirable screening platform for efficient pre-*in vivo* prodyes and prodrugs behavioural examination, prior to the TAU 2nd secondment of ESR11.

1.2.7 Work package 7: Training

Objectives: To coordinate all actions related to the training, supervision and progress monitoring of ESRs.

Work carried out during 1st reporting period:

Task 7.1. Training events organisation containing scientific and complementary training courses

The scientific and complementary skills training of the THERACAT ESRs is being organized through:

(1) Local Scientific Training activities, which are provided by host institutions and that include scientific, methodological and technical aspects relevant for the Individual Research Project of the ESR in which the host group is expert.

(2) Secondments to other partner's laboratories, providing specific scientific training on techniques or knowledge necessary for the development of the Individual Research Project and not available at the host institution, favouring also the development of joint research efforts and the exposure of ESRs to different disciplines and sectors (academic/industrial). See below the list of secondments implemented during this first period (information also included in the "Funding & Tenders" Portal):

- ESR2 – Shreyas Wagle (TAU) to GRO: *Metal catalyst synthesis* (17/02/2020 – 18/03/2020)
- ESR11 – Melissa van de L'Isle (EDI) to BGX: *Gel-based implants* (24/02/2020 – 13/03/2020)
- ESR12 – Maria Vlastara (TAG) to EDI: *Pro-catalysis agent synthesis* (03/02/2020 – 13/03/2020)

Please note that these secondments had to finish earlier than initially planned to comply with the containment measures taken at national level due to the COVID-19 outbreak. The Project Officer was already informed.

(3) Network-wide scientific/complementary skills courses (Training Events), providing a wide and common scientific and technological background to all ESRs of the network and a holistic view of the multi- and interdisciplinary nature of the research being conducted. These Training Events guarantee a common language and knowledge on different scientific fields, facilitating the communication between members of the Network and providing a high-level training baseline to all ESRs, complemented by several sessions to gain key transferable competences. All Training Events included a combination of both technical hard-skills and transferable soft-skills, and special emphasis was given to hands-on training of the ESRs. Find below the Training Events organised during this first reporting period:

- Training Event 1 (4 days): *"Introducing the THERACAT Network & How to plan and start a PhD"*, was held at TUE (Eindhoven, THE NETHERLANDS) – March 2019 (Month 13). The Training Event started with a general introduction of the network and its scientific and training goals, followed by theoretical lectures and practical activities to prepare ESRs for their future PhD studies and research to be conducted in the framework of the project (D7.2).
- Training Event 2 (5 days): *"Chemical Synthesis & Catalysis"*, was held at BAS (Basel, SWITZERLAND) – September 2019 (Month 19). The event introduced the ESRs to the fundamental principles of designing the structure and synthesis of the prodrugs and catalysts, showed the road to bring new molecules to the market and provided basics on IP and commercial exploitation, including also a chemical safety session and a tour at Novartis (D7.4).
- Training Event 3 (4 days): *"Drug Delivery & Microscopy"*, was held at EDI (Edinburgh, UK) – February 2020 (Month 24). During the Training Event, different training sessions were given focused on microscopy techniques (including lab tours), design of delivery systems, in-vitro imaging and cell assays, and included also a complementary skills session on gender balance in academia (D7.6).

All ESRs attended these Training Events, and several sessions involved the participation of external trainers as top specialists in topics of interest for the fellows (such as journal editors or patent experts, among others).

