Photoactivatable surface-functionalized diatom microalgae for colorectal cancer targeted delivery and enhanced cytotoxicity of anticancer complexes

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Abstract: Systemic toxicity and severe side effects are commonly associated with anticancer chemotherapies. New strategies based on enhanced drugs selectivity and targeted delivery to cancer cells while leaving healthy tissue undamaged can reduce the global patient burden. Herein we report the design, synthesis and characterization of a bio-inspired hybrid multifunctional drug delivery system based on diatom microalgae. The microalgae’s surface was chemically functionalized with hybrid vitamin B12-photoactivable molecules and the materials further loaded with highly active rhenium(I) tricarbonyl anticancer complexes. The constructs showed enhanced adherence to colorectal cancer (CRC) cells via transcobalamin (II) receptors and slow release of the chemotherapeutic drugs. The overall toxicity of the hybrid multifunctional drug delivery system was further enhanced by photoactivation of the microalgae surface. Depending on the construct and anticancer drug, a 2-fold increase in the cytotoxic efficacy of the drug was observed upon light irradiation. The use of this targeted drug delivery strategy, together with selective spatial-temporal light activation may lead to lower effective concentration of anticancer drugs, thereby reducing medication doses, possible side effects and overall burden for the patient.

Keywords: diatoms; drug delivery system; HCT-116; porphyrin; vitamin B12; carbon monoxide releasing molecule; rhenium; cancer.

1. Introduction

Targeted drug delivery for the confined treatment of colonic diseases is becoming increasingly important to address locally life-threatening disorders like colorectal cancer (CRC). Colon-focused drug delivery (CFDD) permits direct treatment at the disorder site, lowers drug dosing, and decreases the chance of systemic side-effects. Indeed, most available chemotherapeutics are poorly tolerated by patients and induce severe side effects that are unbearable in many cases. CFDD is a strategy actively pursued to address inflammatory bowel diseases [1-3] and most importantly CRC. Indeed, despite advances in diagnostic and therapeutic modalities, CRC is the third most commonplace cause of cancer-related deaths worldwide. Generally, CRC originates from the inner wall of the colorectal epithelium, develops as a polyp and finally spreads by invading nearby lymph nodes and other organs. As pointed out in recent reviews, issues like the path of the gastro-intestinal (GI) tract, dynamic pH changes and the inability to discern healthy tissues from cancerous ones must be overcome in order to develop effective materials for CFDD treatment [4-6].

Different porous material such as zeolites, nanoparticles, dendrimers/polymers, nano-hydrogels or metal organic frameworks were shown to be prospective tools in the field of drug delivery systems (DDSs) and cancer targeted treatments [7-17]. In terms of material design for colon-focused drug delivery, several approaches have been described. Salleh et al., e.g., evaluated the performance of gelatin-coated type Y zeolite for the controlled release of the anticancer drug zerumbone [18] encapsulated in its pores, showing sustained and prolonged drug release over 24 h [19]. Li and
coworkers addressed CFDD via a nano-in-micro dual drug delivery platform composed of halloysite nanotubes and a pH-responsive polymer. This clay mineral was loaded with atorvastatin and celecoxib, which were released only at pH 7.4 and effectively inhibited colon cancer cell proliferation [20]. Xu et al. recently presented up conversion nanoparticles (UCNPs) to trigger cancer immunotherapy in CRC by NIR-induced photodynamic therapy (PDT) to directly destroy tumor cells and to stimulate immune responses by triggering the maturation of dendritic cells and secretion of cytokines [21]. Others have used gold [22] and folate-coated nanoparticles for active CFDD of nanotherapeutics [23]. Finally, different authors have used reactive oxygen species (ROS) responsive nanoplatorms and polymers as drug delivery system with excellent results [6].

We have recently began to develop CFDD materials based on environmentally sustainable, abundant and inexpensive diatom microalgae (DEMs) [24] with the aim of designing an innovative structure able to simultaneously target the tumor site and release loaded chemotherapeutics in its immediate proximity [25]. DEMs, as the fossil frustules of diatoms, recently gained attention for their use in drug delivery due to their biocompatibility and ability to shuttle and slowly release different drugs [26-29]. Diatoms are enclosed in a three-dimensional highly ordered silica shell (called frustule) and represent an inexpensive and well-engineered source of microporous silica [30-33]. Our original proof of concept relied on the chemical functionalization of the diatom microalgae’s surface with vitamin B₁₂, which allowed specific binding of the material to CRC cells and, therefore, discrimination between healthy and diseased tissues. The next step in our concept is illustrated in Figure 1. This bio-inspired hybrid multifunctional drug delivery system is modified by the introduction of photoactivatable units (orange spheres in Figure 1), which are envisioned to act in concert with the loaded chemotherapeutic drugs (light blue squares) in order to: a) sensitize the tumor and b) lower the overall drug dose needed for effective treatment.

Figure 1. Left. Concept showing the bio-inspired hybrid multifunctional drug delivery system described in this study. Natural diatom microalgae (DEMs) are functionalized at their surface with a photoactivatable elements (orange spheres) linked to vitamin B₁₂ (red spheres) acting as the outer tumor targeting layer. The construct can be loaded with a chemotherapeutic drug (light blue squares) to be selectively delivered to the tumor site in the intestinal tract. Once DEMs are bound to the tumor mass, the chemotherapeutic drug is slowly released. In addition to that, the micro-capsules can be photo-activated to generate a toxic CO or O₂, sensitizing the tumor to the treatment. Right. Photo-induced chemical reactions at the surface of the diatom microalgae (vide infra for more details).

For this purpose, two complex bio-vehicles were synthetized. In both cases, the outer layer of the microalgae was still composed of cobalamin (vitamin B₁₂, red spheres in Figure 1). The inner layers of the constructs were built with either a chemical photosensitizer (for photodynamic therapy, PDT) or a photo-triggered CO-releasing molecule (see right inserts in Figure 1). Photodynamic therapy is a minimally invasive treatment incorporating three different components: a light source, a chemical photosensitizer (PS) and tissue oxygen. This process aims to generate endogenously highly
toxic singlet oxygen (\(1\text{O}_2\)) and reactive oxygen species (ROS) upon light irradiation [34]. The so
toxic species induce cell apoptosis in tumors [35,36]. The advantages of this
technique are the spatio-temporal controlled activity allowed by the photo-activation and the
potential to be combined with other therapeutic treatments as chemotherapy, surgery, radiotherapy
or immunotherapy [37]. As chemical photosensitizer for our hybrid multifunctional drug delivery
system, we chose porphyrins, as they absorb at several wavelengths in the visible region (Soret band
in the blue and Q-bands to the red) and show long-lived triplet states allowing high \(1\text{O}_2\) quantum
yields [38-41]. Furthermore, coordination of metal ions such as \(\text{Zn}^{2+}\), \(\text{Ga}^{3+}\) or \(\text{Si}^{4+}\) allows tuning \(1\text{O}_2\)
generation capacities of porphyrins giving long excited state lifetimes and permitting high singlet
oxygen yields [42]. As photo-triggered CO-releasing molecule we selected a \([2,2’\text{-bipyridine}\]-3,4’-
dicarboxylic acid manganese tricarbonyl complex. Such type of complexes (\textit{vide infra} for detailed
chemistry) are well-known to decompose if photo-irradiated, liberating in the process carbon
monoxide. CO, in turn, is known to exhibit antiproliferative and cytotoxic effects in cancer treatments
[43-47]. In this contribution, we describe the preparation of these new bio-inspired hybrid
multifunctional drug delivery systems. In addition to that, we detail CRC cellular interaction of the
same, their drug loading and releasing properties, and their effects on the proliferation of CRC HT-
116 cells in the dark and when photo-activated.

