pH-Responsive Protein Nanoparticles via Conjugation of Degradable PEG to the Surface of Cytochrome *c*

Elena Steiert,[1] Johannes Ewald,[2] Annika Wagner,[3] Ute A. Hellmich,[3] Holger Frey[2] and Peter R. Wich[1,4]*

[1] Institute of Pharmacy und Biochemistry, Johannes Gutenberg-University Mainz
Staudingerweg 5, 55128 Mainz, Germany

[2] Institute of Organic Chemistry, Johannes Gutenberg-University Mainz
Duesbergweg 10-14, 55128 Mainz, Germany

[3] Institute of Pharmacy und Biochemistry, Johannes Gutenberg-University Mainz
Johann-Joachim Becherweg 30, 55128 Mainz, Germany

[4] Australian Centre for NanoMedicine, University of New South Wales, Sydney,
NSW 2052, Australia

[5] School of Chemical Engineering, University of New South Wales, Sydney, Australia

phone: +61 2 9385 5664

email: p.wich@unsw.edu.au
web: http://www.wichlab.com

Table of Contents

Materials

Cytochrome *c* from horse heart [EC 232.700.9] was purchased from SERVA Electrophoresis GmbH, Germany. Sodium hydride (NaH), Triethylene glycol monomethyl ether (mTEG), Benzophenone, *trans*-2-[3-(4-*tert*-Butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB), Calcium hydride (CaH_2), dioxane, dimethylformamide (DMF), dichloromethane, benzene, tetrahydrofuran (THF), methanol, *N,N′*-Disuccinimidyl carbonate (NHS-DSC), coomassie brilliant blue G, thiazolyl blue tetrazolium bromide (MTT), Dulbecco's modified eagle medium (DMEM) GlutaMAX™, phosphate buffered saline (PBS), hydrogen peroxide solution 30 % were purchased from Sigma-Aldrich, St. Louis. Ethylene oxide (EO) was purchased by Air Liquide, Germany. Dimethyl sulfoxide (DMSO) was purchased from Acros, Germany. Ammonium persulfate (APS), Roti®-Load 1, Rotiphorese® Gel 30 were purchased from Carl Roth, Germany. Tetramethylethylenediamine (TEMED) was purchased from VWR, Germany. 2,2´-azinobis-(2-ethylbenzthiazoline-6-sulfonate (ABTS) and Epoxy butadiene (EPB) were purchased from Alfa Aesar, Germany. The PageRuler Prestained Protein Ladder for SDS-PAGE and Oregon-Green™ 488 dextran (10 kDa) were purchased from Thermo Scientific, Germany. Fetal calf serum (FCS), glutamine, phosphate buffered saline (PBS, for cell culture), pyruvate and penicillin/streptomycin were purchased from Invitrogen. Spectra/PORTM dialysis membranes regenerated cellulose (RC) tubing molecular weight cut-off (MWCO) 1000 Da were purchased from Spectrum Labs. DMSO-d6 was purchased from Deutero GmbH, Germany. All chemicals were used as received without further purification unless stated otherwise. All used organic solvents were purchased from different suppliers and distilled once prior use.

Analytical Instrumentation

^{1}H NMR and spectra were measured using a Bruker AMX400 spectrometer (256 Scans, and B-ACS 60 auto sampler) at 296 K. 2D NMR and ^{13}C NMR spectra were measured on a Bruker Avance II 400 (100.5 MHz, 5 mm BBO probe, and B-ACS 60 auto sampler) at 296 K. All spectra were processed with MestReNova v12 software and referenced internally to residual proton signals of the deuterated solvent. Size exclusion chromatography (SEC) data were obtained using Agilent 1100 Series equipped with PSS HEMA-columns (106/104/102 Å porosity) using DMF with 1 g/L LiBr as an eluent and RI detection. Polydispersity indices ($Đ = Mw/Mn$) were determined with monodisperse linear PEG standards from Polymer Standard Service GmbH (PSS). MALDI-ToF analysis was performed using an rapifleXTM MALDI-TOF/TOF equipped with a 10 kHz scanning Smartbeam 3D Laser (Nd:YAG at

355 nm) and 10 bit 5 GHZ Digitizer. Analysis was performed in reflectormode positive for polymer and in linear mode positive for protein and protein-PEG conjugates. DCTB was used as matrix and dichloromethane as solvent for polymer samples and sinapic acid as matrix and CH_3CN/H_2O 1/1 as solvent was used for protein and protein-PEG conjugates.

1. Polymer Synthesis

2D NMR spectra of P(EG-*co*-EPB) and P(EG-*co*-*iso*EPB) were already reported in a previous publication.[1]