Task 7.2. Personal Career Development and Employment Plans for all ESRs

The Personal Career Development Plan (PCDP) is a valuable tool in the implementation of the training of the ESRs. A template for the PCDP, agreed during the kick-off meeting, was provided to all supervisors and ESRs in order to guide them, including also some instructions on how to prepare it (MS1, MS4). The PCDP contains the ensemble of research objectives and training actions to be undertaken by each researcher of the Network, in particular:

- Scientific objectives and methodology of the Individual Research Project, ensuring originality and feasibility
- Individual secondment plan to expose the ESRs to multidisciplinary, intersectoral and multicultural environments
- Local and network-wide scientific training necessary to ensure the successful completion of the research project

- Transferable skills training actions to be undertaken
- Communication and dissemination activities envisaged
- Details on other actions of interest (networking opportunities, teaching activity, research management, etc.)
- Prospective on the professional career

PCDPs were agreed between ESRs and Supervisors and collected by the Coordinator and the Training Committee after the recruitment of the ESRs, and were further discussed between the ESRs and their Assessment Commission at Network Meeting 1. The final content of each PCDP can be found in D7.1 (updated at month 24, D7.8).

Task 7.3. Periodic individual assessment of ESRs

The day-to-day progress of the Individual Research Projects has been locally monitored by each ESR Supervisor, and network-wide by the Assessment Commissions (AC). The AC oversee the progress of the assigned ESRs every 6 months. The first assessment (D7.3) was performed during Network Meeting 1 and was focused on general questions to ensure that ESRs were properly integrated in the host institution and that had received all relevant information before starting their research (contractual issues, enrolment in PhD Programme, project objectives). Moreover, ESRs discussed their PCDP with the assigned AC. The second assessment was performed virtually at month 18 (D7.5), when ESRs sent a short report summarising the research performed, main obstacles and contingency plans, training received, outreach and dissemination activities performed, and future plans, and the AC provided recommendations for the forthcoming period. The third assessment was performed during Network Meeting 2 and was based on the work and other activities reported by the ESRs during the meeting, with the aim of overseeing the evolution of the ESRs in terms of research, training, PhD studies and future perspectives.

In all cases and after assessing the ESRs periodically every 6 months, it was observed that ESRs are making good progress not only in research but also in training activities (both at local and network level), and that are overall satisfied with the training program received.

Task 7.4. Doctoral studies

All ESRs of the Network have been enrolled in a Doctoral Program at a University except ESR9 (África Gálvez-Flores), who is finally not pursuing a PhD due to SME Biogelx internal regulations. The list of Universities and Doctoral Programs corresponding to each ESR are detailed below (MS5):

ESR	University of PhD enrolment	Doctoral Program (and link)
Michela Vargiu (ESR1-GRO)	Groningen University	PhD in Chemistry (Groningen Graduate School of Science and Engineering)
Shreyas Wagle (ESR2-TAU)	Tel Aviv University	PhD in Chemistry (TAU School of Chemistry)
Krishna Vippala (ESR3-TEVA)	Tel Aviv University	PhD in Chemistry (TAU School of Chemistry)
Anjana Sathyan (ESR4-TUE)	Eindhoven University of Technology	TU/e PhD Programme Chemical Engineering
Stephen Croke (ESR5-EDI)	University of Edinburgh	PhD Cancer (Edinburgh Cancer Research Centre)
Manos Archontakis (ESR6-TUE)	Eindhoven University of Technology	TU/e PhD Program Biomedical Engineering
Alis Olea (ESR7-IBEC)	University of Barcelona	PhD in Nanoscience (UB)

Linlin Deng (ESR8-TUE)	Eindhoven University of Technology	TU/e PhD Programme Chemical Engineering
Africa Galvez-Flores (ESR9-BGX)	N/A	N/A
Boris Lozhkin (ESR10-BAS)	University of Basel	PhD in Chemistry (UniBas)
Melissa van de l'Isle (ESR11-EDI)	University of Edinburgh	PhD Cancer (Edinburgh Cancer Research Centre)
Maria Vlastara (ESR12-TAG)	Radboud University Medical Center	PhD in Molecular Life Science
Daniel Rodríguez (ESR13-TAU)	Tel Aviv University	PhD in Medical Sciences (Sackler Faculty of Medicine)

1.2.8 Work package 8: Dissemination and outreach

Objectives: To promote the efficient and effective awareness of academic and industrial scientists as well as general public about THERACAT, its training potential and results.

