D2.1. Experimental design of the hybrid systems

Artificial PHOTOsynthesis to produce FUELs and chemicals: hybrid systems with microorganisms for improved light harvesting and CO_2 reduction

Grant Agreement Number 101069357

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EXECUTIVE SUMMARY

D2.1 aims to present the experimental design of the hybrid systems for methane and acetate production. During M1-6, UU, TZE and ICCAS had meetings to discuss the experimental design of the proposed bio-hybrid systems concerning photosensitiser Pdots synthesis and preparation (UU and ICCAS), microorganism culture (TZE and ICCAS) and photocatalytic experiments (TZE and ICCAS). UU has prepared and characterised the first batch of designed Pdots and delivered them to TZE and ICCAS. TZE and ICCAS have been working on *Methanosarcina barkeri* and Moorella thermoacetica cultures, respectively. The relevant photocatalytic test and optimisation of Pdots and bio-hybrid systems will be performed during M6-18. All work is going well according to what is proposed in D2.1 deliverable of WP2.

1. INTRODUCTION

1.1 DESCRIPTION OF THE DOCUMENT AND PURSUE

This report describes the deliverable D2.1 in WP2. It includes all work done during M1-6 and work planned for M6-18 at UU, TZE and ICCAS. It contains four parts: Design and Synthesis Pdots for bio-hybrid systems; Development of hybrid microorganism-organic semi-conductor systems to convert CO_2 into methane using Methanosarcina barkeri and sunlight; Development of hybrid microorganism-organic semi-conductor systems to convert CO_2 into acetate using Moorella thermoacetica and sunlight and workflow in WP2.

1.2 WPS AND TASKS RELATED TO THE DELIVERABLE

This deliverable refers to Task 2.1, Task 2.2, Task 2.3 and Task 2.4 in WP2: **Hybrid microorganism organic semi-conductor systems**.

2. Design and Synthesis Pdots for bio-hybrid systems (UU)

Polymer dots (Pdots) have shown excellent light absorption and bio-compatibility with bacteria [1–3]. However, for different microorganisms, the electron pathway is different and therefore requires suitable photosensitisers or redox mediators to deliver electrons in the systems for synthesis. In WP2, two types of microorganisms were proposed to use in this biohybrid systems. *Methanosarcina barkeri* (M. barkeri) is developed for methane production, and *Moorella thermoacetica (M. thermoacetica)* will be used for acetate production. All electron transfer pathways in both microorganisms have been discussed between partners involved in this WP, and various Pdots are therefore designed and prepared to match the energy levels needed for charge delivery.

The plan is that TZE and ICCAS will screen all Pdots provided by UU in their individual system and delivery first-hand experimental data and information to UU for further optimising Pdots or new polymers designed if needed. Once one or more Pdots-based systems are found excellent for photocatalytic performance, UU will do a further photophysical study to understand the charge and energy transfer pathways in Pdots and scale up the Pdots sample for further device tests.

Design principle of organic polymers and Pdots for bio-hybrid systems:

a) Organic polymers with donor-acceptor (D-A) or donor-π linker-acceptor (D-π-A) configuration was chosen in this project (as shown in Figure 1).

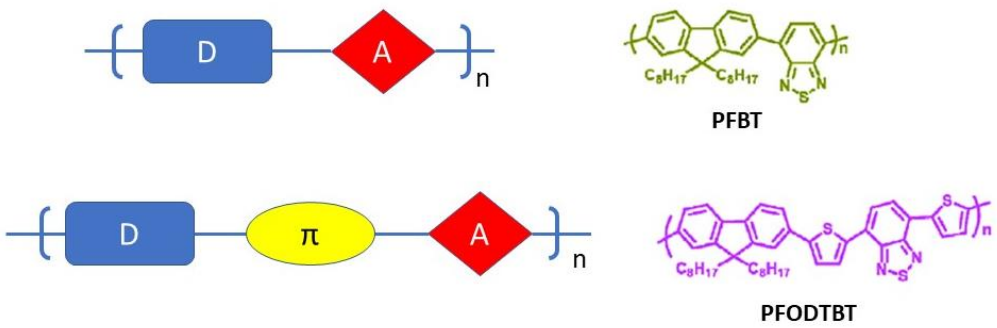

Figure 1. Schematic drawings of D-A and D-π-A types of organic polymers and two organic polymers with corresponding structures.

By varying different subunits, we can adjust the absorption of organic polymers and their energy levels to match the energy required to drive microorganisms (Figure 2). For example, we can increase the HOMO level of the polymers by using more electron-donating units and decrease the polymers' LUMO level by using more electron-withdrawing units. Our previous work showed that PFODTBT, F8T2 and ABA polymers worked well with enzymes or bacteria. Therefore, Pdots based on these polymers will be first tested with *Methanosarcina barkeri* and *Moorella thermoacetica*. In parallel, we have synthesised 12 new batches of Pdots with heterojunction architecture by varying different donor and acceptor

polymers. Hereby, polymers were selected to make efficient electron transfer to microorganisms feasible based on energy level alignment in pair with efficient light harvesting within the entire visible range.