2. Materials and Methods

2.1 Chemicals and Materials

Diatomaceous earth microalgae (DEMs), in the form of Celatom® Fw-14, were obtained from
Applied Minerals Ltd. (Staffordshire, UK). Pyrrole was purchased from Acros Organics (New Jersey)
and 4-carboxybenzaldehyde from Fluka Chemicals. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
(EDC), N-hydroxysuccinimide (NHS), tetra-phenylporphyrin (TPP) and 9,10-dimethylanthracene
(DMA) were purchased from TCI Europe. Triethylamine, Vitamin B\(_12\), 1,1’-Carbonyl-di-(1,2,4-
triazole) (CDT), 4,7,10-trioxa-1,13-tridecanediamine and zinc acetate dihydrate were delivered by
Sigma-Aldrich. (3-aminopropyl)triethoxysilane (APTES), H\(_2\)SO\(_4\) and HCl were purchased from
Honeywell Research chemicals (Bucharest, Romania). H\(_2\)O\(_2\) and glycerol were purchased from
Reactolab SA (Switzerland). All the rhenium complexes used as drug models were synthetized as
described in a previous paper [48].

2.2 Characterization Techniques

NMR analyses were performed with a Bruker Advance III 500 MHz, or 400 MHz respectively. The corresponding \(^1\text{H}\) chemical shifts are reported relative to residual solvent protons and carbons. Mass analyses were performed either using ESI-MS on a Bruker FTMS 4.7-T Apex II in positive mode or MALDI with a Bruker UltraflieXtreme MALDI-TOF. UV-Vis spectra were measured using a Jasco V730 spectrophotometer. Solid-state UV-Vis spectra were measured using a Perkin Elmer UV/VIS/NIR Spectrometer Lambda 900 with a Perkin Elmer 150 mm Int. Sphere. The dry powder samples (app. 5 mg) were dispersed between two quartz slits and stacked before being analyzed. IR spectra were measured using a Perkin Elmer FTIR Frontier Serie 99155 equipped with a PIKE TECHNOLOGIES GladiATR system. Preparative and analytical HPLCs were done with a Merck Hitachi Hitachi L-7000 system, which comprises a Pump L-7100 and a UV-Detector L-7400. For preparative HPLC, a column Macherey-Nagel Nucleodur C18 HTec (5 \(\mu\)m particle size, 110 \(\text{Å}\) pore size, 250 \(\times\) 21 mm) was used. Aqueous trifluoroacetic acid 0.1 % solution and pure methanol were
dispersely used as solvents (A) and (B). The compounds were separated using the following
gradient: 0–5 min (75 % A), 5–35 (75 % A \(\rightarrow\) 0 % A), 35–45 min (100 % B) or 0–5 min (50 % A), 5–30
(50 % A \(\rightarrow\) 0 % A), 30–45 min (100 % B), the flow rate set to 5 mL min\(^{-1}\) and the compounds detected
at 320 nm. For the analytical HPLC, a Macherey-Nagel Nucleodur C18 HTec (5 \(\mu\)m particle size, 110
\(\text{Å}\) pore size, 250 \(\times\) 21 mm) was used. Aqueous trifluoroacetic acid 0.1 % solution and pure methanol
were respectively used as solvents (A) and (B). The compounds were separated using the following
gradient: 0–5 min (75 % A), 5–35 (75 % A \(\rightarrow\) 0 % A), 35–45 min (100 % B) or 0–5 min (50 % A), 5–30
(50 % A → 0 % A), 30–45 min (100 % B), the flow rate set to 0.5 mL min⁻¹ and the compounds detected at 320 nm. Scanning Electron Microscopy (SEM) pictures were recorded using a Tescan Mira3 LM FE SEM. Samples were coated with 4 nm to 10 nm platinum depending the analysis and recorded at 10.0 kV under high vacuum 6x10⁻⁴ Pa. Measurements of Inductively Coupled Plasma with Optical Emission Spectroscopy (ICP-OES) were performed with a Perkin Elmer Optima 7000 DV. The zeta potential was measured with a DelsaMax PRO device from Beckmann Coulter, using the Smoluchowski mode when the samples were diluted in a PBS buffer solution to 0.01 wt% and sonicated before proceeding the measurement. High resolution ESI-MS were performed on a Bruker FTMS 4.7-T Apex II in positive mode.

2.3 DFT Calculations.

All computations were performed with the Gaussian 09 programs. Geometry optimizations were performed employing a solvent continuum dielectric model for water. The hybrid-GGA (GGA = generalized gradient approximation) functional M06 and B3LYP were used in combination with the standard LanL2DZ basis sets for the optimization of B12-TCPP and B12-Mn respectively. For the spin state of the molecules (singlet state in all cases), the default spin formalism was followed in the calculations and default Gaussian 09 values were adopted for the numerical integration grids, self-consistent-field (SCF) and geometry optimization convergence criteria. Geometries were optimized without symmetry restrictions. The calculated molecular geometries were visualized using GaussView.

2.4 Syntheses

5,10,15,20-tetrakis (4-carboxyphenyl) porphyrin (TCPP). TCPP was synthetized via the monopyrrole tetramerization method firstly described by Adler et al. and slightly modified [49,50]. 1.5 g of 4-carboxybenzaldehyde (10 mmol) were added to 50 mL acetic acid, stirred and heated to 80°C for better dissolution of the aldehyde. Afterwards, distilled pyrrole (0.7 mL, 10 mmol) was added, the reaction mixture was brought to reflux and stirred for 2h. After allowing the reaction mixture to cool to room temperature, the reaction flask was placed in the fridge to induce precipitation of the porphyrin. Vacuum filtration of the reaction mixture, washing the residue with DCM (5 times 50 mL) and recrystallization permitted to isolate a dark purple solid (1.1 g, 1.4 mmol, 55% yield) which was dried under vacuum in the oven. 1H NMR (400 MHz, DMSO -d6) δ(ppm) = -2.93 (s, 2 H), 8.32 - 8.36 (d, 8 H), 8.37 - 8.40 (d, 8 H), 8.85 (s, 8 H), 13.08 (br. s., 4 H) which was consistent with previous studies [51] HR-ESI-MS (ESI+) (m/z): [M + H]⁺ = 791.0, calculated for C₄₈H₃₀N₄O₈ = 790.21; UV-Vis (in MeOH, λmax, nm): 415, 512, 546, 588.5, 645.

Vitamin B₁₂ derivative B₁₂-1. Cyanocobalamin was modified by pegylation on the 5'-hydroxyl group of the ribose moiety by slight modification of published procedures [52,53]. Briefly, 100 mg of reduced B₁₂ (0.0739 mmol) were mixed with 75 mg of CDT in 3 mL of DMSO and stirred overnight. The product was then precipitated in ethyl acetate (150 mL) and centrifuged for 10 min at 6'000 rpm in order to recover a red precipitate. The dried precipitate was resolubilized in 1 mL anhydrous DMF and 100 μL of 4,7,10-trioxa-1,13-tridecanediamine in 1 mL anhydrous DMF was added and stirred for 24h. The mixture was precipitated in 3:1 ether: ethyl acetate (150 mL) and centrifuged for 10 min at 6'000 rpm before being purified by preparative HPLC. The fraction corresponding to the pegylated B₁₂ (B₁₂-1) was evaporated under vacuum and further lyophilisated to recover a pure dried red powder.

B₁₂-TCPP. In the dark under a nitrogen atmosphere, to a solution of TCPP (25.5 mg, 3.23x10⁻⁵ mol) in 3 mL anhydrous DMF, EDC hydrochloride (12.3 mg, 6.45x10⁻⁵ mol) and NHS (7.4 mg, 6.45x10⁻⁵ mol) were added. After stirring for 1 h at room temperature, B₁₂-1 (34.4 mg, 2.15x10⁻⁵ mol) in 1 mL anhydrous DMF and 20 μL of triethylamine were added and the reaction mixture was stirred for 3 days in the dark under argon atmosphere. Afterwards, the product was precipitated in 3:1 ether: ethyl acetate (150 mL) and centrifuged for 10 min at 6000 rpm before being purified by preparative HPLC (7.7 mg, 3.23x10⁻⁶ mol, 15% yield). 1H NMR (500 MHz, methanol-d4) δ(ppm) = 0.42 (s, 3 H), 1.05 (s, 3 H), 1.19 (d, J=6.41 Hz, 4 H), 1.26 - 1.37 (m, 15 H), 1.80 (s, 3 H), 2.22 (d, J=9.31 Hz, 6 H), 2.33 (d, J=7.78
B$_2$-ZnTCPP. To a solution of B$_2$-TCPP (3 mg, 1.26-10$^{-5}$ mol) in 3 mL MeOH, 0.5 mg Zn(OAc)$_2$-2H$_2$O were added and the reaction mixture was stirred under reflux for 1 h. Afterwards, the reaction mixture was allowed to cool to room temperature. Vacuum filtration and washing the residue several times with water, allowed removing excess zinc and permitted to isolate a brown-red solid that was dried under vacuum in the oven. UV-Vis (in MeOH, $\lambda_{max}$, nm): 360, 415, 512, 546, 588.5, 645.