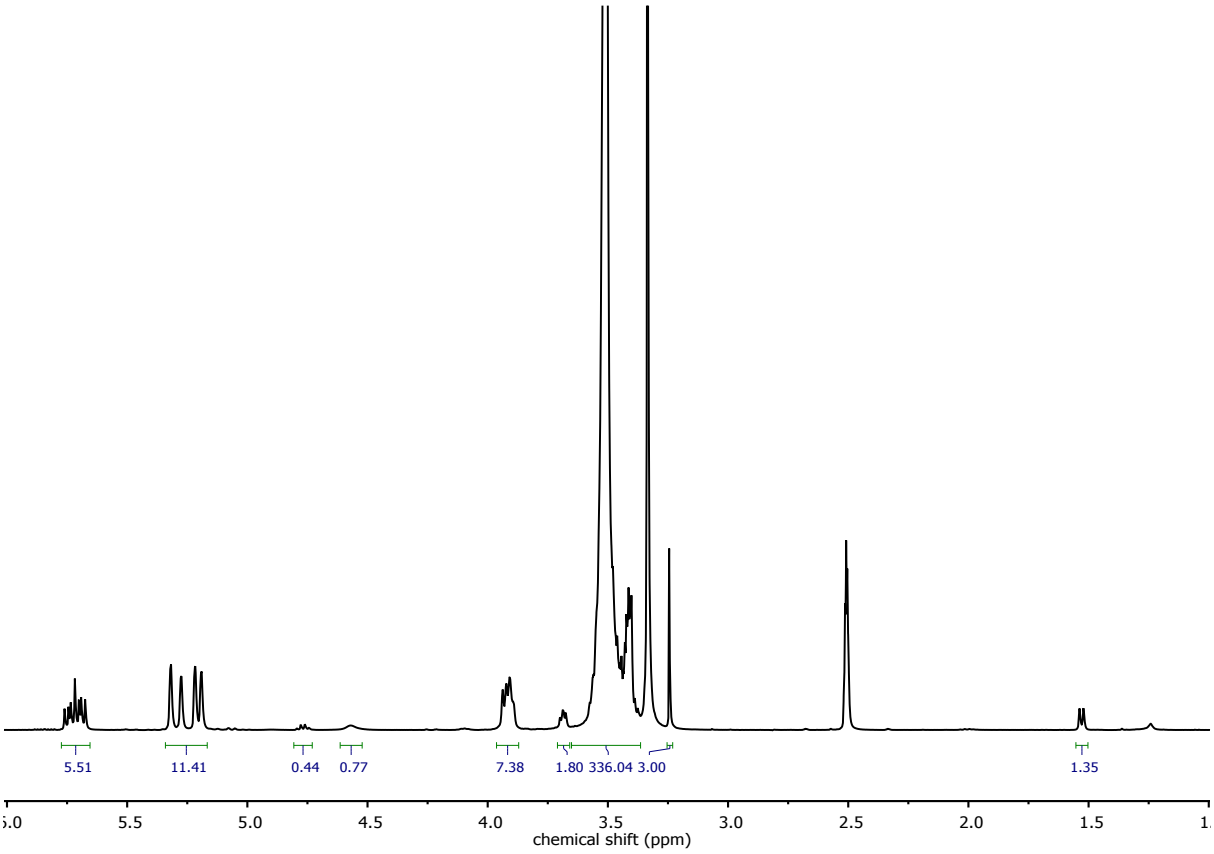

Figure S1: ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of mP(EG_{81}-*co*-EPB_6).

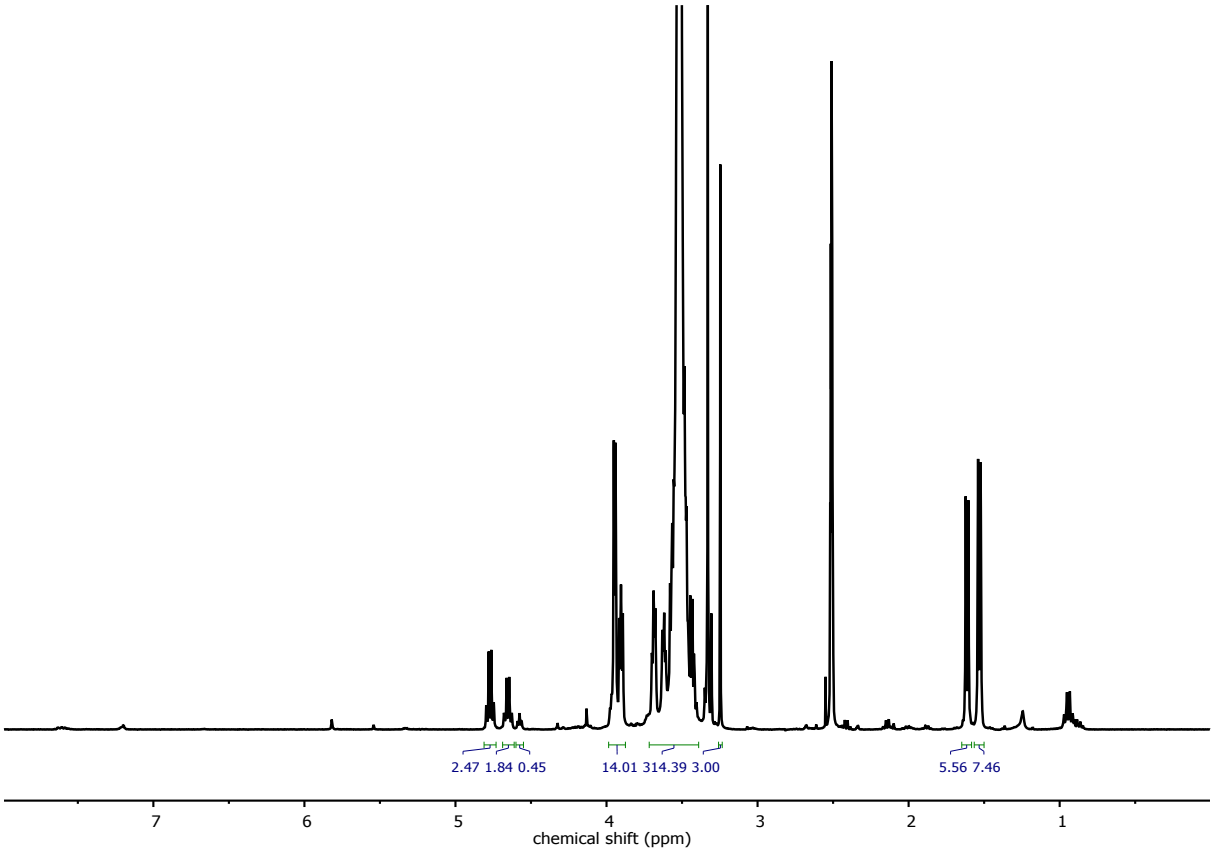

Figure S2: ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of mP(EG_{81}-*co*-*iso*EPB_6).