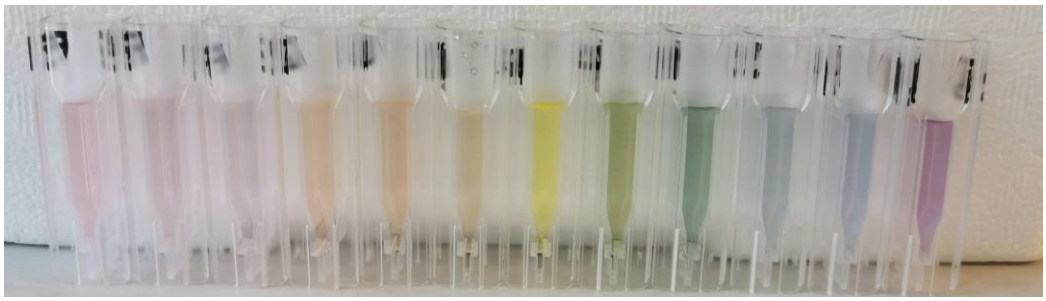

Figure 2. Image of 12 different batches of Pdots that have been already sent to TZE and ICCAS for screening in bio-hybrid systems.

b) Pdots preparation was carried out by the nanoprecipitation method (as shown in Figure 3). Briefly, the organic polymers, along with amphiphilic polymers or other surfactants, will be dissolved in an organic solvent such as THF and then sonicated to ensure the complete dissolving of polymers and to mix between all components. Then this mixture will be poured into water. After mixing, THF will be slowly removed to obtain Pdots' aqueous solution. Using the nanoprecipitation method, it was also feasible for us to prepare heterojunction Pdots, meaning having more than one photoactive component in one particle. With heterojunction design, it is possible to have enhanced charge separation between components and improve charge transfer efficiency from Pdots to bacteria.

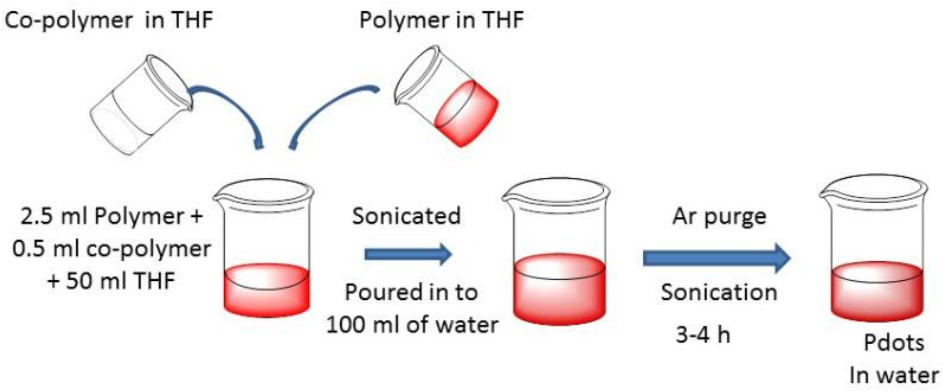

Figure 3. A schematic drawing of preparation of Pdots via nano-precipitation method.[4]

The Pdots were characterised by DLS, UV-vis and electrochemistry to get first-hand information on size and energy levels before sending samples to TZE and ICCAS. The morphology of Pdots could play an important role in charge transfer inside Pdots. Therefore, the morphology will be studied by Cryo-EM if needed.

c) To ensure efficient electron transfer to bacteria and circumvent the extra energy consumption when redox shuttles transmembrane transport, one must consider the interaction of Pdots in bacteria. According to our previous study, the positively charged Pdots can interact well with bacteria systems. So, all 12 batches of Pdots were decorated by the surfactant with a positive charge, such as ABA to promote interaction with the negatively charged surface of bacteria. The amount of ABA will be considered in the following experiments. During M1-6, 12 batches of Pdots samples have been prepared and sent to TZE and ICCAS for a further photocatalytic test. With feedback from TZE and ICCAS, UU will contribute to optimising and designing new Pdots or other photosensitisers to improve the photocatalytic performance of bio-hybrid systems. The final test bio-hybrid systems will be chosen based on all results from UU, TZE and ICCAS. Various spectroscopic tests will also investigate the finally chosen photosensitisers or the entire bio-hybrid systems to get insights into the charge transfer mechanism. The workflow is shown in Figure 4.

