Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Catalytic polymersomes to produce strong and long-lasting bioluminescence

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Experimental Section

Materials. *Gaussia* Luciferase was purchased from Enzo life sciences (Lausen, Switzerland). Water-soluble coelenterazine was purchased from Nanolight technologies (Pinetop, USA). Dulbecco's PBS was purchased from BioConcept (Allschwill, Switzerland). N-Octyl-β-D-glucoside was purchased from Anatrace (Maumee, USA). All compounds and solvents were used as received.

Synthesis of $PDMS_{25}$-$PMOXA_{10}$. *Synthesis of monocarbinol-functionalized poly(dimethyl siloxane) ($PDMS_{25}$-OH).* All reactions were conducted under argon atmosphere in dried glassware. Hexamethylcyclotrisiloxane (D_3) (100 g, 0.450 mol) was added to a 250 mL one-neck round-bottom flask and dried over calcium hydride at 75 °C. After two days, D_3 was distilled under vacuum into a 250 mL three-neck round bottom flask with a yield of 90.32 g (0.406 mol, 13 eq). Dried cyclohexane (150 mL) was added, followed by dropwise addition of *n*-butyl lithium solution (12 mL, 30 mmol, 2.5 M in hexane, 1 eq). After stirring for 4 h, dried tetrahydrofuran (15 mL) was added and the reaction was left stirring for 38.5 h at room temperature. The polymerization was quenched by addition of dimethylchlorosilane (9.46 g, 11.1 mL, 100 mmol, 3.2 eq). After 4 h of stirring, the solution was filtered through a glass frit to remove the precipitated lithium chloride salt. Then, the solvents were evaporated using a rotary evaporator and unreacted D_3 was removed via subsequent vacuum distillation. The remaining hydride-terminated $PDMS_{25}$-H (58.35 g, 31.5 mmol) was dissolved in dried toluene (80 mL), followed by the addition of 2-allyloxyethanol (3.76 g, 3.94 mL, 33.1 mmol) and platinum(0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane complex solution (Pt(dvs), in xylene, 35.9 µL). The flask was equipped with a reflux condenser and the solution stirred at 110 °C overnight. Afterwards, toluene was removed using a rotary evaporator and the crude monocarbinol-functionalized $PDMS_{25}$-OH was dissolved in dichloromethane (100 mL). Activated charcoal was added, the solution stirred for 30 min and filtered through Celite S.

Finally, the solvent was evaporated to yield a colorless $PDMS_{25}$-OH oil (55.07 g, M_n = 2000 g/mol, 27.5 mmol). $PDMS_{25}$-H and $PDMS_{25}$-OH were characterized by ^{1}H-NMR:

$PDMS_{25}$-H: ^{1}H-NMR (500 MHz, $CDCl_3$, 295 K, δ, ppm): 0.07 (m, 150H, -Si(CH_3)$_2$-), 0.53 (m, 2H, -Si-CH_2-), 0.88 (t, 3H, -CH_2-CH_3), 1.32 (m, 4H, -Si-CH_2-CH_2-CH_3), 4.70 (m, 1H, -Si-*H*).

$PDMS_{25}$-OH: ^{1}H-NMR (500 MHz, $CDCl_3$, 295 K, δ, ppm): 0.07 (m, 155H, -Si(CH_3)$_2$-), 0.53 (m, 4H, -Si-CH_2-), 0.88 (t, 3H, -CH_2-CH_3), 1.32 (m, 4H, -Si-CH_2-CH_2-CH_3), 1.63 (m, 2H, -Si-CH_2-CH_2-CH_2-O-), 3.44 (m, 2H, -Si-CH_2-CH_2-CH_2-O-), 3.54 (m, 2H, -O-CH_2-CH_2-OH), 3.73 (m, 2H, -O-CH_2-CH_2-OH).