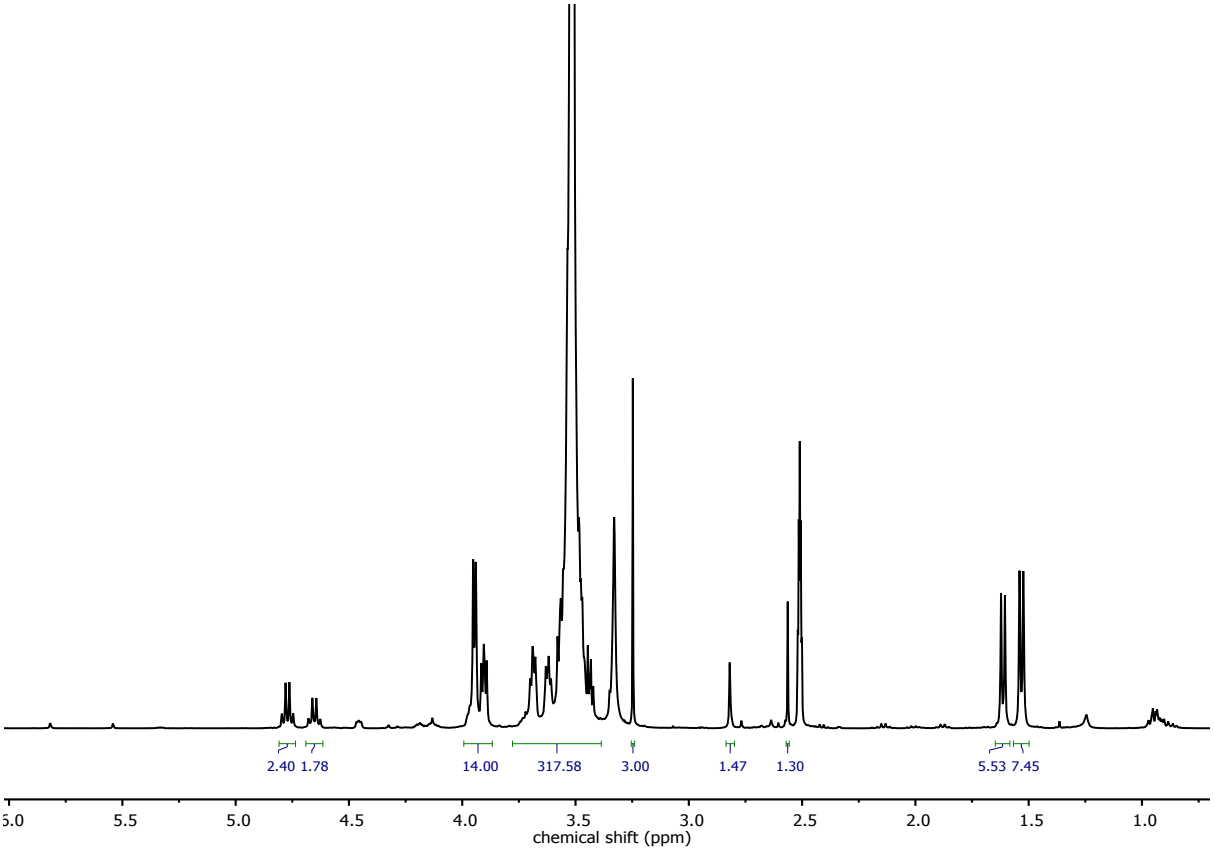

Figure S3: ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of mP(EG_{81}-*co*-*iso*EPB_6)-NHS.

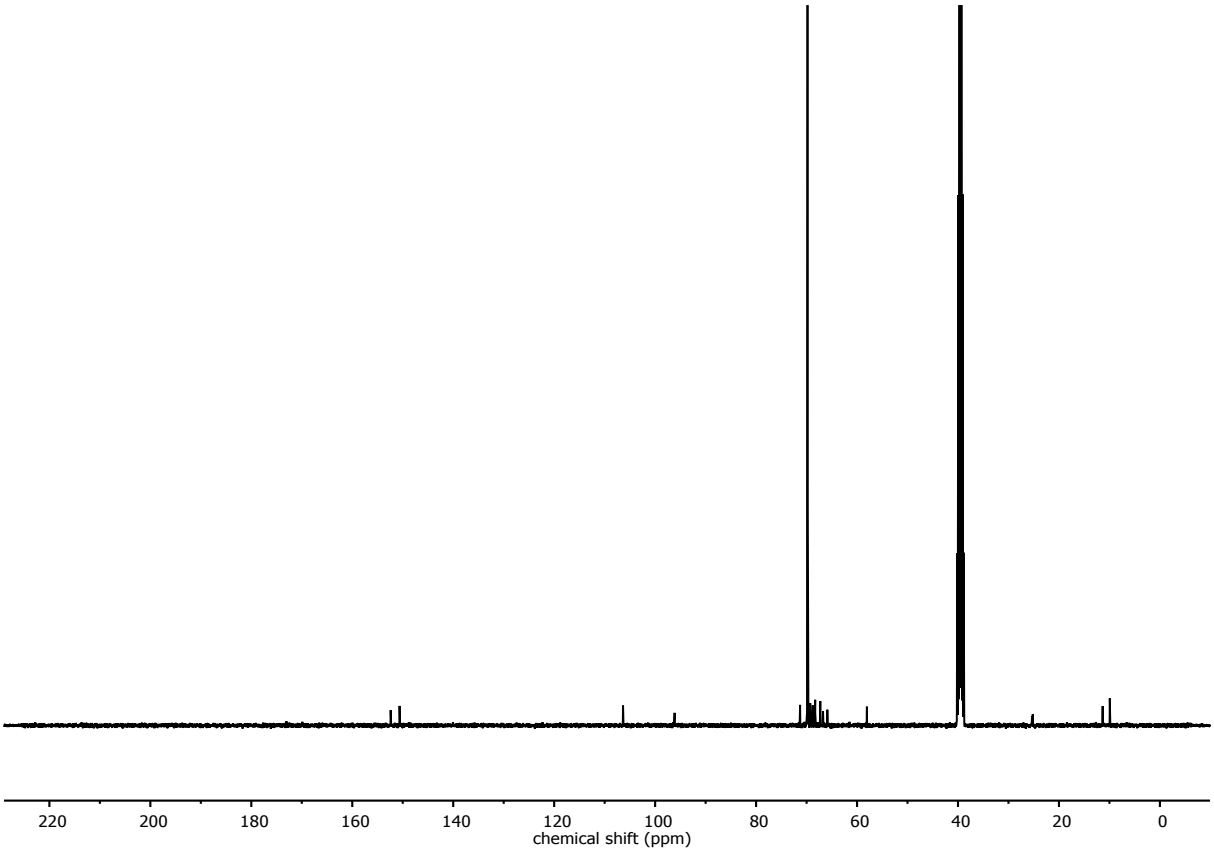

Figure S4: ^{13}C NMR spectrum (400 MHz, DMSO-d_6) of mP(EG_{81}-*co*-*iso*EPB_6)-NHS.