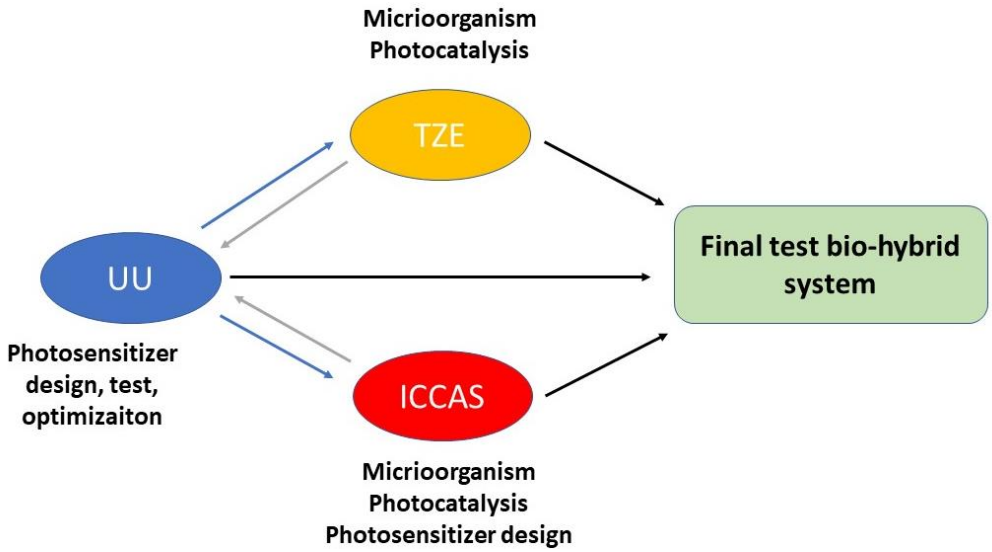

Figure 4. A schematic drawing of workflow between UU, TZE and ICCAS for development and optimisation of bio-hybrid systems.

3. Development of hybrid microorganism-organic semi-conductor systems to convert CO_2 into methane using *Methanosarcina barkeri* and sunlight (TZE)

Methanosarcina barkeri (Figure 5) is a mesophilic hydrogenotrophic methanogenic archaea which can produce methane anaerobically through different metabolic pathways. It can use methanol, acetate and carbon dioxide to produce methane under anaerobic conditions. For the Photo2fuel project, *Methanosarcina barkeri* (DSM 1538) was bought from German Leibniz Institute (DSMZ). As the strain is well described and a wide body of research has been done with the specific strain, it was chosen as the prime candidate for methanogenesis from CO_2. Cultivation followed the respective protocols and media provided by DSMZ. The heterotrophic media (DSM 120) was used for traditional cultivation utilising 80% H_2 and 20% CO_2 gas atmosphere at 2 absolute bar pressure and 37 °C. For the biohybrid experiments, an autotrophic media, based upon DSM120, was developed and tested under various conditions, temperatures and reduction potentials to safeguard stability and cell viability.