TCPP derivative TCPP-1. To a solution of TCPP (30 mg, 3.8-10$^{-5}$ mol) in 5 mL anhydrous DMF EDC hydrochloride (9.1 mg, 4.75-10$^{-5}$ mol) and NHS (5.5 mg, 4.75-10$^{-5}$ mol) were added. After stirring for 2 h at room temperature, 10 $\mu$L of 4,7,10-trioxadodecane and triethylamine (20 $\mu$L) were added and the reaction mixture was stirred for 3 days in the dark under argon atmosphere. The product was precipitated in 3:1 ether: ethyl acetate (600 mL) and centrifuged for 10 min at 6000 rpm before being purified by preparative HPLC (8 mg, 8.06-6 mol, 21% yield). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$(ppm) = -2.88 (br. s., 2 H), 1.76 - 1.85 (m, 2 H), 1.92 (quin, $J=6.63$ Hz, 2 H), 2.84 - 2.93 (m, 2 H), 3.52 (t, $J=6.05$ Hz, 4 H), 3.54 - 3.57 (m, 2 H), 3.58 - 3.62 (m, 8 H), 7.62 (br. s., 2 H), 8.24 - 8.47 (m, 16 H), 8.77 (t, $J=5.56$ Hz, 1 H), 8.86 (s, 8 H) (ESI Figure S2); HR-ESI-MS (ESI+) (m/z): [M + H]$^+$ = 993.3, [M + 2H]$^{2+}$/2 = 497, calculated for C$_{58}$H$_{52}$N$_6$O$_{10}$ = 993.09; UV-Vis (in MeOH, $\lambda_{max}$, nm): 410, 517, 601.

ZnTCP-1. To a solution of 10 mg TCPP (1.0-10$^{-5}$ mol) in 5 mL DMF, 2.65 mg Zn(OAc)$_2$-2H$_2$O (1.2-10$^{-5}$ mol) were added and the reaction mixture was stirred under reflux for 2 h. Afterwards, the reaction mixture was allowed to cool to room temperature. Vacuum filtration and washing the residue several times with water, allowed removing excess zinc and permitted to isolate a dark green solid that was dried under vacuum in the oven (10 mg, 9.47-10$^{-6}$ mol, 94.7% yield). UV-Vis (in MeOH, $\lambda_{max}$, nm): 415, 512, 546, 588.5, 645.

fac-[Mn(I)(Br(CO))(4,4-carboxyl-2,2-bipyridin)] (Mn). The manganese(I) photoCORM complex was synthesized by slight modification of a published procedure [54]. Bromopentacarbonylmanganese(I) (1 mg, 0.55 mmol, 1 eq.) with 1.1 eq of the 4,4′-Bipyridine-4′-dicarboxylic acid (148 mg, 0.61 mmol) were stirred in THF (20 mL) under dark conditions overnight. Afterwards, the mixture was filtered and the supernatant containing the complex (Mn) was dried under vacuum. Mn was then purified via HPLC before being evaporated to dryness in a lyophilizer. An orange-yellow solid was recovered. Yield 67.9 mg (26%). UV-Vis spectrum in methanol solution: $\lambda_{max}$ = 410 (ESI Figure S13); FTIR (ATR, cm$^{-1}$): $\nu$C≡O = 2026, 1943, 1901, 1733 (ESI Figure S8). ESI-MS (pos$^+$): [M-Br]$^+$ = 383.01, calculated for C$_{15}$H$_{8}$MnN$_2$O$_7$ = 382.97. All other analytical data are in agreement with what reported previously [54].

Vitamin B$_2$ derivative B$_2$-Mn. Cyanocobalamin was modified by pegylation on the hydroxyl function of the ribose part (5′-OH) of the molecule as previously described.[25,55] The pegylated B$_2$ was then reacted with the manganese complex Mn to give the B$_2$-Mn. For this purpose 1.6 mg (0.0035 mmol, 1.5 eq.) of Mn were activated in MES buffer pH 5.5 with 46 $\mu$L EDC during 30 min, in the dark, before adding 17 mg of NHS for another 30 min and finally pouring dropwise 3.7 mg (0.0023 mmol, 1.0 eq) of pegylated B$_2$ in 1 mL MES buffer pH 5.5. For the next 5 h, 15 mg of EDC were added each hour. Two hours after the last EDC addition, 50 $\mu$L of triethylamine were added to the mixture that was allowed to react overnight in the dark. The crude was finally purified by preparative HPLC. Each manipulation was done in dim light due to the photo-sensitivity of Mn. Yield 3.7 mg (79%). $^1$H NMR (400 MHz, MeOD-{d$_4$}): $\delta$ = 9.11 (t, $J=5.5$ Hz, 2H), 8.88 (d, $J=7.4$ Hz, 2H), 8.16 (d, $J=4.3$ Hz, 2H), 7.79 (t, $J=5.64$ Hz Hz, 1H)$^*$, 7.23 (s, 1H), 6.98 (s, 1H), 6.42 (s, 1H), 6.19 (d, $J=2.5$ Hz, 1H), 6.02 (s, 1H), 4.77-4.72 (m, 2H), 4.58 (d, $J=10.0$ Hz, 1H), 4.22-4.16 (m, 3H), 3.87 (q, $J=5.33$ Hz, 1H), 3.63-3.60 (m, 10H), 3.55 (t, $J=5.47$ Hz, 2H), 3.51 (q, $J=1.65$ Hz, 1H), 3.49 (s, 1H), 3.46 (s, 1H), 3.38-3.32 (m, 8H), 2.93-2.86 (m, 1H), 2.80-2.73 (m, 2H), 2.64-2.54 (m, 4H), 2.53-2.39 (m, 14H), 2.28 (s, 6H), 2.08-1.93

Vitamin B$_2$ derivative B$_2$-Mn. Cyanocobalamin was modified by pegylation on the hydroxyl function of the ribose part (5′-OH) of the molecule as previously described.[25,55] The pegylated B$_2$ was then reacted with the manganese complex Mn to give the B$_2$-Mn. For this purpose 1.6 mg (0.0035 mmol, 1.5 eq.) of Mn were activated in MES buffer pH 5.5 with 46 $\mu$L EDC during 30 min, in the dark, before adding 17 mg of NHS for another 30 min and finally pouring dropwise 3.7 mg (0.0023 mmol, 1.0 eq) of pegylated B$_2$ in 1 mL MES buffer pH 5.5. For the next 5 h, 15 mg of EDC were added each hour. Two hours after the last EDC addition, 50 $\mu$L of triethylamine were added to the mixture that was allowed to react overnight in the dark. The crude was finally purified by preparative HPLC. Each manipulation was done in dim light due to the photo-sensitivity of Mn. Yield 3.7 mg (79%). $^1$H NMR (400 MHz, MeOD-{d$_4$}): $\delta$ = 9.11 (t, $J=5.5$ Hz, 2H), 8.88 (d, $J=7.4$ Hz, 2H), 8.16 (d, $J=4.3$ Hz, 2H), 7.79 (t, $J=5.64$ Hz Hz, 1H)$^*$, 7.23 (s, 1H), 6.98 (s, 1H), 6.42 (s, 1H), 6.19 (d, $J=2.5$ Hz, 1H), 6.02 (s, 1H), 4.77-4.72 (m, 2H), 4.58 (d, $J=10.0$ Hz, 1H), 4.22-4.16 (m, 3H), 3.87 (q, $J=5.33$ Hz, 1H), 3.63-3.60 (m, 10H), 3.55 (t, $J=5.47$ Hz, 2H), 3.51 (q, $J=1.65$ Hz, 1H), 3.49 (s, 1H), 3.46 (s, 1H), 3.38-3.32 (m, 8H), 2.93-2.86 (m, 1H), 2.80-2.73 (m, 2H), 2.64-2.54 (m, 4H), 2.53-2.39 (m, 14H), 2.28 (s, 6H), 2.08-1.93
(m, 8H), 1.91-1.74 (m, 12H), 1.74-1.65 (m, 2H), 1.52 (s, 1H), 1.55 (s, 1H), 1.46 (s, 3H), 1.53 (d, 1H), 1.34 (s, 3H), 1.26 (d, J = 6.3 Hz, 3H), 1.22 (s, 3H), 1.50 (s, 3H), 1.01-0.88 (m, 2H), 0.40 (s, 3H) ppm. *amide, disappear with proton exchange (ESI Figure S3); UV/Vis spectrum in methanol solution: λmax = 361, 415, 519, 551 (ESI figure S14); FTIR (ATR, cm⁻¹): νC≡O = 2043, 1961, 1944 (ESI Figure S9).