Work carried out during 1st reporting period:

Task 8.1. THERACAT webpage including a private intranet for internal communication

The THERACAT project website was launched at month 6 (D8.1, MS3), and is regularly updated with inputs from all partners (https://theracat.eu/). It was designed following two complementary objectives. First, an extranet dedicated area is devoted to the communication and dissemination of the project and its results to the general public. Therein, it is described and promoted, in plain English, the research being conducted within the framework of the project, with special emphasis on the concept of bio-orthogonal catalysis and how it can provide novel anti-cancer prodrugs. It also includes presentations of the ESRs, events coverage and dissemination and communication actions performed so far. Second, an intranet area of the webpage was developed to support the network management and contribute to have an effective internal communication (access is restricted to Supervisory Board members and ESRs). This area contains a detailed schedule of activities and of the network plan, as well as a repository of documents which is continuously updated (including deliverables, meeting minutes and presentations, reports, guidelines and templates, among others).

Task 8.2. Open-access publications in high-impact journals and patents

Despite the progresses made in the Individual Research Projects of the ESRs, no paper in a peer-reviewed journal has been published yet. Patents have not been filled neither. However, **ESR7-IBEC** is currently preparing a manuscript in collaboration with Prof. Amir from TAU containing the results obtained so far to be published in an international peer-reviewed journal. This work focuses on the study of the behaviour in cells of the micelles produced by TAU using advanced optical imaging. **ESR10-BAS** is also finalizing a manuscript describing the methodology developed within THERACAT to generate monosubstituted benzene derivatives via ring-closing metathesis, followed by spontaneous 1,2-elimination, which proved widely applicable and compatible with physiological conditions.

Task 8.3. Work presented in international conferences and workshops

During the first 18 months, the THERACAT project, consortium and unique approach was made visible to the scientific community, mainly through its website (https://theracat.eu/) and through presentations and plenary sessions by the supervisors at international conferences (such as the RSEQ Biennial Meeting held in May 2019 in San Sebastian, the Bioorthogonal &

Bioresponsive 2019 Conference held in June 2019 in Edinburgh, and the Unconventional Catalysis, Reactors and Applications Conference held in October 2019 in Zaragoza - *more details in D8.2 and D2.6*). Moreover, beneficiary EDI co-organised the Bioorthogonal & Bioresponsive 2019 Conference (Edinburgh, UK; June 6th-7th, 2019), having Asier Unciti-Broceta as the Chair and ESR5 (Stephen Croke) and ESR11 (Melissa van de L'Isle) as Delegates; this conference was attended by most of the THERACAT ESRs.

As soon as the ESRs started obtaining relevant results, they have presented their work in different conferences and workshops (D8.3):

(1) Conferences

Poster presentation:

- Authors: **Africa Galvez-Flores (ESR9, BGX)**. Title: 3D Hydrogels and Bioinks for Realistic In-vitro Cancer Modelling. Conference: Goodbye Flat Biology (Berlin, GERMANY). 11/11/2019

Oral presentation:

- Authors: **Africa Galvez-Flores (ESR9, BGX)**. Title: 3D Hydrogels and Bioinks for In-vitro modelling in Advanced Therapies. Event: Joint 3DBioNet IBIN Meeting (London, UK). 20/01/2020