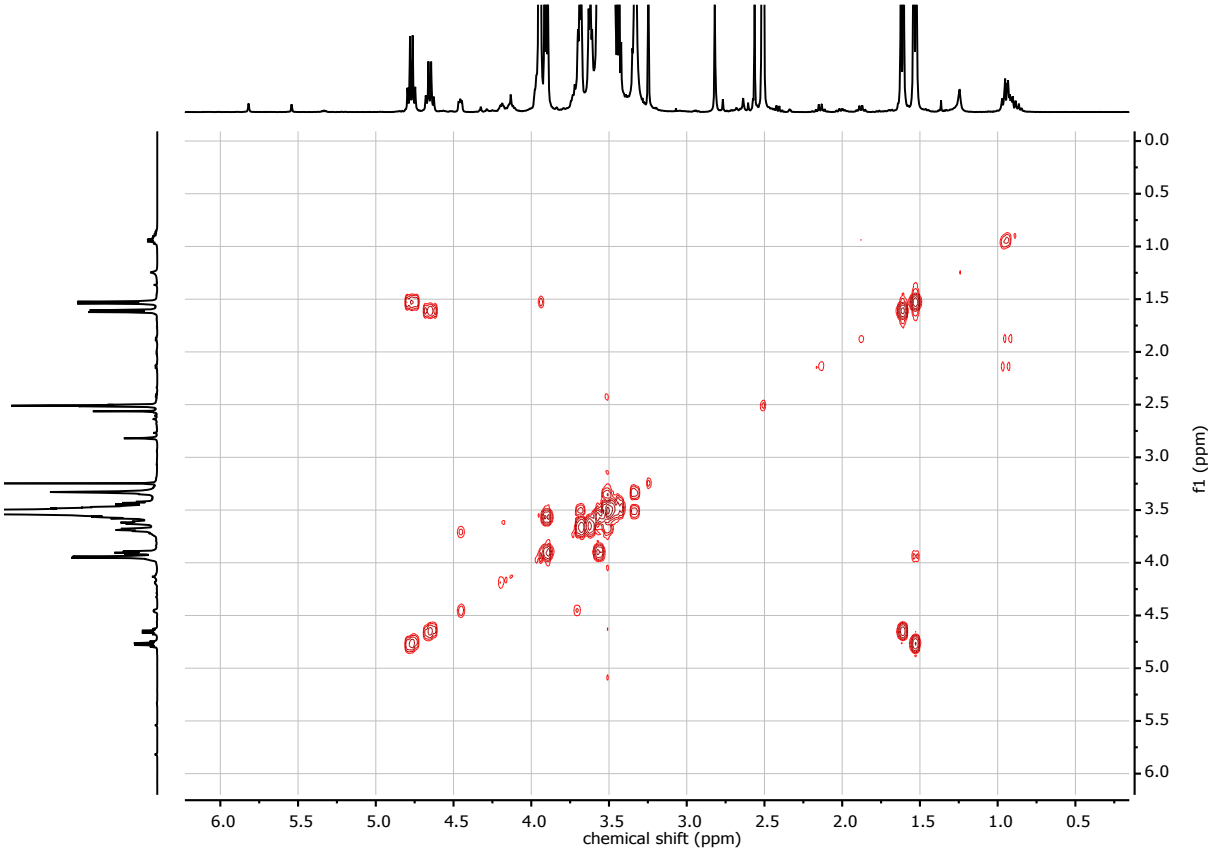

Figure S5: COSY NMR spectrum (400 MHz, DMSO-d_6) of mP(EG_{81}-*co*-*iso*EPB_6)-NHS.