2.5 DEMs Functionalizations

The isolation, purification and surface activation of DEMs were done as previously described by Delasogie et al. [25]. The aminated DEMs surface was generated by APTES condensation.

**B12-TCPP-DEMs.** To a solution of B12-TCPP (2.5 mg, 1.05∙10⁻⁶ mol) in 1 ml anhydrous DMF, EDC hydrochloride (0.4 mg, 2.1∙10⁻⁶ mol) and NHS (0.26 mg, 2.1∙10⁻⁶ mol) were added. After stirring for 1 h in the dark at room temperature, ≈10 mg of aminated DEMs in 0.2 ml anhydrous DMF and triethylamine (20 μL) were added and the reaction mixture was stirred for 3 days in the dark at room temperature. Afterwards, the functionalized DEMs were separated by centrifugation and washed several times with MeOH and water, yielding a slightly reddish powder. The material was characterized by UV solid-state and zeta potential (Figure 6 and Table 1).

**B12-ZnTCPP-DEMs.** B12-TCPP-DEMs in MeOH were stirred under reflux in the presence of Zn(OAc)₂·2H₂O (1 mg) for 1 h. Afterwards, the functionalized DEMs were separated by centrifugation and washed several times with MeOH and water to yield a slightly brownish powder. The material was characterized by UV solid-state and zeta potential (Figure 6 and Table 1).

**Mn-DEMs.** In order to functionalize the aminated DEMs with Mn, 0.5 mg of Mn were dissolved in 1 mL DMF with 1 mg of EDC under dim light and stirred for 30 min. Then 1 mg of NHS was added and let react for the next 30 min. After direct addition of ≈ 10 mg of aminated DEMs and 20 μl TEA, the mixture was reacted overnight at RT in the dark. The mixture was then centrifuged, the supernatant discarded, and this was repeated with 10 mL DMF and 10 mL methanol thrice. The sample was finally dried under vacuum. The recovered powder was denoted as Mn-DEMs.

**B12-Mn-DEMs.** To achieve the functionalization of aminated DEMs with B12-Mn, typically 0.8 mg of B12-Mn was added to 0.15 mg of EDC chloride and 0.10 mg of NHS in 1 ml DMF for 1 h dim. light. Then 2 mg of DEMs in 0.5 ml DMF was added to the mixture. 20 μl of TEA were added directly after. The mixture was reacted at RT in the dark overnight. The mixture was then centrifuged and the supernatant discarded. The recovered powder was washed with 20 ml DMF and with 20 ml methanol thrice and finally dried under vacuum overnight. The recovered powder was denoted as B12-Mn-DEMs.

2.6 Photodynamic Measurements

For fluorescence quantum yield and lifetime measurements, diluted solutions of ZnTCPP, ZnTCPP-1 and B12-ZnTCPP in DMSO were prepared in 1 cm quartz cuvettes. Fluorescence spectra were recorded with a FS5 Spectrofluorometer from Edinburgh Instruments. Fluorescence quantum yields were measured with the same instrument, equipped with an integrating sphere. The fluorescence lifetime of the samples were determined with a time-correlated single photon counting (TCSPC) LifeSpec II instrument from Edinburgh Instruments equipped with a EPL-405 picosecond pulsed diode laser from Edinburgh Photonics. The detection was set to emitted photons at a wavelength of 660 nm. Both quantum yields and lifetime data were elaborated with Fluoracle software provided by Edinburgh.

Singlet oxygen quantum yield (ΦΔ) were determined via assessment of light absorption decrease based on the oxidation of 9,10-dimethylanthracene (DMA) generating an endoperoxide in a [4+2]-reaction. Tetraphenylporphyrin (TPP) in DMSO (ΦΔ = 0.52) was used as reference to calculate ΦΔ for the PS [56]. A DMA solution (1.45∙10⁻⁴ M in DMSO) was mixed with the PS in 1 cm quartz cuvette and bubbled with oxygen for 5 minutes. The absorbence of the reaction mixture was taken at 401 nm. The cuvette was irradiated by a 100 W LED-light at 420 nm (LUMOS 43 from Atlas Photonics) and the decrease in the absorbance of DMA at 401 nm was followed. The measurements were done in triplicate. The kinetics of DMA photo-oxidation permits to calculate the singlet oxygen generation of the PS. Following equation is used:
\[
\Phi_S = \Phi_R^R \frac{K_S}{K_R I_S}
\]

Where \(K^R\) and \(K^S\) are the slopes of the kinetic plot of the difference in absorbance vs. irradiation time of DMA photooxidized by the reference and sample respectively, \(I^R\) and \(I^S\) the total light intensities absorbed by the reference and sample and \(\Phi_S\) the singlet oxygen quantum yield of the sample. Light intensities were calculated according to the following equation:

\[
I = I_0 (1 - 10^{-A(\lambda=420nm)})
\]

Where \(I\) is the absorbed light intensity from the sample, \(I_0\) the light intensity from the light source and \(A(\lambda = 420nm)\) the absorbance of the photosensitizer at the excitation wavelength.

2.7 Kinetic and Equivalent CO Released by Photoirradiation.

For both Mn and B12-Mn complexes, the kinetic of CO release was evaluated by monitoring the spectral changes at a specific wavelength (410 and 365 nm respectively) by UV/Vis spectroscopy. For this purpose, the complexes were firstly dissolved in DMSO (1 % v/v, final concentration) before being diluted in 0.1 M PBS. Samples were irradiated with a 420 nm light. Then, the equivalent of CO release was evaluated under the conditions of myoglobin assay for all complexes. The solutions Mn (15 μM) and B12-Mn (10 μM), measured in 0.1 M PBS buffer at pH 7.4, 10 mM dithionite, with 3 equivalent of myoglobin (respectively 45 and 30 μM), under argon, were irradiated with a 420 nm light. The stability of each compound in the presence dithionite was evaluated prior to each experiment and none of them showed spontaneous CO release. The concentration of Myoglobin-CO complex c(Mb-CO) formed through time due to CO release from the photoCORMs upon irradiation at 420 nm was monitored, and the CO release equivalent was calculated as described by Atkin et al. [57]. Due to its poor solubility, Mn was previously dissolved in DMSO (1 % v/v, final concentration).