Synthesis of poly(dimethyl siloxane)-block-poly(2-methyl-2-oxazoline) ($PDMS_{25}$-b-$PMOXA_{10}$). All reactions were conducted under argon atmosphere in dried glassware. $PDMS_{25}$-OH (10 g, 4.89 mmol, 1 eq) was added to a 250 mL three-neck round-bottom flask and dried overnight at 100 °C under vacuum. After addition of dried hexane (85 mL) and dried and distilled trimethylamine (0.643 g, 0.886 mL, 6.36 mmol, 1.3 eq), the solution was cooled with an ice/sodium chloride/acetone bath and stirred for 15 min. Then trifluormethanesulfonic anhydride (triflic anhydride, 1.66 g, 0.987 mL, 5.87 mmol, 1.2 eq) in hexane (15 mL) was added dropwise over 30 min under cooling. After 4 h stirring, the solution was filtered through a glass frit under inert atmosphere in order to remove the precipitated triflate salt. The resulting triflate-activated PDMS ($PDMS_{25}$-OTf) was obtained after evaporation of the solvent. Subsequently, dried ethylacetate (100 mL) and 2-methyl-2-oxazline (4.58 g, 4.56 mL, 53.8 mmol, 11 eq) were added and the solution was heated to 40 °C. After 63 h, the solution was cooled to room temperature and water (5 mL) and trimethylamine (5 mL) were added in parallel to quench the reaction. After 6 h, the solvents were evaporated using a rotary evaporator. For purification, the crude copolymer was dissolved in 300 mL methanol and centrifuged (4000

rpm, 1664 rcf, 10 min) in order to remove the remaining PDMS homopolymer precipitates. After transferring the supernatant to a round bottom flask, the solvent was evaporated. Subsequently, the copolymer (13.91 g) was dissolved in a 1:1 mixture of ethanol and water and dialysed against ethanol:water (1:1, 2 L in total) with 5 changes over two days, followed by a last dialysis step against water (2 L). The resulting copolymer precipitate was lyophilized. The purified copolymer (11.82 g) was not able to undergo self-assembly into vesicular structures. Therefore, it was extracted in a 1:1 mixture of methanol and hexane (each 200 mL). The solvent from the bottom methanol-enriched phase was evaporated and the purified, $PDMS_{25}$-*b*-$PMOXA_{10}$ was obtained as a slightly yellow gel (10.74 g, M_n = 2850 g/mol, 3.77 mmol). $PDMS_{25}$-OTf and $PDMS_{25}$-*b*-$PMOXA_{10}$ where characterized by ^{1}H-NMR:

$PDMS_{25}$-OTf: ^{1}H-NMR (500 MHz, $CDCl_3$, 295 K, δ, ppm): 0.07 (m, 157H, -Si(CH_3)$_2$-), 0.54 (m, 4H, -Si-CH_2-), 0.88 (t, 3H, -CH_2-CH_3), 1.32 (m, 4H, -Si-CH_2-CH_2-CH_3), 1.62 (m, 2H, -Si-CH_2-CH_2-CH_2-O-), 3.45 (m, 2H, -Si-CH_2-CH_2-CH_2-O-), 3.74 (m, 2H, -O-CH_2-CH_2-OH), 4.61 (m, 2H, -O-CH_2-CH_2-OTf).

$PDMS_{25}$-*b*-$PMOXA_{10}$: ^{1}H-NMR (500 MHz, $CDCl_3$, 295 K, δ, ppm): 0.07 (m, 152H, -Si(CH_3)$_2$-), 0.51 (m, 4H, -Si-CH_2-), 0.87 (t, 3H, -CH_2-CH_3), 1.31 (m, 4H, -Si-CH_2-CH_2-CH_3), 1.57 (m, 2H, -Si-CH_2-CH_2-CH_2-O-), 2.14 (m, 30H, -N-CO-CH_3), 3.46 (m, 43H, -O-CH_2-CH_2-N-CH_2-CH_2-N-), 3.80 (m, 2H, -N-CH_2-CH_2-OH).