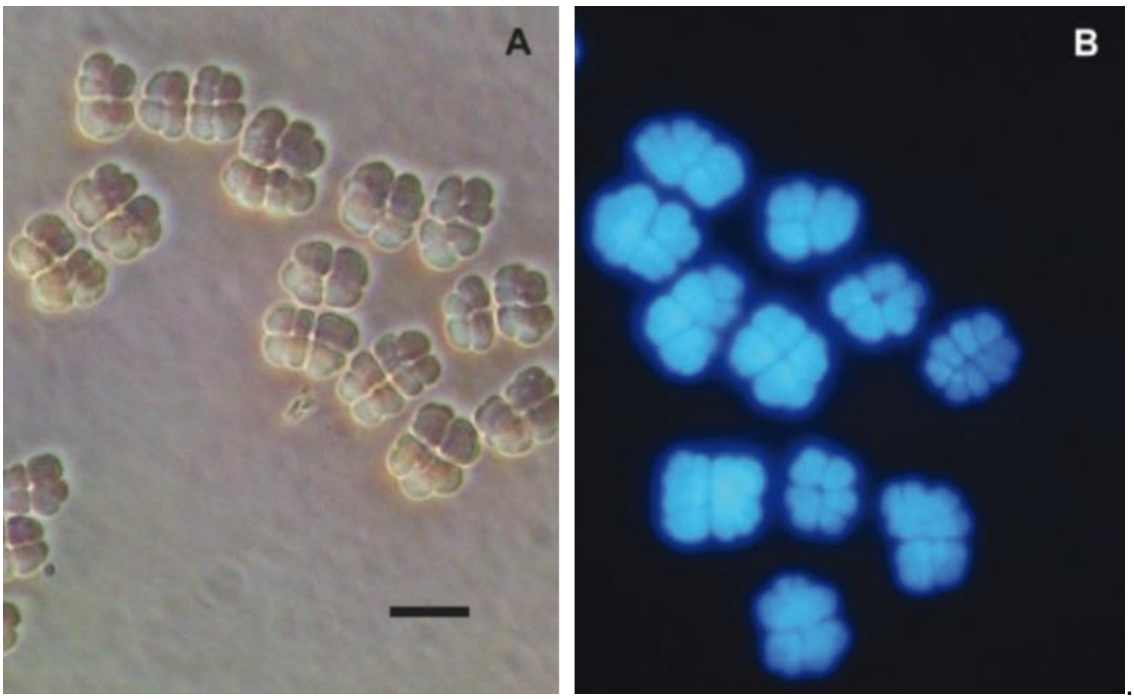

Figure 5 Methanosarcina barkeri (DSM800) : Phase contrast (A) Epifluerescence (B) image scale 10µm https://www.dsmz.de/fileadmin/Bereiche/Microbiology/Dateien/Kultivierungshinweise/Kultivierungshinweise_neu_CD/dt_Methanogene_Update.pdf

M. barkeri is a mesophilic archaea species with optimum metabolic activity at 37 °C. As photosensitisers may be negatively affected by high temperatures, *M. barkeri* was chosen to safeguard the stability of these compounds. Moreover, the generation time of this strain is slow, in the range of days in fully autotrophic conditions, so the hybrid system is expected to be more stable over a prolonged time.

For the microorganism-organic semi-conductor systems to convert CO_2 into methane by using sunlight, special organic photosensitisers have to be designed, which can interact with the specific *M. barkeri* metabolic pathways. In cooperation with the UU the specific metabolic pathways of *M. barkeri* to produce methane and the respective electron transfer pathways were discussed and analysed in detail according to the literature shown in Figure 6.

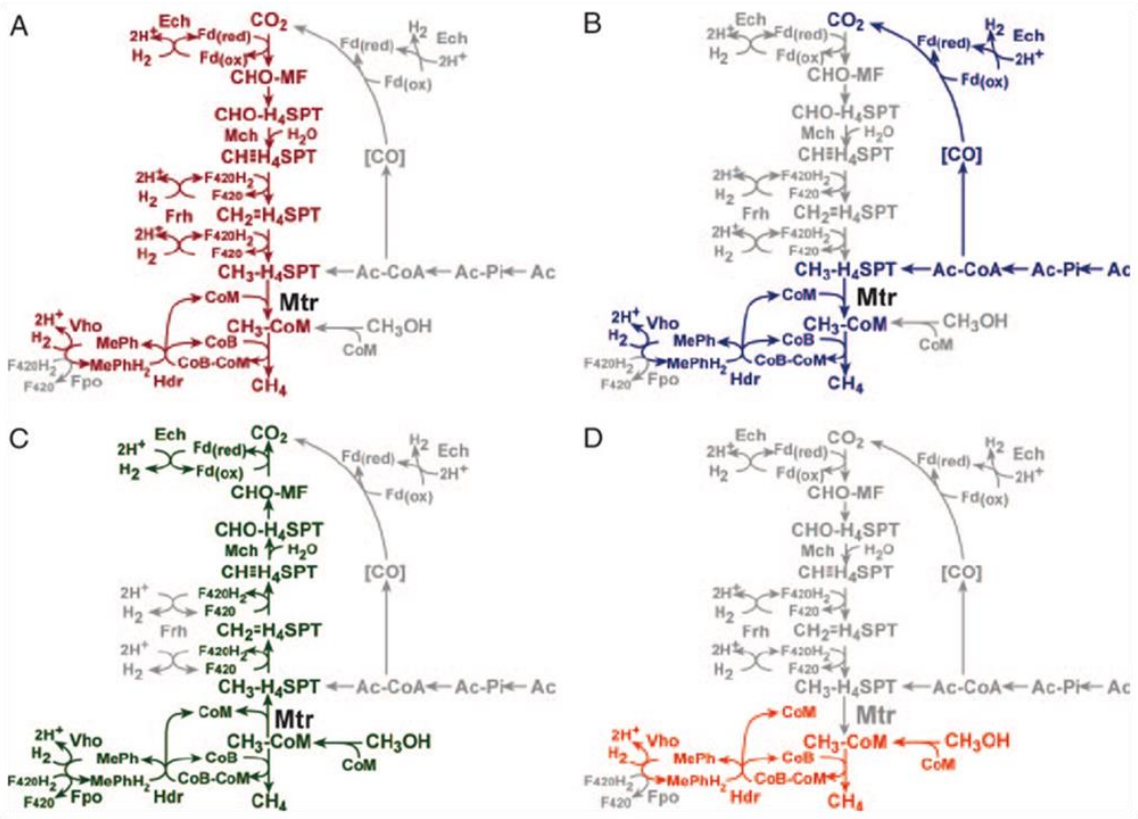

Figure 6: Four overlapping methanogenic pathways found in M. barkeri [5]

As a result, 12 polymer dots (Pdots) were designed and characterised by the UU and delivered to the TZE for application to the *M. barkeri* surface, forming a hybrid photosensitiser-archaea system. Based on the artificial photosynthesis concept, the hybrid system will be exposed to sunlight and CO_2 to produce methane via photocatalytic reduction. To mimic the sunlight in the experimental setup, the light intensity and the light spectrum of a sunlight spectrum-mimicking lamp was analysed.