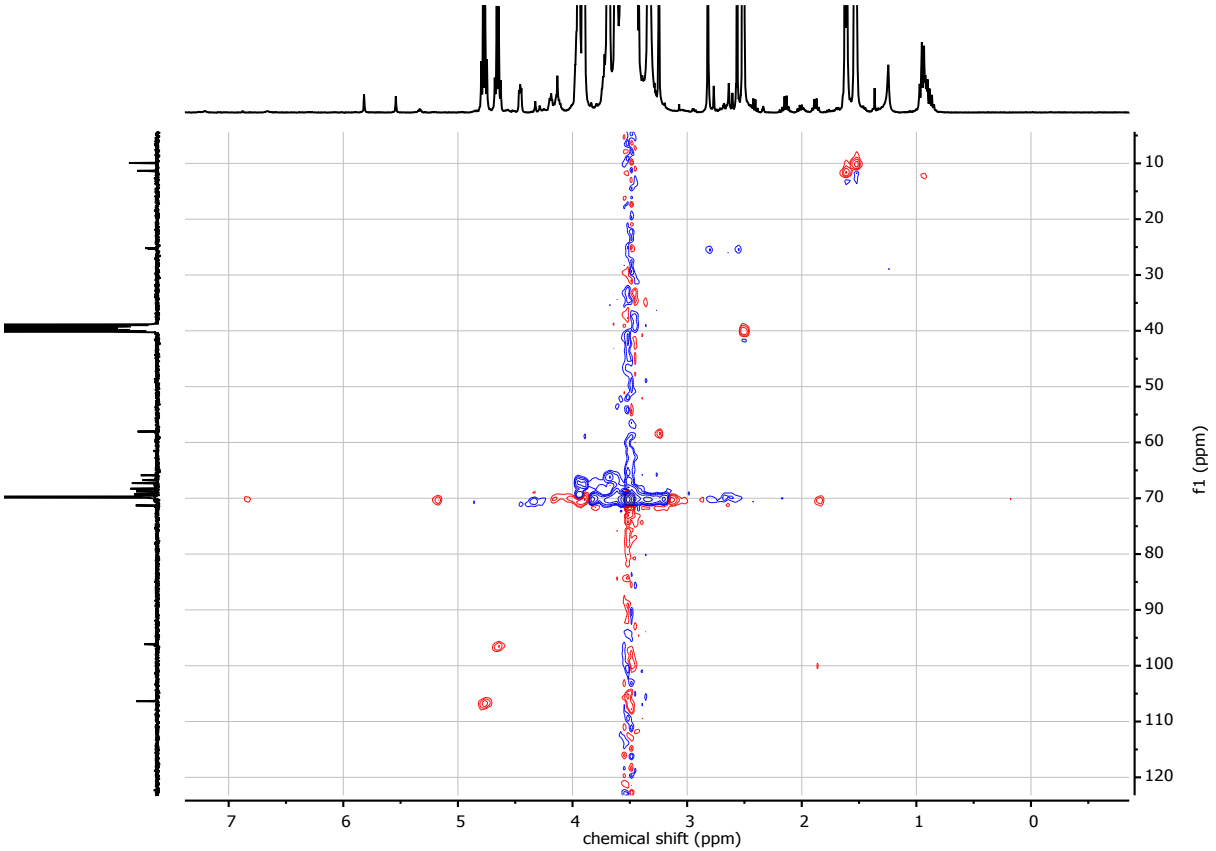

Figure S6: HSQC NMR spectrum (400 MHz, DMSO-d_6) of mP(EG_{81}-*co*-*iso*EPB_6)-NHS.

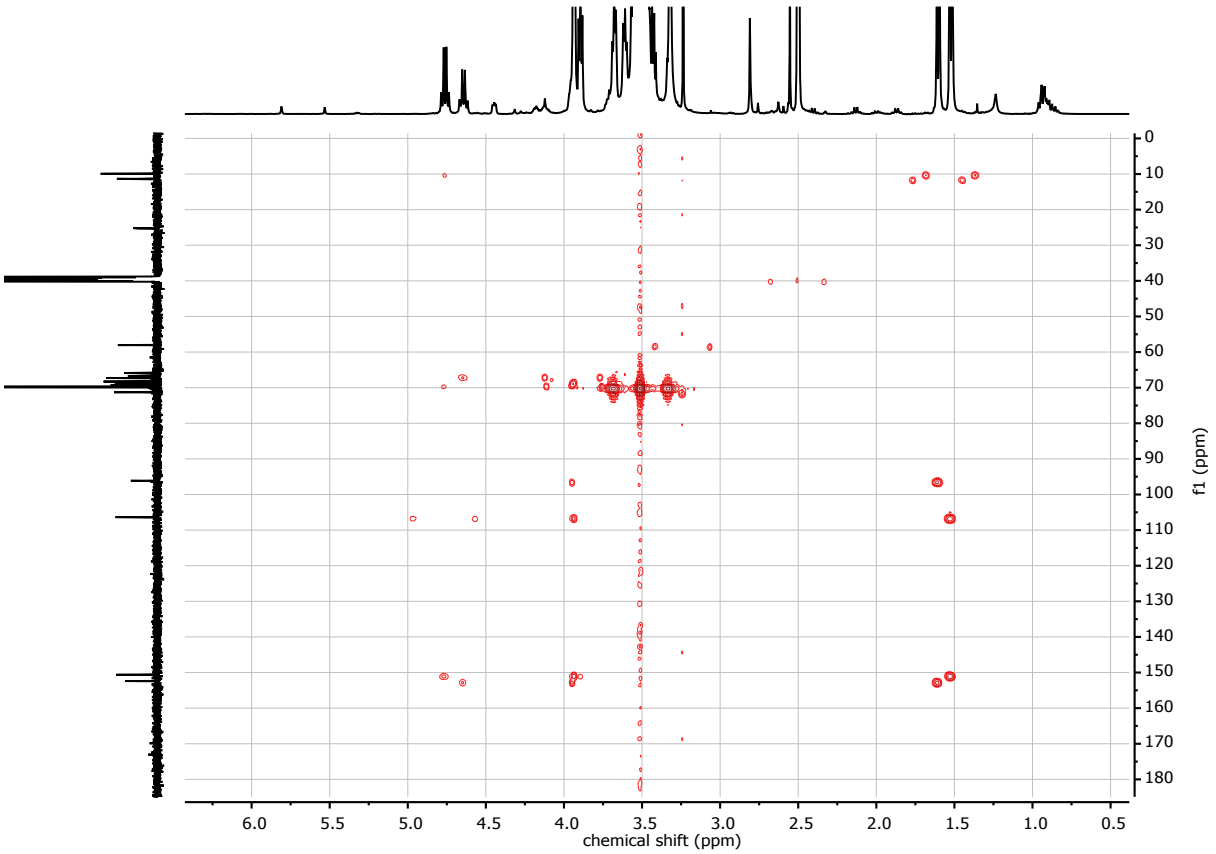

Figure S7: HMBC NMR spectrum (400 MHz, DMSO-d_6) of mP(EG_{81}-*co*-*iso*EPB_6)-NHS.