Expression and extraction of Outer Membrane Protein F (OmpF). Recombinant wild-type OmpF was produced according to a previously reported protocol,[1] with the following modifications: Transformed *E.coli* were grown for 6 h at 30 °C in Terrific Broth (TB; Difco, U.S.A.), and ultracentrifugation was performed at room temperature.

Preparation of GLuc Ncomp. $PDMS_{25}$-$PMOXA_{10}$ block copolymers were dissolved in ethanol to yield a stock solution with a concentration of 10 mg/mL. 600 µL of this solution

was transferred into a 5 mL round-bottom flask and dried in a rotary evaporator (170 mbar, 40 ˚C, 75 rpm). The resulting thin polymer film was rehydrated with a solution composed of 322 µL PBS, 200 µL *Gaussia* Luciferase (GLuc; 0.1 mg/mL in PBS) and 78 µL dialyzed OmpF solution (0.8 mg/mL) which yields the polymer/OmpF ratio previously used for the preparation of PDMS-PMOXA catalytic polymersomes[2]. The resulting solution was stirred overnight at 4 °C, and then extruded (under sterile conditions) 15 times through a polycarbonate (PC) membrane with a 200 nm diameter pore size using an Avanti mini-extruder (Avanti Polar Lipids, Alabama, USA) to unify the size of the polymersomes. The unencapsulated enzymes were removed by size-exclusion chromatography (SEC) eluted with PBS.

Preparation of polymersomes. Control polymersomes were prepared as described for GLuc Ncomp (600 µL of 10 mg/mL $PDMS_{25}$-$PMOXA_{10}$ solution; overnight stirring at 4 °C) with the following modifications: empty polymersomes were rehydrated with PBS only and GLuc Ncomp without OmpF were rehydrated with a solution of GLuc and a solution of dialyzed 3 % OG (to match the traces of OG ≃ 0.05 % present after dialysis of OmpF).

Dynamic light scattering (DLS). The apparent diameter D_H values of polymersomes were determined on a Zetasizer Nano ZSP (Malvern Instruments Inc., UK) at 25 °C at an angle of 173°. Each sample was diluted with PBS to 0.2 mg/mL final concentration. A cuvette was filled with 500 µL sample and subjected to 11 measurement runs with three repetitions.

Static light scattering (SLS). Multi-angle dynamic light scattering (DLS) and static light scattering (SLS) were performed on a setup from LS Instruments (Switzerland), equipped with a 21 mW He-Ne laser (λ = 632.8 nm) for scattering angles from 30° to 150° at 25 °C. All samples were diluted in order to avoid multiple scattering. Second-order cumulant analysis for various angles was performed to obtain the hydrodynamic radius (Rh). The radius of gyration (Rg) was obtained from the SLS data using a MIE fit.[2]

Transmission electron microscopy (TEM). 5 µL aliquots of polymersomes (0.1 mg/mL) were adsorbed to 400 mesh square copper grids. Excess liquid was blotted and grids were negatively stained with 2% uranyl acetate. Micrographs of nanostructures were recorded on a Philips CM100 transmission electron microscope at an accelerating voltage of 80 kV.

Estimation of GLuc encapsulation efficiency. The concentration of non-encapsulated GLuc was determined from the fraction of free GLuc present in the solution after SEC purification of GLuc Ncomp. Empty polymersomes with OmpF formed under the same conditions as GLuc Ncomp (but without GLuc) were used as a blank. After UV-vis (280 nm) absorbance measurements using a NanoDrop 2000 spectrophotometer (Thermofisher), we determined the amount of GLuc molecules encapsulated for 1mL of polymersomes ($1.21*10^{13} \pm 5.52*10^{12}$) by calculating the difference between the total amount of GLuc used for film rehydration and the total amount of enzyme that was not encapsulated. In parallel, the concentration of vesicles ($1.7*10^{12}$ vesicles/mL) was determined via single nanoparticle tracking analysis (NTA) using a NanoSight NS300 instrument from Malvern Analytical (Malvern, United Kingdom). The number of encapsulated GLuc molecules was divided by the number of vesicles, obtaining a value of 7 ± 3 GLuc molecules encapsulated per vesicle. An approximate percentage of GLuc encapsulated has been calculated to be 12 ± 5 %, which is consistent with the values reported in the literature for the encapsulation efficiency of molecules inside polymersomes.[3-5] After SEC purification, the concentrated GLuc Ncomp solution (2 mg/mL) was obtained, corresponding to a concentration of GLuc of 0.80 µg/mL.