2.8 Drug Loading and Release from DEMs

Around 2 mg of drug were weighed and dissolved in 20 μL of DMSO, then 10 μL of this solution of 100 mg/mL were diluted (100 fold) to 1000 μL with aqueous buffer PBS pH 7.4 (1 % v/v, DMSO) giving the first standard solution (S1 = 1,0 mg mL\(^{-1}\)). Dilutions of S1 in PBS pH 7.4 (1 % v/v, DMSO) gave the standards used for the calibration curves (5 standards, \(R^2 > 0.9\)). The standards and samples were analyzed by UV-Vis spectroscopy. For each measurement, the sample was quickly vortexed before being transferred for UV-Vis measurement. The drug loadings (DL) were performed by weighting around 10 mg of the drug and the same amount of DEMs in an Eppendorf. Afterwards, 1 mL of acetone was added to obtain a 10 mg mL\(^{-1}\) drug solution. The samples were shaken on the plate for 48 h. The mixtures were then centrifuged and the supernatant removed. A quick wash was performed by adding 500 μL of acetone, centrifuging 5 s and removing supernatant. The samples were then evaporated under argon to dryness. Drug release experiments were performed by adding 1000 μL of PBS pH 7.4 (1 % DMSO) to the drug loaded DEMs samples. Samples of 100 μl were taken at each time point and replaced with fresh buffer. The concentrations of these samples were evaluated by UV-VIS spectroscopy by monitoring the absorption of the complexes at specific wavelengths. The values for the cumulative drug release in percent were calculated from the linear regressions previously established. The graphs were plotted using Origin 7.5. The DL were calculated as the mass percentage (wt%) of drug over the sum of drug and DEMs.

2.9 Bioactivity Assays

Rhenium anticancer complexes 1 and 2 were freshly dissolved first in DMSO and then in RPMI 1640 medium (Merck, Munich, Germany), while the other analysed compounds were immediately dissolved in RPMI 1640 medium and used for bioactivity assessments. In vitro cytotoxicity in terms
of antiproliferative effects was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [58] on colorectal cancer HCT116 cell line, obtained from American Type Culture Collection (ATCC). The cells, cultured in the complete RPMI 1640 medium as a monolayer (1×10^4 cells per well) were incubated with test compounds for 48 h in humidified atmosphere of 95% air and 5% CO2 at 37 °C and the MTT assay was carried out two times in four replicates. The extent of MTT reduction was measured spectrophotometrically at 540.0 nm using Tecan Infinite 200 Pro multiplate reader (Tecan Group Ltd., Männedorf, Switzerland) and the cell survival was expressed as percentage of the control arbitrarily set to 100%. Cytotoxicity was expressed as the concentration of the compound inhibiting cell growth by 50% (IC50) in comparison to untreated control. For the photostimulation experiment, Osram L30W/840 cool white light has been used (Lumilux) two times for 10 min, immediately upon the addition of compounds to the cells and 1 h after.

3. Results and Discussion

3.1 Synthesis and Characterization of Surface coating Molecules

Vitamin B12 (tumor targeting agent) surface coating molecules bearing either a [2,2'-bipyridine]-3,4'-dicarboxylic acid manganese tricarbonyl complex (photoCORM, B12-Mn) or a zinc of 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin molecule (PDT agent, B12-ZnTCPP) were prepared according to the synthetic procedure illustrated in scheme 1. Vitamin B12 (represented by a simplified structure) was first modified with an aminated PEG-chain on the 5'-OH ribose moiety and then reacted directly with the manganese complex (Mn, giving B12-Mn) or with 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin (TCPP) respectively. The latter was finally metalated with zinc(II) acetate giving B12-ZnTCPP. Alternatively B12-ZnTCPP could be prepared by PEGylation of TCPP followed by metalation and coupling to vitamin B12. We note here that the length of the selected PEG-chain allows vitamin B12 derivatives to be still recognized by the transcobalamin receptors (TC(II) and TC(II)-R) leading to increased adherence towards cancer cells [25,59,60]. DFT calculations of B12-Mn and B12-TCPP performed in water (polarizable continuum model, vide infra Figure 2) indicate a distance of ca. 18 Å between the molecular units. Thus, the PEG chain does not only act as a linker but also provides a certain degree of freedom to the system. Indeed, we expected this distance to minimize steric hindrance in the final composite materials, allowing the different components to play their role with minimal interferences [61]. Once grafted onto the surface of diatom microalgae, this arrangement facilitates B12 being complexed by the transcobalamin receptors TC(II)-R and still act as a tumor targeting agent. At the same time, photo-activation of the surface allows for CO release from B12-Mn or 1O2 generation from B12-ZnTCPP respectively. Both surface coating derivatives were characterized by standard techniques. 1H-NMR, visible spectroscopy and MS data are fully consistent with the proposed molecular structures of B12-Mn and B12-ZnTCPP. Particularly revealing were the 1H-NMR spectra of the molecules (ESI Figure S1 to S3). As expected, the homotopy of the aromatic protons of the [2,2'-bipyridine]-3,4'-dicarboxylic acid and TCPP of B12-Mn and B12-ZnTCPP respectively, was lost in upon coupling to B12-1 (i.e. PEGylated vitamin B12, see scheme 1). In the case of B12-ZnTCPP (e.g.) the symmetric 1:1 doublets of TCPP split into three doubles of relative intensity of 4:3:1; the latter being assigned to the 4-carboxyphenyl protons in ortho-position to the newly formed amide bond (ESI Figure S1). Metalation of B12-TCPP was confirmed by UV-Vis spectroscopy. Indeed, the Soret band of the porphyrin undergoes a bathochromic shift from 415 to 423 nm upon reaction with Zn(OAc)2∙2∙H2O (Figure 2). Moreover, upon metalation, the degeneracy in the porphyrin orbital causes the coalescence of the four TCPP Q-bands (512, 546, 588 and 645 nm) into two Q-bands in B12-ZnTCPP (560 and 601 nm) as previously described [62,63]. The peaks at 360 nm and 517 nm were respectively attributed to the α-band and β-band of vitamin B12 (Figure 2).
Scheme 1. Reaction steps for the synthesis of B12-Mn and B12-ZnTCPP. Reagents and conditions: (i) 2 h, acetic acid under reflux; (ii) CDT, 24 h, DMSO, then PEG, 24 h, anhydrous DMF; (iii) EDC/NHS, 1 h, anhydrous DMF, dim light, RT; (iv) 72 h, anhydrous DMF, RT; (v) Zn(OAc)\(_2\)·2·H\(_2\)O, 1 h, MeOH under reflux; (vi) EDC/NHS, 2 h, anhydrous DMF, RT; (vii) Zn(OAc)·2·H\(_2\)O, 1 h, MeOH under reflux; (viii) CDT, DMSO, 12 h / anhydrous DMF, 24 h.

Figure 2. (A) and (B) represent the UV-Vis absorption spectra of TCPP, PEG-TCPP, PEG-ZnTCPP and B12, B12-TCPP, B12-ZnTCPP respectively; (C) and (D) show optimized structures of B12-TCPP and B12-Mn respectively, calculated in silico by DFT (polarizable continuum model with water as solvent). C-atoms are grey, N-atoms blue, O-atoms red, P is orange and Co dark blue. H-atoms are omitted for clarity.
3.2 \( ^1\text{O}_2 \) Generation and CO Release of Surface Coating Molecules

Fluorescence quantum yield (\( \phi_{fl} \)), lifetime (\( \tau_f \)) and singlet oxygen (\( ^1\text{O}_2 \)) quantum yield (\( \phi_{\Delta} \)) were assessed for PEG-ZnTCPP and B12-ZnTCPP in DMSO (Table 1). Fluorescence spectra of PEG-ZnTCPP and B12-ZnTCPP in DMSO show emission bands around 612 and 666 nm (ESI Figure S6) and the fluorescence quantum yields and lifetimes are similar for the two species. The results are consistent with values reported in literature for comparable porphyrin analogues, and support the hypothesis that the structural change at the periphery of the porphyrin does not drastically affect \( \phi_{fl} \) and \( \tau_f \) since the fluorescence phenomenon arises from the inner \( \pi \)-electron system on the macrocycle [63-65]. Nevertheless, it is known that introducing electron donating or withdrawing groups onto porphyrin center will slightly influence the fluorescence properties [66]. Longer fluorescence lifetime and improved inter-system crossing positively influence the singlet oxygen quantum yield, but the same is affected by many other parameters such as solubility or tendency of the fluorophore to form aggregates. However, \( \phi_{\Delta} \) does not correlate to the fluorescence lifetime and quantum yield improvement. Porphyrins can easily aggregate in solution thereby lowering their efficiency in energy transfer with \(^1\text{O}_2\) resulting in lower \(^1\text{O}_2\) \( \phi_{\Delta} \) [67,68]. The solubility and aggregation propensity of the zinc(II) porphyrin are substantially modified by introducing a hydrophilic PEG chain or a large and highly water soluble structure like vitamin B12. The coupling of vitamin B12 to ZnTCPP slightly increases the singlet oxygen quantum yield by 15\%. Indeed, vitamin B12 covalently linked to the porphyrin enhances the solubility of the structure and lowers its tendency to aggregate, thereby leading to an efficient generation of \(^1\text{O}_2\) singlet oxygen. B12-ZnTCPP is stable to light. We observed no decomposition of the molecule under irradiation, suggesting the great potential of B12-ZnTCPP in PDT. Pseudo half-life and equivalents of CO released were measured for B12-Mn and its constituent photOCORM complex (Mn) [69] according to the method described by Atkin et al. (Table 1) [57]. To this end, changes in the UV-Vis spectrum at 410 and 365 nm were monitored as a function of irradiation time for Mn and B12-Mn respectively (ESI Figures S19 to S22). The stabilities of both complexes were demonstrated by the absence of spontaneous CO releases in the dark in the presence of sodium dithionite. Both molecules, upon light irradiation, released \textit{ca.} one equivalent of CO (ESI Figures S17 and S18).