2. Protein Modification and Analysis

2.1 SDS-PAGE

The protein analysis by SDS-PAGE was performed like described elsewhere.[2] A 15% polyacrylamide gel (Rotiphorese® 30 gel mix) with a thickness of 0.75 mm (T Spacer, Hoefer, USA) was prepared. Native cytochrome *c* (1 mg/mL) and Cyt_{degPEG} (8 mg/mL) were dissolved in water. Denaturation of 15 μL of the protein solutions were achieved by adding 5 μL of Roti®-Load 1 (Carl Roth) and heating in a boiling water bath for 15 min. These 20 μL mixtures were loaded into separated pockets of the gel and 5 μL of Pre-Stained Protein Ladder (10-170 kDa) was used as marker. After gel running at 90 V for 60 min, and 60 min at 200 V, the gel was stained with Coomassie Brilliant Blue G. The SDS-PAGE was imaged with FUSION PULSE TS (Vilber Lourmat Deutschland GmbH) and processed with the 'Image Master' assistant™ Software (camera filter 2).

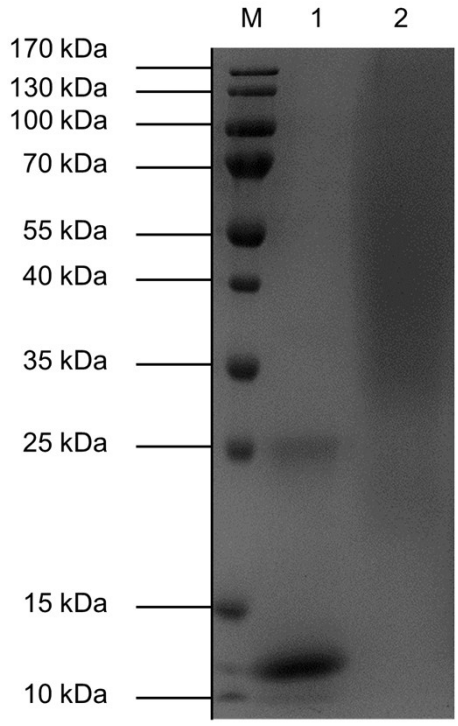

Figure S8: SDS-PAGE (15%) of cytochrome *c* (lane 1) and PEGylated cytochrome *c* (lane 2). Pre-Stained Protein ladder (10–170 kDa) was used as marker. 120 μg of the modified protein and 15 μg of the native cytochrome *c* were analyzed.

2.2 Circular Dichroism (CD)

Jasco J-815 Circular Dichroism spectrometer was used for CD measurements. These measurements were carried out in quartz cells with a path length of 1 mm and recorded at 20 °C using the Spectra Manager 2.08.04 software. Native cytochrome *c* and Cyt_{degPEG} were analyzed with concentrations of 0.1 mg/mL in 10 mM potassium phosphate/50 mM Na_2SO_4 pH 7 buffer. All data points were collected with a resolution of 0.1 nm and measurements were performed in triplets. The spectrum of the buffer was subtracted as background from each measurement. Secondary structure was determined with DichroWeb[3, 4] using the analysis program CONTIN (reference set 7[5]). The detailed comparison of secondary structure is in Table **S1**

Table S1: Calculated secondary structure elements for native Cyt and Cyt_{degPEG} (in %) by DichroWeb using CONTIN.

	Cyt	Cyt_{degPEG}
α-helix	64.4	63.1
β-sheet	3.9	2.5
turns	14.1	14.7
unordered	17.7	19.8

2.3 Enzymatic Activity

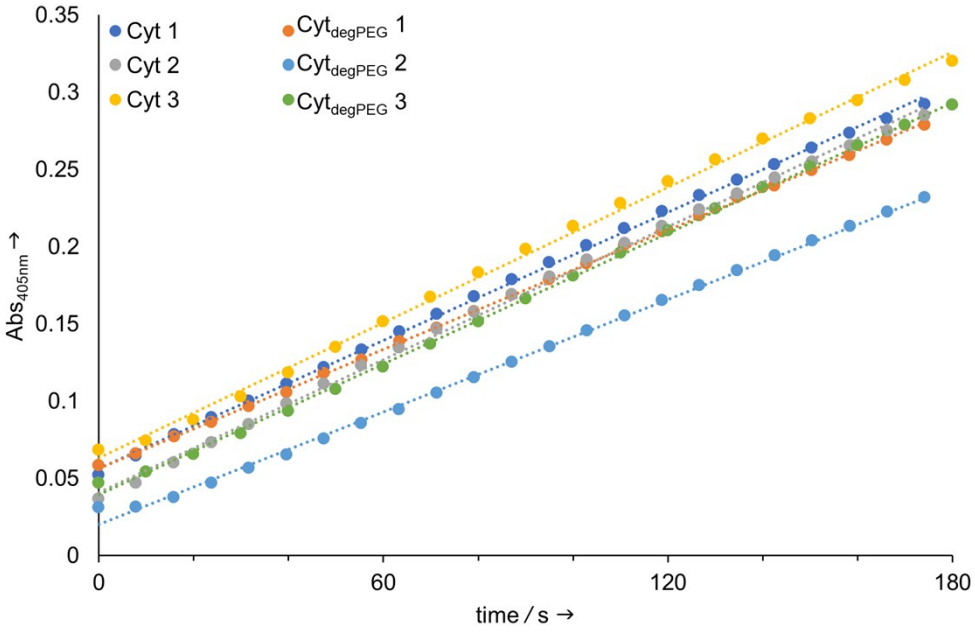

Figure S9: Results of the enzymatic activity assay of native Cyt compared to Cyt_{degPEG} in three individual measurements. The dark blue, grey and yellow straights show the enzymatic activity of native lysozyme and the orange, light blue and green straight of Cyt_{degPEG}.