In the next steps, the designed Pdots have to be applied to *M. barkeri* and screened for methane production, cell viability and surface binding etc. Then, to construct the microorganism-organic semi-conductor systems, *M. barkeri* will be mixed with different concentrations of the Pdots samples, and the mixture will be injected into Balch vials containing the autotrophic media based on DSM120.
The concentration of the Pdots will be measured, and the specific cell density of *M. barkeri* will be analysed via OD600. *M. barkeri*, which is standard cultured in

DSM120, will be harvested by centrifugation at 4500 rcf for 3 min and washed three times with autotrophic DSM120. Then, the specific concentration of *M. barkeri* can be added to different concentrations of Pdots and adjusted by 5 ml autotrophic DSM120 and cultivated under anaerobic conditions (2 absolute bar pressure, CO_2) for a defined time period at 37 °C. If necessary, the hybrid system will be centrifuged and washed again to remove unbound Pdots. Then the hybrid system will be incubated in an autotrophic Medium in anaerobic conditions under light exposure or with day and night cycle (12h/12h) at 37 °C. To analyse the efficiency of the microorganism-organic semi-conductor systems, methane production will be measured after defined time points via gas chromatography and pressure measurements. Moreover, fluorescent microscopy and OD measurements will safeguard the cell viability and cell growth of *M. barkeri*. The binding interaction of the microorganism-organic semi-conductor systems through electrostatic or hydrophobic interactions, which is beneficial to transferring a photoexcited electron from polymer nanoparticles to *M. barkeri* will be analysed in cooperation with the partners. Due to this experimental setup, the best parameters and working protocol for the artificial photosynthesis process are finally defined. Moreover, screening the 12 Pdots samples will demonstrate which Pdot design is the best and most efficient for interacting with *M. barkeri* regarding electron transfer and methane production. In the next step, the most appropriate Pdot will be improved by the UU and analysed at the TZE under the defined experimental conditions for the microorganism-organic semi-conductor systems to improve methane production.

4. Development of hybrid microorganism-organic semi-conductor systems to convert CO_2 into acetate using *Moorella thermoacetica* and sunlight (ICCAS)

In our previous work (*ACS Appl. Mater. Interfaces 2023, 15, 2183−2191*), we have shown that Pdots can work well with Ralstonia eutropha H16 (*RH16*) bacteria to produce Poly-3-hydroxybutyrate (PHB) which is a promising biomedical material due to its good mechanical durability, biodegradability and biocompatibility, making it a degradable plastic. In that work, PFODTBT Pdots were used as a photosensitiser and NR as an electron shuttle carrier (Figure 7a). In the presence of light, Pdots incubated on the bacterial surface generate electrons and holes. After receiving electrons from reduced neutral red (NRred), bacteria initiate the Calvin cycle to fix CO_2 and produce PHB via acetyl-coenzyme A and 3HB-coenzyme A. We analysed the energy levels and electron transfer mechanisms of Pdots in bacteria using cyclic voltammetry (see Figure 7b). We could find that photo-generated electrons from Pdots promote a proportion of nicotinamide adenine dinucleotide phosphate (NADPH) through NR, driving the Calvin cycle of *RH16* to convert CO_2 into PHB, with a yield of 21.3 ± 3.78 mg/L, almost three times higher than that of original *RH16*. This work provides the concept of an integrated photoactive biological factory based on organic semi-conductor polymer sites/bacteria that can produce valuable chemicals using only solar energy as energy input. This work is a good reference work for us to adapt the Pdots to other bacteria.