Luminescence assay. GLuc Ncomp, free GLuc enzyme and control polymersomes were assayed using a spectrophotometer equipped with an injector (SpectraMax ID3 microplate reader, Molecular Devices, U.S.A.) in luminescence mode using black 96-well plates. 50 µL GLuc Ncomp (1 mg/mL), control polymersomes (1 mg/mL), or free GLuc enzyme (0.4 µg/mL, corresponding to 1 mg/mL of GLuc Ncomp) was added to each well. Subsequently, 50 µL/well

of a freshly prepared coelenterazine substrate (80 µM in PBS) was added by means of the automatic injector to ensure the direct recording of luminescence. To determine the kinetics, luminescence was measured every 30 seconds for 1 hour.

Luminescence assay in cell supernatants. MCF-7 cells were seeded in a 96-well plate (3000 cells in 100 µL /well) and cultured overnight at 37°C. Then, 50 µL of the cell supernatant was removed and replaced by 50 µL of fresh medium and 100 µL of GLuc Ncomp (2 mg/mL), free GLuc (0.8 µg/mL), or control polymersomes (2 mg/mL). After 3 days of culturing at 37°C, 50 µl of cell supernatant was collected and assayed for luminescence production as described above.

Stability assays. After storage of GLu Ncomp, GLuc Ncomp without OmpF and free GLuc for 3, 7 and 14 days at 4 °C or 37 °C in PBS or cell medium, samples with a consistent enzyme concentration of 0.40 µg/mL were assayed for luminescence production in the same manner as described above. The activity recorded on the day of polymersome preparation (Day 0) in PBS or cell medium was set to 100%. The activity after different time periods of storage was determined in comparison to the activity of Day 0. Luminescence production at the time point where the luminescence signal is the highest (t = 10 min for GLuc Ncomp and t = 0 for free GLuc enzymes) was used to compare the activity of GLuc over time of storage.

Cell culture. MCF-7 (epithelial breast cancer, human; ATCC, HTB-22) were routinely cultured in Eagle's Minimum Essential Medium (EMEM, Gibco Life Sciences) supplemented with 10% fetal bovine serum (BioConcept), 100 units/mL penicillin and 100 µg/mL Streptomycin (Sigma Aldrich). Cells were maintained in a humidified atmosphere at 37 ^{o}C and 5% CO_2.

Cell viability assay (MTS). CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Invitrogen) was used to determine cell viability. In accordance with the supplier's

protocol, MCF-7 cells were seeded at a concentration of 3000 cells/well in a 96 well plate. After 24 h, 50 µL of medium was removed and replaced by 50 µL of fresh medium and 100 µL of GLuc Ncomp (2 mg/mL), free GLuc, or control PBS. The cells were incubated in the presence of GLuc Ncomp, free GLuc or PBS control for 3 more days in a humidified atmosphere at 37 °C and 5% CO_2. The MTS reagent (20 µL) was added to each well and absorbance at 490 nm was measured after 2 h at 37 °C using a Spectramax plate reader. The data was normalized to PBS treated control cells after background absorbance removal.