### Table 1. Fluorescence quantum yield (\( \phi_{fl} \)), lifetime (\( \tau_f \)) and singlet oxygen quantum yield (\( \phi_{\Delta} \)) of the porphyrin complexes in DMSO. Photo-induced CO-release kinetics of the manganese complexes in PBS buffer pH 7.4.

|                  | \( \phi_{fl} \) | \( \tau_f \pm 0.1[\text{ns}] \) | \( \phi_{\Delta} \) |
|------------------|-----------------|--------------------------------|---------------------|
| ZnTCPP           | 0.009           | 4.7                            | 0.77 \( \pm \) 0.01 |
| PEG-ZnTCPP       | 0.046           | 8.6                            | 0.66 \( \pm \) 0.10 |
| B12-ZnTCPP       | 0.035           | 5.7                            | 0.90 \( \pm \) 0.03 |
| \( t_{1/2} \) s | \( a \)         | \( \text{equiv. of CO released} \) \( b \) |
| Mn \( c \)       | 1.6 \( \pm \) 1.1 | 1.3 \( \pm \) 0.1              |
| B12-Mn           | 2.6 \( \pm \) 1.4 | 1.3 \( \pm \) 0.1              |

\( a \) Pseudo half-life, determined from UV/Vis spectral studies. \( b \) Determined via myoglobin assay. \( c \) [2,2'-bipyridine]-3,4’-dicarboxylic acid manganese tricarbonyl complex.

### 3.3 Surface Functionalization, Characterization and DEMs Cellular Interaction

Surface functionalization of DEMs with B12-ZnTCPP and B12-Mn was achieved via amide-bond formation with one of the free carboxylic function available on the molecules and the previously aminated surface of the DEMs (Figure 3). In the case of B12-TCPP and B12-ZnTCPP, the presence of free carboxylic functions on the porphyrin macrocycle in both cis- and trans-position to the PEG arm, offers the possibility of several binding modes to DEMs. Given the fact that B12-ZnTCPP-DEM interact strongly with cancer cells (\textit{vide infra}, Figure 3) we posit either that the trans-4-carboxyphenyl
is preferentially favored or that the system is flexible enough so that steric hindrance (i.e. in the case of cis-4-carboxyphenyl binding) does not play a significant role in TCII-mediated B12-ZnTCPP-DEMs cell interaction.

Successful surface functionalization of diatoms microalgae was assessed via solid state UV-Vis spectroscopy (Figure 3D and E). For B12-TCPP-DEMs, comparison of the spectra of the new material to the non-functionalized DEMs, shows new absorbance peaks at 362 nm (Soret band of vitamin B12), 423 nm (Soret band TCPP), 518, 552, 591, and 648 nm (Q-bands of TCPP). By further reacting B12-TCPP-DEMs with zinc acetate in methanol under reflux, metalation of the porphyrin centers occurred leading to B12-ZnTCPP-DEMs. The solid-state UV-Vis spectrum of B12-ZnTCPP-DEMs compared to the non-metalated equivalent shows a bathochromic shift of the TCPP Soret band to 432 nm and coalescence of the Q-bands to two peaks at 561 and 601 nm (Figure 3E). This evidence confirmed the successful metalation of the material. Similarly, solid-state UV-Vis spectroscopy was performed to evaluate the newly synthesized material B12-Mn-DEMs. When comparing the DEMs spectra before and after surface functionalization with pure Mn, B12 and B12-Mn, the appearance of new peaks corresponding to each compound could be observed (Figure 3D). For the Mn-DEMs (sulfonated to assess the feasibility of the surface coating), a peak at 431 corresponding to the MLCT absorbance spectrum of the Mn complex is detected. In the case of B12-DEMs [25], peaks at 361 nm, 521 nm and 545 nm are consistent with absorbance of pure cyanocobalamin [70]. Finally, the spectrum of B12-Mn-DEMs shows peaks corresponding to cobalamin at 363 nm, 515 nm and 545 nm as well as a very small peak at 423 nm attributed to the presence of Mn. The solid-state UV-Vis spectrum of B12-Mn-DEMs is consistent with the spectrum of B12-Mn in solution (Figure S14) which shows a signal at 420 nm, very low in intensity when compared to the peaks attributed to the vitamin B12 at 361, 521 and 545 nm.

Further direct characterization of successful DEMs surface functionalization came from zeta potential (ζ-potential) analysis of B12-ZnTCPP-DEMs, and inductively coupled plasma atomic emission spectroscopy (ICP-OES) of B12-Mn-DEMs. Zeta potential of different samples of DEMs were recorded in PBS buffer at pH 7.4. Clean hydroxylated diatoms (DEMs-OH) expressed a negative potential of -23.4 ± 0.5 mV, which shifted to positive +9.5 ± 0.4 mV upon surface amination with APTES. This positive shift is indicative of amine functions on DEMs, which can be easily protonated in the buffer [25,71-73]. Further functionalization with TCPP moves the ζ-potential back towards negative value of -53.0 ± 0.4 mV logically deriving from the presence of carboxylic acid groups. The same potential slightly increases to -48.8 ± 0.3 mV for B12-TCPP-DEMs and remained virtually the same following Zn metalation for B12-ZnTCPP-DEMs (-47.1 ± 0.2 mV).

The surface functionalization of B12-Mn-DEMs was assessed by ICP-OES. Naturally occurring diatoms microalgae may contain small traces of cobalt in their frustules composition but considerable traces of manganese [74]. For example, in Cyclotella meneghiniana and Stephanodiscus hantzschii, common fresh water diatom species, zinc and manganese compete for cell wall incorporation with Mn accumulating preferentially at the girdle band of frustules [75] [76]. In contrast, cobalt can only replace zinc when this ion is totally absent from the growth medium [77]. ICP-OES measurements of the cobalt and manganese content in clean DEMs, indicated that values for the ions were below the limit of quantification (4 ppm). However, in B12-Mn-DEMs, cobalt and manganese content were found at concentrations of 32 ± 6 and 61 ± 1 ppm respectively, confirming enrichment of the two ions in our material. The difference in the total amount of both Co and Mn measured among the sample arise from the difficulty to generate Co ions from cobalamin in the plasma [78]. The amount of B12-Mn bound to DEMs, calculated from the manganese content, was found to be 2263 ± 45 ppm.