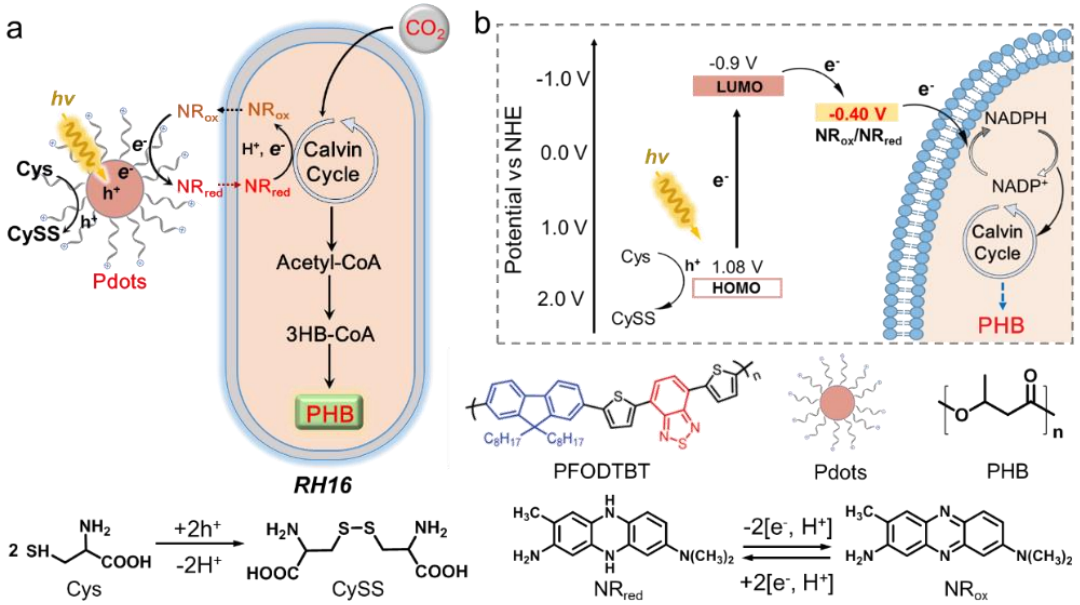

Figure 7. *Schematic of RH16/NR/Pdots photosynthetic biohybrid factory. (a) The metabolic pathway of CO_2 reduction into PHB in RH16. 3HB-CoA: 3-hydroxybutyryl-CoA, PHB: polyhydroxybutyrate, h+: electron hole, e−: electron, Cys: cysteine, CySS: cystine, NRox: the oxidised state of NR, NRred: the reduced state of NR. (b) Schematic diagram for Pdots promoting PHB production of RH16.* [3]

Recently, we studied an organic semi-conductor–bacteria biohybrid photosynthetic system, which will perform CO_2 reduction to produce acetic acid through non-photosynthetic bacteria *M. thermoacetica,* ATCC 39073 (*Angew Chem Int Ed. 2020, 59(18):7224-7229*). Therefore, according to our previous published reports (*ACS Appl. Mater. Interfaces 2023, 15, 2183−2191; Angew Chem Int Ed. 2020, 59(18):7224-7229*), we have developed a work plan for biohybrid photosynthetic system utilising organic semi-conductor and non-photosynthetic bacteria of M. thermoacetica (ATCC 39073) to reduce CO_2 for acetic acid. M. thermoacetica (ATCC 39073) has been obtained from the Global Bioresource Centre ATCC. The defined photosynthesis medium (DPM) was 0.4 g/L NaCl, 0.64 g/L K_2HPO_4, 1.5 g/L KH_2PO_4, 0.4 g/L NH_4Cl, 0.33 g/L $MgSO_4$• $7H_2O$, 0.05 g/L CaCl, 0.25 g/L KCl, 2.5 g/L $NaHCO_3$, trace mineral mix and wolfe' s vitamin mix. The heterotrophic medium comprises 2 g/L tryptone, 2 g/L yeast extract, 50 mM glucose and DPM. The DPM, glucose solution and Cys·HCl solution were sterilised by passage through a 0.2 µm SFCA filter. The heterotrophic medium, anaerobic bottles, and tubes were autoclaved for 15 min at 121°C and cooled to room temperature. Late log cultures were cryopreserved at -80 °C with 30% sterilised glycerine as a cryoprotectant.

We are particularly interested in the potential of π-conjugated organic semi-conductors in various biological systems due to their biocompatibility with organisms, electrochemical capabilities for photoelectronic conversion, and ability to fine-tune frontier molecular orbital energy levels and optical band gap.

To begin our research, we will carry out photocurrent response experiments of 12 Pdots prepared from UU to check for the successful construction of the p-n heterojunction layer on the surface of M. thermoacetica, and perform electrochemical analyses to obtain further photophysical information, such as energy level (HOMO and LUMO) and electrical impedance.