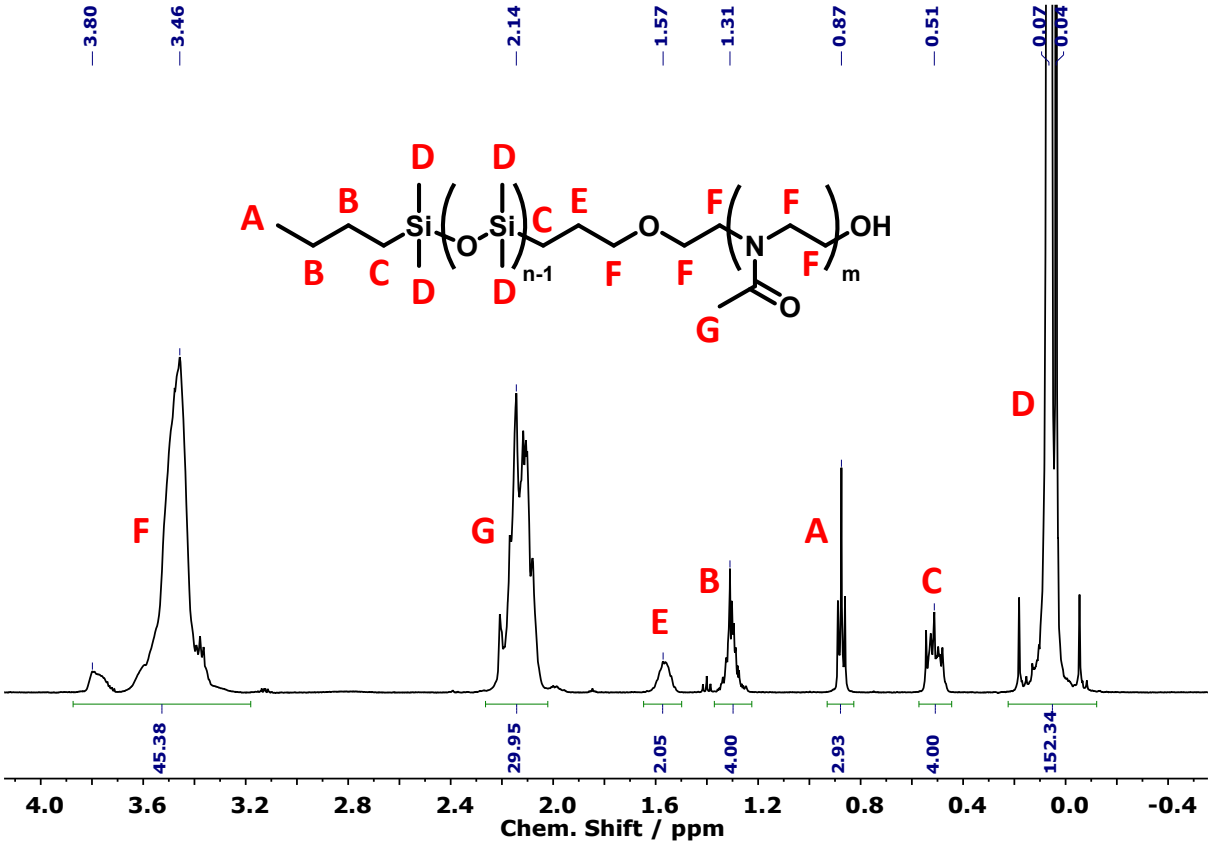

Figure S1. ^{1}H-NMR of $PDMS_{25}$-*b*-$PMOXA_{10}$ in $CDCl_3$.

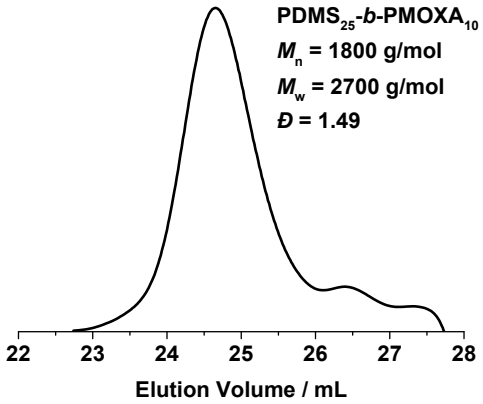

Figure S2. GPC elugram of $PDMS_{25}$-*b*-$PMOXA_{10}$ in tetrahydrofuran, measured *via* refractive index detector.