Scanning electron microscopy was used to assess the adherence of B12-ZnTCPP and B12-Mn on colorectal cancer HT-116 cells. In order to demonstrate cellular adherence, cells were treated with either unmodified (DEMs) and B12 functionalized diatoms under identical conditions (200 μg biomaterial mL⁻¹, 1 h exposure). After intensive washes, cells exposed to B12-ZnTCPP and B12-Mn still presented and retained a large amount of micro-particles (Figure 3I and L) while in the sample of unmodified DEMs, almost all the micro-particles were removed via the washing steps.
3.4 In vitro Drug Loading and Release from Diatom Microalgae

We have recently described the anti-proliferative efficacy of a series of rhenium(I) tricarbonyl N-derivatized \( N-(\text{2,2'-bipyridin}-6-\text{ylmethyl}) \)-complexes of increasing lipophilicity against different cell lines [48]. Among the series of these compounds the \( \text{fac-}[\text{Re(CO)}_3\text{Br}] \) complexes with 6-(bromomethyl)-2,2'-bipyridine (1 in this study, Figure 4) and \( N-(\text{2,2'-bipyridin}-6-\text{ylmethyl})\)-N-isobutyl-2-methylpropan-1-amine (2, Figure 4) showed excellent \textit{in vivo} toxicity profiles and \textit{in vivo} anticancer/antimetastatic efficacy and effective inhibition of angiogenesis in the zebrafish-human CRC tumour xenograft model. These two complexes were selected for the present study. The molecules were successfully loaded into DEMs according to the vacuum infiltration method described by Vasani \textit{et al.} [79] (giving \( 1@\text{B}_{12}\)-ZnTCPP-DEMs and \( 2@\text{B}_{12}\)-Mn-DEMs respectively) with respective loading degrees of 3.7 (1) and 1.5 (2) wt\% (Table 2). These are in the typical range of drugs loaded into DEMs biosilicas [25,26,80-82]. Following successful encapsulation, the drug release in PBS buffer pH 7.4 (1 % v/v, DMSO), was initiated by addition of solvent and monitored by UV-VIS spectroscopy over period of 5 days in both cases. As often observed in different studies an important initial burst release occurs during the first hours (Figure 4) [27,29]. Ca. 60 % and 50% of complexes 1 and 2 were respectively released in solution after the first hour. A second more gradual and sustained released was then observed over the course of a few days. Indeed, 1 and 2 were nearly completely discharged from \( \text{B}_{12}\)-ZnTCPP-DEMs and \( \text{B}_{12}\)-Mn-DEMs after 2 and 5 days respectively.
Table 2. Loading degrees of rhenium complexes 1 and 2 in % wt into DEMs and in vitro cytotoxicity (HCT-116 cells, IC_{50}, µM) of complexes and when administered free, in DEMs in the dark (@-B12-#-DEMs) and in DEMs following photo-activation of their surface (@B12-#-DEMs-λν).

| Compound | µg drug/mg DEMs | IC_{50}, µM |
|----------|-----------------|-------------|
| 1@B_{12}-ZnTCPP-DEMs | 38.9 (3.7%) | 4.7 ± 0.1 |
| 1@B_{12}-ZnTCPP-DEMs-λν | 38.9 (3.7%) | 1.2 ± 0.1 |
| 2@B_{12}-Mn-DEMs | 15.9 (1.6%) | 6.0 ± 0.2 |
| 2@B_{12}-Mn-DEMs-λν | 15.9 (1.6%) | 3.1 ± 0.1 |

Figure 4. (A) Representative loading of B_{12}-ZnTCPP-DEMs with complex 1 (giving 1@B_{12}-ZnTCPP-DEMs) and graph showing the release of 1 from B_{12}-ZnTCPP-DEMs over a period of 5 days (insert shows release in the first 5 hours). (B) Representative loading of B_{12}-Mn-DEMs with complex 2 (giving 2@B_{12}-Mn-DEMs) and graph showing the release of 2 from B_{12}-Mn-DEMs over a period of 5 days (insert shows release in the first 5 hours).

3.5 PDT and Cytotoxicity Assessment

The cytotoxicity of the 1@B_{12}-ZnTCPP-DEMs and 2@B_{12}-Mn-DEMs formulations in dark and under light irradiation were assessed using the human colorectal HT-116 cell line (Figures 5 and 6). In the case of 1@B_{12}-ZnTCPP-DEMs, light activation promotes singlet oxygen generation by the ZnTCPP component. Photo-activation of 1@B_{12}-ZnTCPP-DEMs increased the cytotoxicity of the construct up to 2.4-fold for the most concentrated sample (λν 200 µg/mL (+ IC_{50}), Figure 5 and Table 2) when compared to the effects of the administration of 1 alone. Notably, 1@B_{12}-ZnTCPP-DEMs containing only 25% of the IC_{50} concentration value of 1, caused a 45% increase in cell death upon light exposure in comparison to dark conditions, essentially reaching the cell killing effects of the IC_{50} value of 1 at ¼ the complex concentration. 1@B_{12}-ZnTCPP-DEMs with 2.5% the IC_{50} of 1 caused an increase of 14% of cell death under tested similar conditions. Overall, as it can be appreciated from the graphs in Figure 5, the data suggest that lower amounts of cytotoxic compounds are needed in
combination with the photoactivatable surface-functionalized diatom microalgae for an equally
effective drug formulation or cytotoxic effect. Unmodified natural DEMs, either in the dark or
exposed to light, showed no toxicity at concentrations >100 μg/mL, while both DEMs containing
adsorbed 1 and B12-ZnTCPP-DEMs particles alone showed slightly higher activity upon the light
exposure (Figure 5). The photo-activity of complex 1 was somewhat surprising. We posit that is may
be due to activation of the pending α-dimine ligand-CH2-Br arm towards nucleophilic substitution
with bio-available amines via reactions of the complexes previously [48].

For 2@B12-Mn-DEMs, light activation induces CO release from the photoCORM Mn component
at the surface of the microalgae. CO released from this type of molecules is known to promote pro-
apoptotic effects in different cancer cell lines [83-86] [87] [88] via CO-mediated attenuation of
 glutathione and nuclear metallothionein levels [89] and inhibition of cystathionine β-synthase [90].
When HT-116 cells were exposed to 2@B12-Mn-DEMs, photo-activation of the material similarly
increased the overall cytotoxicity effect of complex 2. Light irradiation of 2@B12-Mn-DEMs increased
the cytotoxicity of the construct by ca. 2.0-fold in the case of the most concentrated sample (λν 200
μg/mL (+ IC50), Figure 6 and Table 2) when compared to the same sample in the dark or to the effects
of the administration of 2 alone. In particular, 2@B12-Mn-DEMs containing 25% of the IC50
concentration value of 2, caused a 31% increase in cell death upon light exposure in comparison to
dark conditions. Photo-activation of 2@B12-Mn-DEMs containing ½ the IC50 concentration value of 2,
was more effective than administration of 2 alone at its IC50 concentration or B12-Mn-DEMs, inducing
total 64% cell death. Photo-activation of 2@B12-Mn-DEMs containing only 2.5% of the same IC50 was not
effective but still induced a 13% increase of cell death compared to the same concentrations of 2 or
B12-Mn-DEMs. As concluded for 1@B12-ZnTCPP-DEMs, the data for 2@B12-Mn-DEMs also suggest a
potential of this poly-systemic approach in cancer treatment.

**Figure 5.** (A) Histograms representing the effects on HCT-116 colorectal cancer cells % survival by
different doses of 1@DEMs, B12-ZnTCPP-DEMs and 1@B12-ZnTCPP-DEMs when administered in the
dark (empty bars) and under the same conditions following light activation of the microalgae surface
(filled colored bars). The red square brackets on the graph are meant to highlight the enhanced
cytotoxicity of the material upon photo-irradiation. (B) Conceptual representation of the experiment,
structure of complex 1 and detail of the photochemical reaction taking place at the microalgae surface
sites (ZnTCPP-induced 1O2 generation).
Figure 6. (A) Histograms representing the effects on HCT-116 colorectal cancer cells % survival by different doses of 2@DEMs, B12-Mn-DEMs and 2@B12-Mn-DEMs when administered in the dark (empty bars) and under the same conditions following light activation of the microalgae surface (filled colored bars). The red square brackets on the graph are meant to highlight the enhanced cytotoxicity of the material upon photo-irradiation. (B) Conceptual representation of the experiment, structure of complex 2 and detail of the photochemical reaction taking place at the microalgae surface sites (CO release from Mn complex).

Finally, by plotting the delta percentage of cell death measured upon light irradiation of raw DEMs, B12-ZnTCPP-DEMs, B12-Mn-DEMs and their respective combinations with rhenium anticancer complexes 1 and 2 (Figure 7) clearly demonstrates the improved efficiency of this approach (data not shown for raw DEMs alone which are non-toxic). Data confirm the synergistic effect of drugs 1 and 2 together with the O2 generating porphyrin and the CO releasing photoCORM functionalized microalgae surface respectively.