Table S2: Slopes of absorbance change at 405 nm over time of individual measurements. Through the mean of the three individual Cyt_{degPEG} measurements we obtained 91.4±5.0 % of the initial enzymatic activity.

	slope · 10^{-3}	slope / %
Cyt 1	1.3827	100
Cyt_{degPEG} 1	1.2852	92.95
Cyt 2	1.4332	100
Cyt_{degPEG} 2	1.2147	84.75
Cyt 3	1.4615	100
Cyt_{degPEG} 3	1.4119	96.61

3. Nanoparticle Preparation

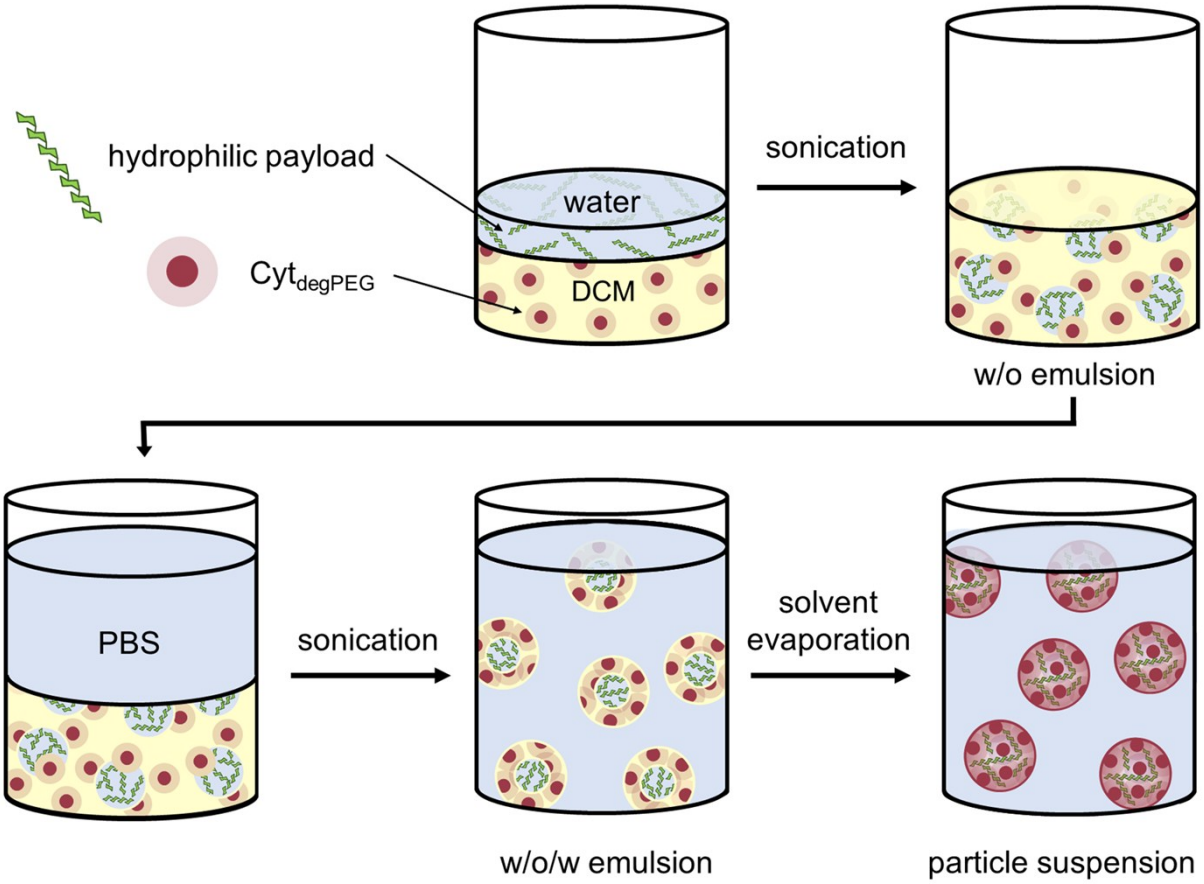

Figure S10: Nanoparticle preparation by double emulsion. Cyt$_{degPEG}$ is dissolved in DCM, covered with a small amount of water, containing the hydrophilic payload OGD. After sonication a w/o emulsion is obtained. Further addition of an aqueous phase and a second ultrasonic treatment results in a w/o/w emulsion. After DCM removal a stable nanoparticle suspension is obtained.

3.1 OGD Loading in Nanoparticles

After purification of the nanoparticle suspensions (Cyt$_{degPEG}$-NP) by dialysis (Float-a-Lyzer®G2 Dialysis Device, MWCO 100 kDa, Spectrum Labs) for 4 hours, the OGD content of the particles was determined by measuring the fluorescence (ex. 490 nm, em. 527 nm) of the particle suspension in comparison to a OGD standard in triplets of 100 μL using an Infinite® 200 PRO (Tecan) plate reader. The fluorescence of the background (PBS pH 7.4) was subtracted from each measurement.