(2) Workshops/Seminars

- Authors: **Maria Vlastara (ESR12, TAG)**. Title: In-vivo click and click-to-release strategies for in vivo catalyst activation. Event: Presentation at the Nuclear Medicine Department, Radboud University Medical Center (Nijmegen, THE NETHERLANDS). 28/05/2019
- Authors: **Africa Galvez-Flores (ESR9, BGX)**. Title: An In-vitro 3D Model for Chemotherapy Testing. Event: Biomaterials Seminar Series at the Centre for the Cellular Microenvironment (University of Glasgow, UK). 26/11/2019
- Authors: **Anjana Sathyan (ESR4, TUE)**. Title: Single chain polymeric nanoparticles as catalyst carriers for prodrug activation. Event: Colloquium at the TU Eindhoven (Eindhoven, THE NETHERLANDS). 14/12/2019
- Authors: **Stephen Croke (ESR5, EDI)**. Title: Targeting Cancer Through the Local Destruction of Proteins. Event: Seminar at Institute of Genetics & Molecular Medicine (Edinburgh, UK). 21/01/2020
- Authors: **Linlin Deng (ESR8, TUE)**. Title: A study on the stability of single chain polymeric nanoparticles in complex media. Event: Colloquium at the TU Eindhoven (Eindhoven, THE NETHERLANDS). 17/02/2020

Task 8.4. THERACAT social media account creation and management

During this first period, IBEC and EDI have been in close contact with Partner Organisation Cancer Research UK (CRUK) in order to design the best strategy on communication activities to reach general public, to be implemented all along the project. CRUK is the world's leading cancer charity dedicated to saving lives through research, and its Research Information & Engagement team has an outstanding experience in communicating science to the general public. A plan for future communication activities to be conducted was discussed during Network Meeting 1 (see D8.2) and several activities have been already performed (Task 8.5). Regarding the appearance of THERACAT in social media, find below communication activities performed so far:

- https://www.linkedin.com/posts/biogelx-limited_biogelx-tumor-model-for-chemotherapy-testing-activity-6597807130659684352-agGU/
- https://www.linkedin.com/posts/biogelx-limited_goodbye-flat-biology-2019-biogelx-tumor-activity-6597110360078192640-ZHeY/
- https://www.linkedin.com/posts/biogelx-limited_goodbye-flat-biology-2019-biogelx-tumor-activity-6600685886327918592-7k7S/
- https://www.linkedin.com/posts/biogelx-limited_advanced-biomaterials-a-key-focus-in-the-activity-6544558341656588288-ed5G/
- https://www.youtube.com/watch?v=p48Un3EUR9Q&feature=youtu.be&utm_content=108117114&utm_medium=social&utm_source=linkedin&hss_channel=lcp-3042346
- https://www.facebook.com/plugins/post.php?href=https%3A%2F%2Fwww.facebook.com%2Fbiogelx%2Fposts%2F1394863554031498%3A0
- https://www.facebook.com/plugins/post.php?href=https%3A%2F%2Fwww.facebook.com%2Fbiogelx%2Fposts%2F1357338224450698%3A0
- https://www.facebook.com/plugins/post.php?href=https%3A%2F%2Fwww.facebook.com%2Fbiogelx%2Fposts%2F1355342501316937%3A0
- https://twitter.com/biogelx/status/1111667118821519360
- https://twitter.com/biogelx/status/1088791120635707394
- https://twitter.com/biogelx/status/1017697911818407936
- https://twitter.com/biogelx/status/1204366040592982017
- https://twitter.com/morenorse/status/1199284377177464832
- https://twitter.com/biogelx/status/1194920240335007744
- https://twitter.com/biogelx/status/1192041436319043584
- https://twitter.com/biogelx/status/1191344668853915648

Moreover, a THERACAT social media account will be launched within the next few months to communicate the first project results obtained so far and to explain science behind the project to the general public, aiming to encourage students to undertake research careers as well.