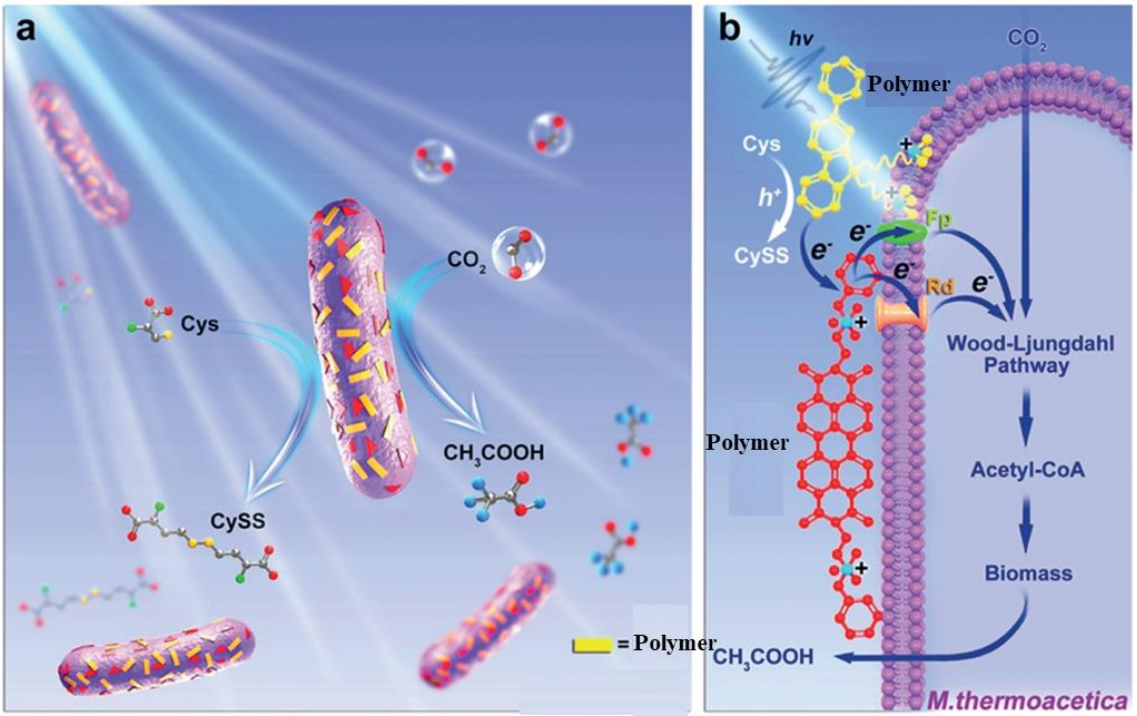

Figure 8. *a) Diagram of the Polymer/M. thermoacetica photosynthesis hybrid system and b) the photoexcited electron generated from Polymer under illumination transferred by the membrane protein and finally passed on to the Wood–Ljungdahl pathway for CO_2 reduction.*[1]

To construct the photosynthesis biohybrid system, M. thermoacetica will be mixed with different concentrations of polymer nanoparticles, and the mixture will be injected into culture tubes containing DPM and incubi (see figure 8a). After calculation for 24 hours, the *M. thermoacetica* could be harvested by centrifuging at 7200 rpm for 3 min and washed with PBS three times. Then, 0.2 OD600 of *M. thermoacetica* could be mixed with different concentrations of Pdots and adjusted by 5 mL DPM injected into culture tubes for 120 mins at 52 °C. The π-conjugated semi-conductors possess an excellent ability to harvest solar energy and generate electrons, driving the metabolic processes of organisms. As for the preparation of the biohybrid system, 50 mM glucose and 0.1 wt.% cysteine was supplemented into 50 mL heterotrophic medium, which was agitated at 52 °C in anaerobic bottles. The headspace of each anaerobic bottle was pressurised to 150 kPa with 80:20 N_2:CO_2. Before photosynthesis measurement, 0.2 wt% cysteine was added to the above tubes. Then each tube was stirred magnetically at 300 rpm and heated to a temperature of 52 °C. Also, polymer nanoparticles (such as P100, P160A, P160B, P160C, P160F1, P160F3, P160L1, P160L2, P160M, P160N, P160P, and P161) may interact with the surface of *M. thermoacetica* through electrostatic or hydrophobic interactions, which is beneficial to the transfer of a photoexcited electron from polymer nanoparticles to *M. thermoacetica*. Furthermore, the interaction of conjugated molecules and *M. thermoacetica* should be confirmed by using Laser scanning confocal: microscopy (CLSM), scanning electron microscope (SEM), and isothermal titration calorimetry (ITC).