Figure 7. (A) and (B) Histograms representing the effect of light activation on different doses of 1@B12-ZnTCPP-DEMs and 2@B12-Mn-DEMs respectively on HCT-116 colorectal cancer cells.

4. Conclusion

In this study we have reported the synthesis and characterization of two bio-inspired hybrid multifunctional drug delivery systems based on diatom microalgae. Both photoactivatable materials, either bearing a tetracarboxyphenyl porphyrin PS or tricarbonyl bipyridyl Mn(I) photo-CORM, are able to bind to HT-116 colorectal cancer cells via the outer cobalamin layer, and slowly release highly active rhenium(I) tricarbonyl anticancer complexes. Furthermore, the potentiation effect of the
chemotoxic drug by the different light-activated molecules at the surface of diatom microalgae was
demonstrated by MTT assay under dark/light conditions on the HCT-116 cell line. At least 2-fold
increase of cytotoxicity toward HCT-116 was observed upon light irradiation for both constructs (at
200 μg ml⁻¹, incubated with drug concentration corresponding to respective IC₅₀ values) with cell
survival falling from ca. 40% to ca. 20%. Our results are in line with what reported earlier in literature
for different constructs [91-95], indicating that synergistic chemophotodynamic therapy mediated
by diatom microalgae is a strategy worthwhile pursuing in colon-focused drug delivery. It is well
known that undesirable side effects of most chemotherapeutics like cisplatin are dose dependent [96],
thus this approach can reduce the need of chemotherapeutics concentration for equal effectiveness
hence leading to diminished undesirable side effects for the patient. Future efforts for the
optimization of this hybrid multifunctional drug delivery system will be directed towards coating
formulations (e.g. pH-dependent polymers such as cellulose acetate phthalates, hydroxypropyl
methyl-cellulose phthalate or biodegradable liposomes) that can respond to dynamic pH changes of
the gastro-intestinal (GI) tract in order to expose the active material in the colon only.

**Supplementary Materials:** Figure S1: ¹H-NMR of B12-TCPP, Figure S2: ¹H-NMR of TCPP-1, Figure S3: ¹H-NMR
of B12-Mn, Figure S4: MS spectrum of B12-TCPP, Figure S5: MS spectrum of TCPP-1, Figure S6: Fluorescence
spectra of PEG-ZnTCP and B12-ZnTCP, Figure S7: Fluorescence lifetime decay curves of B12-ZnTCP and
ZnTCP-1, Figure S8: IR of Mn, Figure S9: IR of B12-Mn, Figure S10: HPLC chromatogram of B12-TCPP, Figure
S13: UV/Vis of Mn, Figure S14: UV/Vis of B12-Mn, Figure S15: Solid state UV/Vis of DEMs, Mn-DEM, B12-DEM
and B12-Mn-DEM, Figure S16: Solid state UV/Vis of DEMs, B12-TCPP-DEM and B12-ZnTCP-DEM, Figure S17:
Concentration of Mb-CO upon light irradiation of Mn, Figure S18: Concentration of Mb-CO upon light
irradiation of B12-Mn, Figure S19: Spectral changes at 410 nm of Mn upon light irradiation, Figure S21: Mn
absorption decrease at 410 nm regarding irradiation time, Figure S20: Spectral changes at 365 nm of B12-Mn upon
light irradiation, Figure S22: B12-Mn absorption decrease at 365 nm regarding irradiation time.

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Electronic Supporting Information

Photoactivatable surface-functionalized diatom microalgae for colorectal cancer targeted delivery and enhanced cytotoxicity of anticancer complexes

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Figure S1. $^1$H-NMR of B$_{12}$-TCPP. 500 MHz NMR in MeOD-d4 ($\star$ = solvent signal).
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Figure S4. MS spectrum of B$_{12}$-TCPP. Measured with MALDI-TOF, 2,5-dihydroxybenzoic acid (DHB) used as matrix.

Figure S5. MS spectrum of TCPP-1. Measured with ESI-MS (positive mode) in MeOH.
Fluorescence Spectroscopy

Figure S6. Fluorescence spectra of ZnTCPP-1 and B_{12}-ZnTCPP. Measured in DMSO.

Figure S7. Fluorescence lifetime decay curves of B_{12}-ZnTCPP and ZnTCPP-1. The samples were prepared in DMSO and measurements were recorded with a TCSPC spectrophotometer.
Infrared Spectroscopy

All the IR spectra were measured with ATR system.

Figure S8. IR of Mn.

Spectra of Mn correspond to previously described analysis in literature¹.

Figure S9. IR of B₁₂-Mn.

¹ A. Ruggi and F. Zobi, “Quantum-CORMs: Quantum Dot Sensitized CO Releasing Molecules,” *Dalton Trans.* 44, no. 24 (2015): 10928–31, https://doi.org/10.1039/C5DT01681A; Jeremie Rossier et al., “Organometallic Cobalamin Anticancer Derivatives for Targeted Prodrug Delivery via Transcobalamin-Mediated Uptake,” *Dalton Trans.* 46, no. 7 (2017): 2159–64, https://doi.org/10.1039/C6DT04443C.
HPLC Analyse

Figure S10. HPLC chromatogram of $\text{B}_{12}$-TCPP. Aqueous trifluoroacetic acid 0.1% solution and pure methanol were respectively used as solvents (A) and (B). The purified compound was injected and following gradient was used: 0–5 min (50% A), 5–30 (50% A → 0% A), 30–45 min (100% B), the flow rate set to 5 mL min$^{-1}$ and the compound detected at 320 nm. The retention time of the compound corresponds to 37.2 minutes.

UV/Vis Spectrometry

In solution

Figure S13. UV/Vis of Mn.  
Figure S14. UV/Vis of $\text{B}_{12}$-Mn.
In solid-state

**Figure S15.** Solid state UV/Vis of DEMs, Mn-DEM, B$_{12}$-DEM and B$_{12}$-Mn-DEM.

**Figure S16.** Solid state UV/Vis of DEMs, B$_{12}$-TCPP-DEM and B$_{12}$-ZnTCPP-DEM.
Equivalent of CO release

Here below are shown the concentration of Myoglobin-CO complex \( c(Mb-CO) \) formed through time with the CO release from either Mn5 (15 \( \mu \)M) or B\(_{12}\)-Mn5 (10 \( \mu \)M) upon irradiation at 420 nm under Argon atmosphere and determined from UV/Vis spectroscopy as described by Motterlini et al\(^2\). The solution were measured in 0.1M PBS buffer at pH 7.4, 10 mM dithionite, with respectively 45 and 30 \( \mu \)M Myoglobin for Mn and B\(_{12}\)-Mn assays. Due to its poor solubility, Mn was previously dissolved in DMSO (1% final concentration).

![Figure S17. Concentration of Mb-CO upon light irradiation of Mn.](image)

![Figure S18. Concentration of Mb-CO upon light irradiation of B\(_{12}\)-Mn.](image)

\(^2\) Roberto Motterlini and Leo E. Otterbein, “The Therapeutic Potential of Carbon Monoxide,” Nature Reviews Drug Discovery 9 (September 1, 2010): 728.
Half life

Monitoring of the spectral changes in the electronic absorption spectrum of compounds Mn and B\textsubscript{12}-Mn in 0.1 M PBS upon irradiation with 420 nm light. The complexes Mn was previously dissolved in DMSO (1% final concentration).

Figure S19. Spectral changes at 410 nm of Mn upon light irradiation.

Figure S20. Spectral changes at 365 nm of B\textsubscript{12}-Mn upon light irradiation.

Figure S21. Mn absorption decrease at 410 nm regarding irradiation time.

Figure S22. B\textsubscript{12}-Mn absorption decrease at 365 nm regarding irradiation time.
Motterlini, Roberto, and Leo E. Otterbein. “The Therapeutic Potential of Carbon Monoxide.” Nature Reviews Drug Discovery 9 (September 1, 2010): 728.

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