Task 8.5. Communication activities incl. cancer-related charity events, science festivals, European Researchers' Night, general press articles, THERACAT video

The THERACAT ESRs and institutions have been strongly committed to communicate science and encourage public engagement through the following actions (D8.2, D8.3):

(1) News

Announcements on the institution's beneficiary websites:

- http://www.ibecbarcelona.eu/training-the-next-generation-of-experts-in-bio-orthogonal-catalysis-for-cancer-therapy_/
- https://www.biogelx.com/theracat-the-use-of-bio-orthogonal-catalysis-for-cancer-therapy/
- https://www.biogelx.com/introducing-biogelxs-new-cell-biologist-africa-galvez-flores/
- https://www.biogelx.com/the-theracat-project-gets-off-to-a-flying-start/
- https://www.ed.ac.uk/cancer-centre/news-and-events/news-2019/european-funding-success

- https://www.biogelx.com/goodbye-flat-biology-biogelx-tumor-model-for-chemotherapy-testing/
- https://www.biogelx.com/advancing-3d-models-for-defeating-cancer/
- https://www.biogelx.com/biogelx-tumor-model-for-chemotherapy-testing/
- https://www.biogelx.com/advanced-biomaterials-key-focus-in-development-of-bioorthogonal-and-bioresponsive-strategies/
- https://www.biogelx.com/3d-hydrogels-and-bioinks-for-in-vitro-modelling-in-advanced-therapies-our-experience-at-ibin-3dbionet-meeting/

Mention in the description of beneficiaries' activities:

- https://nanomedspain.net/institut-de-bioenginyeria-de-catalunya-ibec/

Newspapers:

- Interview ESR2 Shreyas Wagle (newspaper Loksatta, India): https://www.loksatta.com/viva-news/jagachya-pativar-article-shreyas-wagle-abn-97-1996446/

(2) Outreach events

- **Alis Olea (ESR7, IBEC)**. Participation in "(Re)search4Talent" event for undergraduate and master students interested in a research career at IBEC (Barcelona, SPAIN). 21/05/2019
- **Manos Archontakis (ESR6, TUE)**. Demonstration of microscope facilities for BSc students at TUE (Eindhoven, THE NETHERLANDS). 11/2019
- **Maria Vlastara (ESR12, TAG).** Hands-on-sessions at the Radboud University Medical Center Summer school "State of the art Radionuclide Imaging and Therapy in Oncology" (Nijmegen, THE NETHERLANDS). 12-16/08/2019
- **Stephen Croke (ESR5, EDI)**, **Melissa van de L'Isle (ESR11, EDI), Asier Unciti-Broceta (EDI).** Short stay of High School student (Eva Russo) from Algeciras (Spain) that won a Diverciencia scholarship to visit EDI lab and learn on bioorthogonal chemistry (Edinburgh, UK). 02-06/09/2019. https://www.europasur.es/algeciras/omar-rodriguez-eva-russo-diverciencia_0_1360364158.html
- **Boris Lozhkin (ESR10, BAS), Thomas Ward (BAS)**. Chemistry Olympiad Workshop for gifted Swiss high school students (Basel, SWITZERLAND). 23/11/2019

(3) Cancer-related charity events

- **Africa Galvez-Flores (ESR9, BGX)**. Participation in 2020 Scottish 10K Run, fundraising for Cancer Research UK (Edinburgh, UK). https://fundraise.cancerresearchuk.org/page/africagalv
  Of note, due to the COVID-19 confinement measures it may be that the run itself is cancelled (scheduled September 20th 2020), but the fundraising event is still active and people are donating to Africa's fundraising page for CRUK.

(4) THERACAT video (D8.4)

A group of fellows (**ESR4, ESR6, ESR7 and ESR13**) established a working group to create a video to illustrate the concept of THERACAT. The ESRs are planning the

content and sketching the visualization. The video will be realized with the collaboration of the ICMS animation studio of the Eindhoven University of Technology.

Task 8.6. ETN international conference

The THERACAT Conference is planned to be held in Month 42 and therefore this task has not started yet. However, ESRs have been already informed that may propose some external speakers so that they may start identifying experts of interest.

1.3 Impact

In the DoA it is described how the THERACAT project will contribute to the expected impacts on four different areas:

a) Enhancing the career perspectives and employability of researchers and contribution to their skills developments

b) Contribution to structuring doctoral/early-stage research training at the European level and to strengthening European Innovation capacity

c) Exploitation and dissemination of results to the scientific community

d) Communication of the activities to different target audiences

Considering that the project has evolved, in general terms, as planned in the proposal both in research and training aspects, and that deviations from the original plan have been already addressed (more details in section 5), the information contained on the impact section of the DoA remains fully valid and does not need to be updated.