To better understand the mechanism of photoexcited electron transfer, we will use cyclic voltammetry (CV) to determine the charge separation capability of M. thermoacetica incubated with polymer nanoparticles. This will help us demonstrate the potential for in situ generation of photo-generated electrons on microbial surfaces, which can then transfer to the Wood-Ljungdahl pathway and drive acetic acid synthesis from CO_2 (see Figure 8b). The Wood–Ljungdahl pathway is a set of biochemical reactions used by some bacteria and archaea called acetogens. It is also known as the reductive acetyl-coenzyme A (Acetyl-CoA) pathway. This pathway enables these organisms to use hydrogen as an electron donor, and carbon dioxide as an electron acceptor and as a building block for biosynthesis. In this pathway carbon dioxide is reduced to carbon monoxide and formic acid or directly into a formyl group. The formyl group is reduced to a methyl group and then combined with the carbon monoxide and Coenzyme A to produce acetyl-CoA. Two specific enzymes participate on the carbon monoxide side of the pathway: CO Dehydrogenase and acetyl-CoA synthase. The former catalyses the reduction of the CO2 and combines the resulting CO with a methyl group to give acetyl-CoA. The pathway occurs in bacteria and archaea, e.g., methanogens and in acetate-producing bacteria such as Clostridium. Unlike the Reverse Krebs and Calvin cycles, this process is not cyclic. A recent study of the genomes of a set of bacteria and archaea suggests that all cells' last universal common ancestor (LUCA) was using the Wood–Ljungdahl pathway in a hydrothermal setting.

Finally, we will measure the photosynthetic production of acetic acid in the biohybrid system under light illumination and calculate the quantum yield. All photosynthesis measurements were conducted with DPM. Prior to photosynthesis, an 0.2 wt% cysteine was added to the above tubes. Then each tube was stirred magnetically at 300 rpm and heated to a measured temperature of 52 °C employing oil bath. During the photosynthesis measurements, the headspace of each tube remained 150 kPag with 80:20 N_2:CO_2. A Xenon fibre optic lamp with filters larger than 420 nm was employed: with a measured photon flux of 1.48×10^{15} s^{-1} cm^{-2}. The system simulated sunlight with light-dark cycles in 3 days. Concentrations of photosynthesis acetic acid were measured by H-NMR with sodium 3-(trimethylsilyl)-2,2',3,3’-tetradeuteropropionate (TMSP-d4) as the internal standard in D_2O. Samples were taken out every 12-hour interval time with a disposable syringe to the centrifuge tube, and then they were separated by centrifugation at a speed of 7200 rpm for 3.0 min. 450 µL of the supernatant and 50 µL D2O were mixed and carefully injected into the NMR tube, and then the 1HNMR measurements were performed.

Polymer/M. thermoacetica reaction equations:

$$h\nu \xrightarrow{\textbf{Polymer}} e^{-} + h^{+}$$

$$H^{+} + e^{-} \xrightarrow{\textbf{\textit{M. thermoacetica}– Polymer} } [H]$$

$$2CO_2 + 8[H] \xrightarrow{\textbf{M. thermoacetica– Polymer}} CH_3COOH + 2H_2O$$

$$2h^{+} + 2Cys \xrightarrow{\textbf{\textit{M. thermoacetica}– Polymer}} 2H^{+} + CySS$$

$$8hv + 2CO_2 + 8Cys \xrightarrow{\textbf{\textit{M. thermoacetica}– Polymer}} CH_3COOH + 2H_2O + 4CySS$$

Figure 9. Polymer/M. thermoacetica reaction equations

5. Workflow in WP2

The work that was already done (up to M6) and the work planned from M6-M18 are shown in Table 1.

Table 1. Experimental design of the hybrid systems in chronological order.

Objectives	M2	M4	M6	M8	M10	M12	M14	M16	M18
Photosensitiser design (UU)	X	X	X						
M. barkeri culture and preparation of photocatalytic system (TZE)	X	X	X						
M. thermoacetica culture and preparation of photocatalytic experiments (ICCAS)	X	X	X						
Photocatalytic experiments on M. barkeri system (TZE)									
Feedback from TZE to UU									
Photocatalytic experiments on M. barkeri system (TZE)									
Feedback from TZE to UU									
Optimisation of Photosensitizer									
Optimisation of final bio-hybrid systems by optimising photocatalytic condition (TZE, ICCAS)									
Optimisation of final bio-hybrid systems by optimising photosensitiser (UU, ICCAS)									
Scaling up microorganism and photosensitiser for device test (UU, TZE, ICCAS)									

6. CONCLUSION

During M1-M6, all partners extensively discussed the design of the bio-hybrid system to understand possible charge transfer pathways involved in the bio-hybrid systems and then plan the experiments accordingly.

As a result, UU has designed and characterised various Pdots with positively charged surfaces. TZE has worked on *Methanosarcina barkeri* culture and relevant tests. ICCAS has worked on *Moorella thermoacetica* culture and relevant tests. All Pdots samples were delivered to and received by TZE and ICCAS in December 2022.

The bio-hybrid systems have been well-designed to properly achieve the project goals and previous plan (according to the Grant Agreement). Therefore, all the activities performed from M1-M6 and the next experiments (M6-M18) were well planned and executed and were/will be in accordance with the experimental design presented here.

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