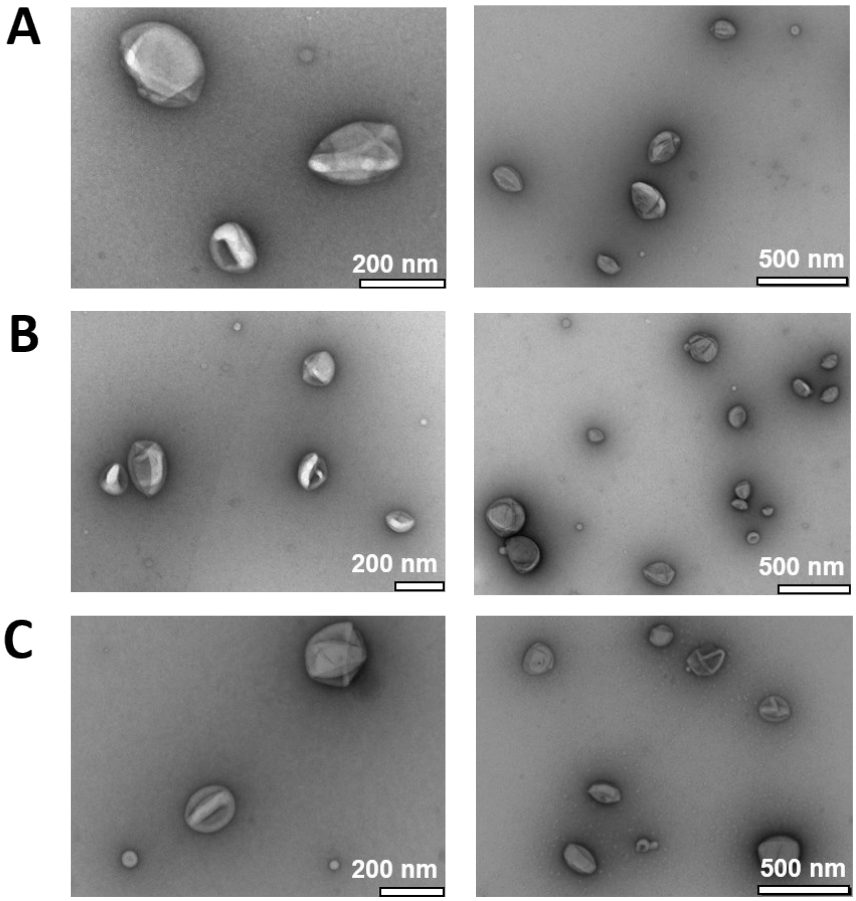

Figure S3. TEM micrographs of (A) empty $PMOXA_{19}$-$PDMS_{25}$ polymersomes, (B) empty polymersomes with OmpF, (C) GLuc Ncomp without OmpF.