2. Update of the plan for exploitation and dissemination of result (if applicable)

In the DoA it is described a plan for exploitation and dissemination of results, always considering potential protection of eventual intellectual property rights and/or exploitation before disseminating any result. This approach is supported by the expertise and guidance of the IP & Innovation Committee.

The plan for exploitation and dissemination described in the DoA remains globally valid and does not need to be updated. However, for the forthcoming period, as more solid data and results will be obtained by the ESRs, efforts will be focused on increasing the dissemination actions to reach the scientific community by means of publications in peer-reviewed journals, posters/oral communications in conferences and announcement of the main scientific results through partners' websites and THERACAT social media account (always considering potential exploitation prior to dissemination).

3. Update of the data management plan (if applicable)

N/A

4. Follow-up of recommendations and comments from previous review(s) (if applicable)

Find below the recommendations received from the Project Officer after the Mid-term check meeting in Edinburgh on June 5th, 2019 and how they were addressed:

- More visibility to the EU funding should be given through the website (namely display the EU emblem and correct funding sentence) as well as all printed material.

  A bigger EU emblem and the correct funding sentence were added to the THERACAT website, and consortium members are continuously reminded in every Network Meeting to properly acknowledge EU funding on any dissemination/communication activity performed.

- All information displayed in the project website should be updated. There are some sections incomplete, in particular those included under fellows tab which are currently empty.

  Webpage was duly updated, including also the presentation of all ESRs in the fellows tab.

- Summary for publication appears as empty in Sygma. Please upload the publishable summary.

  Summary for publication was uploaded in Sygma as requested.

- VISAs should be requested well in advance to avoid any delay with the secondments and trainings outside the fellows' host institutions.

  VISAs were and will continue to be requested in advance to ensure that all fellows can conduct their activities outside the host institution (secondments, training events) as planned. So far, all fellows have obtained all the needed VISAs on time.

- Please ensure that all fellows are paid in accordance to the MSCA-ITN rates, in particular those recruited in TUE, GRO and TAG. This will be checked at the end of the project.

  All beneficiaries were reminded that fellows must be paid in accordance to the MSCA-ITN rates detailed in Network Meetings.

- Any change in the planned secondments should be informed to the PO in advance. Any deviation from the GA will require the prior approval of the PO.

  All changes in planned secondments had been informed and justified to the PO through Formal Notifications and were not implemented until obtaining the approval of the PO.

- Information provided in the Progress report should be updated, in particular information referred to secondments, Fellows Committee and delay in the delivery of some milestones. Progress report will be reopened for modification.

  Progress Report was duly updated following PO recommendations and the new version was submitted.

- According to the Progress report, dissemination to the general audience seems to be limited to two beneficiaries: IBEC and BIOGELX. All beneficiaries are encouraged to take active part of the dissemination and outreach activities.

  We reminded partners the importance to actively participate in dissemination and communication actions, and we are continuously doing it in Network Meetings and Training Events as well as encouraging ESRs through personalised e-mails.

- Information provided in the Progress Report regarding the submission of the results obtained so far to international peer-reviewed journal, should be clarified since,

according to what it was discussed during the meeting, no publication has been prepared yet.

This information was clarified with the involved beneficiary and amended in the last version of the Progress Report submitted.

- Beneficiary 7-TEVA: Some issues have been detected with this beneficiary since the start of the project including delays in the signature of the Grant Agreement and the Consortium Agreement due to size-down of this beneficiary; change in the PI in charge and delay in the recruitment of ESR3. The beneficiary TEVA has been advised to speed up the recruitment process and to issue the contract to ESR3 as soon as possible. Please, ensure that the recruitment of the fellow is completed. The remaining time until the end of the project is 33 months, which means that ESR3 can be contracted for a maximum period of 33 months.