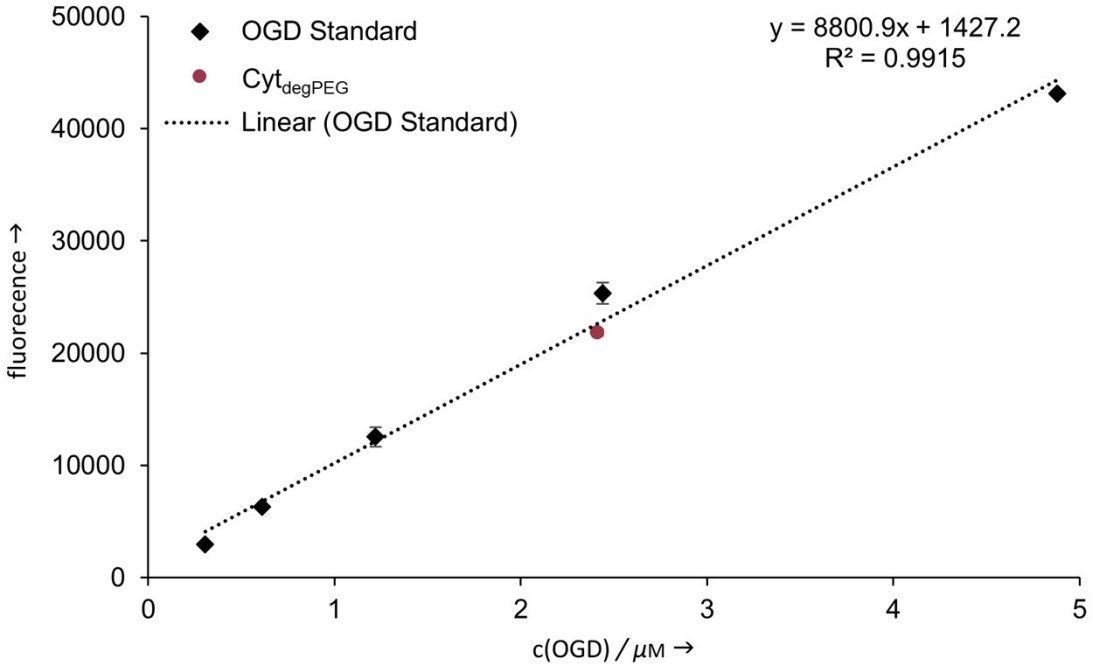

Figure S11: Determination of encapsulated OGD in Cyt_{degPEG}-NP (red dot) using OGD as standard (black squares). The amount of encapsulated OGD was determined to be 2.41±0.16 μM (24.13 μg/mL). The concentration of the particle material (Cyt_{degPEG}) is assumed to be the initial concentration which was used for the particle preparation (58.76 μM, 2.75 mg/mL). This results in a loading ratio of 0.04 mol OGD per 1 mol particle material (Cyt_{degPEG}).

The encapsulation efficiency (EE) and the loading content (LC) were calculated with equations S1 and S2.

$$EE\ (mol\%) = \frac{n_{OGD,encapsulated}}{n_{OGD,feed}} \cdot 100\% \qquad \textbf{(eq. S1)}$$

$$LC\ (wt\%) = \frac{m_{OGD,encapsulated}}{m_{OGD,encapsulated} + m_{CytdegPEG}} \cdot 100\% \qquad \textbf{(eq. S2)}$$

The encapsulated OGD was calculated with $n_{(OGD,encapsulated)}$ of 4.83 ± 0.32 nmol. The initial amount of $n_{(OGD,feed)}$ of the unpurified Cyt_{degPEG}-NP suspension was 9.76 nmol. This results in an encapsulation efficiency of 49.46 ± 3.23%.

The loading content (LC) was calculated with $m_{(OGD,encapsulated)}$ of 48.26 ± 3.15 µg ($n_{(OGD,encapsulated)}$ = 4.83 ± 0.32 nmol, $M_{(OGD,encapsulated)}$ = 10000 g/mol) and $m_{(CytdegPEG)}$ of

2.75 mg ($n_{(CytdegPEG)}$ = 117.52 nmol, $M_{(CytdegPEG)}$ = 23400 g/mol), which results in a loading content of 1.72 ± 0.11%.

3.2 Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis (NTA) was performed on a NanoSight LM 14 equipped with a green laser (532 nm) and a marlin charged coupled device (CCD) camera. Samples were diluted (1:25) in appropriate buffers and loaded into the measurement cell. Movements of particles in the samples were recorded as videos for 30 seconds at 25 °C. The videos were analyzed with the NanoSight NTA 3.1 software showing the mean values of three individual measurements.

3.3 Zeta-Potential

ζ-potential measurements were performed with a Zetasizer Nano ZS instrument (Malvern). Three measurements with automatic measurement duration (between 10 and 100 runs) were performed. The refractive index (RI) of the dispersant (preset: water) was set to 1.330 and the viscosity to 0.8872 cP. The RI of the particles was set to 1.45 with a dielectric constant of 78.5. Cyt_{degPEG}-NPs were analyzed in a clear disposable folded capillary cell at 25 °C. The results are summarized in Table S3.

Table S3: ζ-potential measurement of Cyt_{degPEG} nanoparticles. Nanoparticles show a slightly negative ζ-potential due to the PEGylation of the surface amines on the proteins.

sample	ζ-potential (mV)
Cyt_{degPEG}-NP	-1.98 ± 0.21

3.4 Transmission Electron Microscopy (TEM)

Cyt_{degPEG}-NPs were drop-casted on a 300-mesh copper carbon grid from Plano GmbH for TEM measurements (5 μL). The image acquisition was done with a transmission electron microscope Tecnai T12 (FEI, acceleration voltage: 120 kV, electron source: LaB6 BIO-TWIN cathode) equipped with a 4K CCD camera (Tietz).

3.5 Toxicity of Protein Material and Nanoparticles (MTT-Assay)

HeLa cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 1% pyruvate and 1% penicillin/streptomycin. Cell incubations were performed in a humidified incubator at 37 °C with 5% CO_2 atmosphere. All used buffers were either autoclaved, sterile filtered or already sterile when supplied and were preheated to 37 °C. Cells were grown in 75 cm^2 or 25 cm^2 standard cell culture flasks.

Cells were seeded in 96 well plates with a density of $1.5 \cdot 10^4$ cells per well. After cell attachment overnight, the cell culture media was removed and 100 μL of different dilutions of the samples were added as triplets to the well plate. For this, native Cyt, Cyt_{degPEG} and Cyt_{degPEG}-NP were diluted with culture media to concentrations of 23.50–0.73 μM. After an incubation time of 48 h (37 °C, 5% CO2) a solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) in medium (40 μL, 3.0 mg/mL) was added to each well and incubated for additional 30 min. After removal of the medium a mixture of DMSO (200 μL/well) and 0.1 M glycine buffer (25 μL/well, pH 10) was added. 50 μL of each well of this purple DMSO solution was added to fresh clear-bottom 96-well assay plate (Greiner Bio-One) containing a mixture of glycine buffer (17 μL/well, pH 10) and DMSO (133 μL/well). With an Infinite® 200 PRO (Tecan) plate reader the absorbance at 570 nm and 690 nm (background) were measured and the background was subtracted. The cell viability of sample treated cells were compared to untreated cells.

4. Acknowledgements

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5. Literature

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