  IBEC as Coordinator continuously reminded TEVA the importance of conducting all required actions on time, and especially the recruitment, always offering its support to accelerate required actions. Unfortunately, the internal restructuring process underwent at TEVA before the start of the project difficulted the timely achievement of the abovementioned issues. ESR3 was finally incorporated at TEVA at month 19 (more details in section 5).

5. Deviations from Annex 1 and Annex 2 (if applicable)

Some of the recruited ESRs were incorporated later than planned in the original DoA due to the difficulties to find suitable and competitive candidates, and in some cases the lengthy procedures to obtain their visas delayed even more their incorporation. However, all ESRs were recruited before month 12 (except ESR3 – *more details in the paragraph below* – and ESR8, who was incorporated with a 10-days delay). Therefore, the original workplan was slightly readjusted considering the actual starting date of each ESR, including rescheduling of the secondments planned and associated deliverables. Such modifications were intended at aligning the workplan with the progress of the ESRs' research projects, thus contributing to maximise their scientific productivity and to take full advantage of the research to be performed during secondments. All modifications proposed were notified and duly justified to the Project Officer through Formal Notifications, and were not implemented until the official approval from the Project Officer was received.

The major deviation from the DoA was the delay in the incorporation of ESR3, recruited by TEVA. Notably, the selection process finished on time and the selected candidate was promptly notified his admission into the THERACAT Network, only pending to sign the contract with TEVA. In an effort to ensure that ESR3 would be enrolled in a Doctoral Programme at Tel Aviv University – TAU, TEVA supervisor (Dr. Avramovitch) coordinated with TAU supervisor (Dr. Amir) to prepare the required paperwork. However, this process took longer than expected due to the complexity of having, for the first time, an ESR from an industrial beneficiary (TEVA) enrolling in a PhD program at TAU. This unique situation demanded special arrangements and understandings between and within the beneficiaries that, despite the efforts of Dr. Amir and Dr. Avramovitch to accelerate the process, difficulted the timely incorporation of ESR3 who finally started working at month 19. Despite this delay, ESR3 was quickly and successfully integrated into the Network, having already prepared his PCDP together with his supervisor, receiving the training materials (presentations, minutes) of the Training Event 1 that he could not attend, being enrolled in the PhD Programme at TAU and receiving the corresponding local training, as well as participating in all Network events starting from Training Event 2.

Two other modifications from the DoA were performed after the approval of the corresponding Amendment by the EC, which were: (1) modification of TEVA's Principal Investigator (PI) to be Dr. Avramovitch, since the PI initially appointed to the THERACAT project (Dr. Hila Barash) was no longer working for the company due to a substantial internal restructuring process underwent at TEVA; (2) relocation of ESR6 from IBEC to TUE. The later change was prompted by the new affiliation of the supervisor, Dr. Albertazzi, at TUE, holding a double appointment position between IBEC and TUE. In practical terms, part of Dr. Albertazzi's research activities remain at IBEC while part of them are being performed at TUE, to maximize the scientific performance of the research group, in the framework of a collaborative agreement between the two institutions. In this collaborative framework and thanks to the new appointment of Dr. Albertazzi, TUE invested a substantial budget in microscopy that is at Dr. Albertazzi's disposal. For this reason, Dr. Albertazzi considered beneficial for the project and for the fellow that one of the ESRs initially appointed at IBEC, ESR6, would be recruited and trained at TUE under his supervision. This has been undoubtedly beneficial for the ESR in light of the activities and investments in Dr. Albertazzi's group at TUE in the field of single molecule microscopy, the main focus of the ESR6 Individual Research Project.

Finally, the PIs from three other institutions had to be replaced by other researchers from the same institution due to internal staff changes, namely: (1) Dr. Laura Goldie was substituted by Dr. Chris Allan at beneficiary BGX; (2) Fionnuala Ratcliffe was substituted by Sarah Thomas at partner organisation CRUK; (3) Dr. Joana Gallego was substituted by Dr. Maria Prats at partner organisation UAB. These modifications were also notified to the Project Officer by means of a Formal Notification and were not implemented until obtaining the official approval.

5.1 Tasks

In general terms, tasks and activities proposed in the DoA proceed as planned. This fact reflects a strong commitment of all beneficiaries and partner organisations towards the successful implementation of the project, and its overall success. Only minor modifications were performed to the workplan such as rescheduling of secondments, as explained above. Another minor adaptation from the proposed actions in the DoA refers to the topic of ESR12-TAG secondment to EDI, which was modified to be focused on synthesis of a pro-catalysis agent instead of a pro-PET agent. This change was agreed between the fellow and supervisors (from host and receiving institution), with the aim of aligning the secondment with the ESR12 status of research and ensuring that relevant results could be obtained during secondment; the pro-catalysis agent involves conjugating a catalyst to a tumor targeting agent which is of high relevance to the consortium goals and, moreover, it directly prepares for the secondment from ESR5-EDI to TAG, where the agent can be further evaluated in vivo, with PET imaging.

Finally, there was another minor modification in Task 4.2 (*Synthesis of fluorescent dyes such (rhodamines, cyanines) protected with propargyl/allyl groups*): this task was originally assigned to ESR6 in IBEC; however, due to the change of institution of ESR6 (from IBEC to TUE), the task is now assigned to TUE and in particular to ESR4. This is the optimal choice from the scientific point of view due to the background and previous experience with synthesis of ESR4.

Regarding deliverables and milestones scheduled for the first reporting period, most of them have been already submitted/reached. Only a few of them will be delayed (please find below the corresponding justification and corrective measures that have been/will be applied):

- D7.7. ESRs periodic short reports and AC recommendations (M24):

  Despite that the assessment of the ESRs was performed as scheduled during the last Network Meeting in Edinburgh, the original documents containing the results of the assessment were left at IBEC facilities, which cannot be accessed due to the State of Alarm declared in Spain from March 14th, 2020. Deliverable 7.7 will be submitted as soon as IBEC facilities are re-opened after the confinement.

- D8.4. THERACAT video:

  The THERACAT video planned earlier is a bit delayed from the schedule. This is partially due to some organizational difficulties (some ESRs were moving due to secondments and had to adapt to the new institution before being fully operational) and to the delay associated to the COVID-19 restrictions (the ICMS animation studio is only partly operational). Deliverable 8.4. will be submitted as soon as ICMS facilities are fully operational again.

- MS10. Establishments of the protocol for super resolution imaging in cells (IBEC):

  The original plan for ESR7 consisted in the development of fluorescence and super-resolution protocols for the imaging of nanocatalysts carriers. The development of the super-resolution part has been delayed as the protocol for classical fluorescent microscopy showed to provide interesting results and it has been decided with the fellow to investigate this a bit deeper and postpone the super-resolution analysis. This modification does not impact on other tasks and objectives, which remain unaltered and as described in the DoA.

- MS16. Synthesis and evaluation of the first cell-targeted nanocarrier (TUE):

  Despite the synthesis of a series of saccharide containing nanocarriers for cell targeting purposes was finished in November 2019 by TUE (Task 5.1), their testing was planned to be performed during secondment of ESR8 at IBEC (Spain). Such secondment had to be cancelled due to the State of Alarm declared in Spain from March 14th, 2020 and therefore the corresponding experiments will not be conducted until the COVID-19 restrictions finish and the secondment can be performed. This modification does not impact on other tasks and objectives, which remain unaltered and as described in the DoA.

Therefore, all deviations from the original plan have been already identified and properly addressed to ensure that the project objectives are fully achieved and aligned with the DoA.