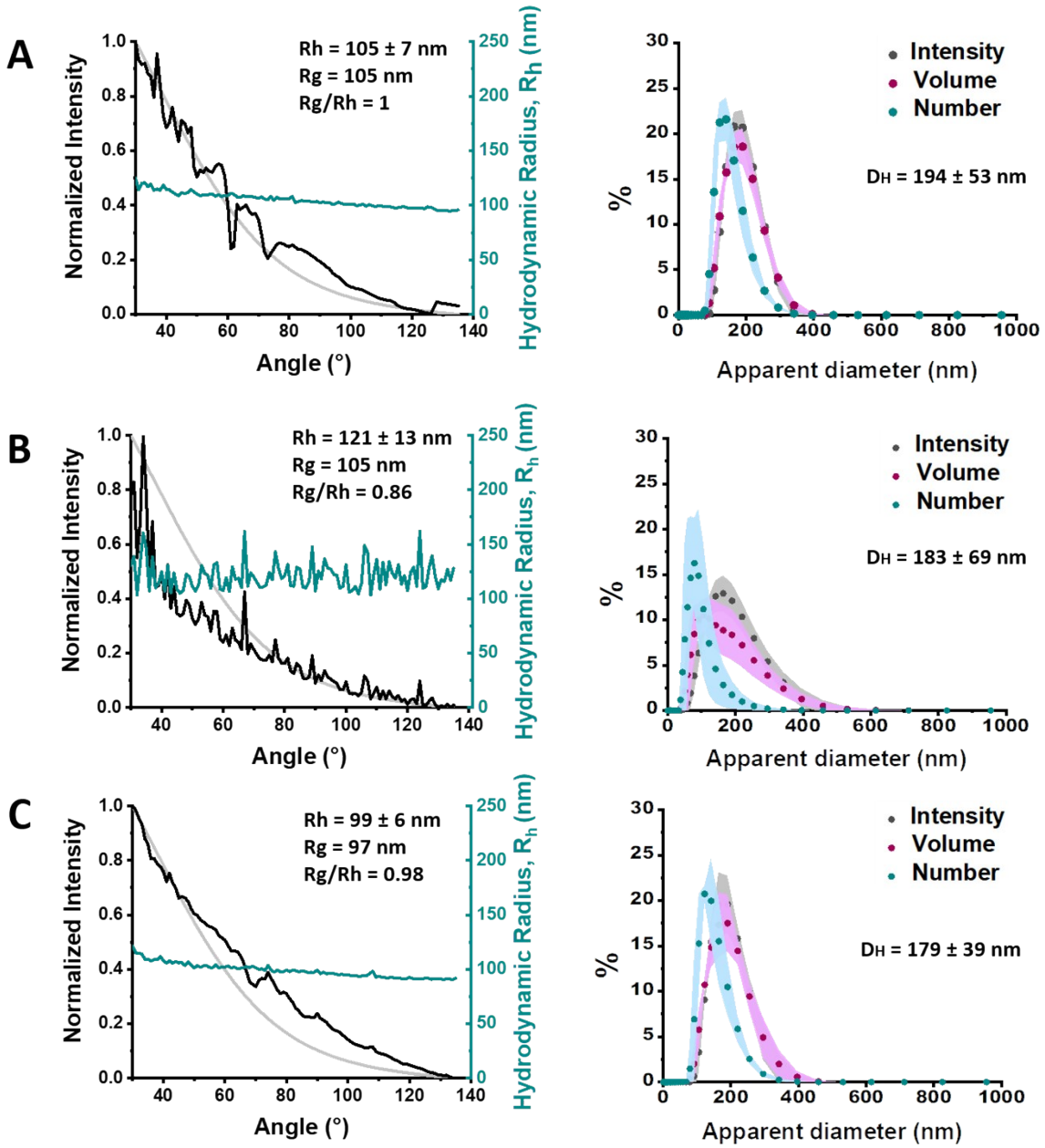

Figure S4. Size determination via SLS (left) and DLS (right) of (A) empty $PMOXA_{19}$-$PDMS_{25}$ polymersomes (PDI = 0.163), (B) empty polymersomes with OmpF (PDI = 0.170), (C) GLuc Ncomp without OmpF (PDI = 0.159). SLS measurements show the DLS profile representing the hydrodynamic radius (Rh) of polymersomes at different angles (blue), the intensity at different angles (black) and the suitable MIE fit (grey) used to determine the radius of gyration (Rg). The ratio $0.775 < Rg/Rh \leq 1$ indicates a hollow sphere structure. DLS graphs show the

Rh of polymersomes, measured at 173° angle, by percentage of intensity (grey), volume (purple) and number (blue).

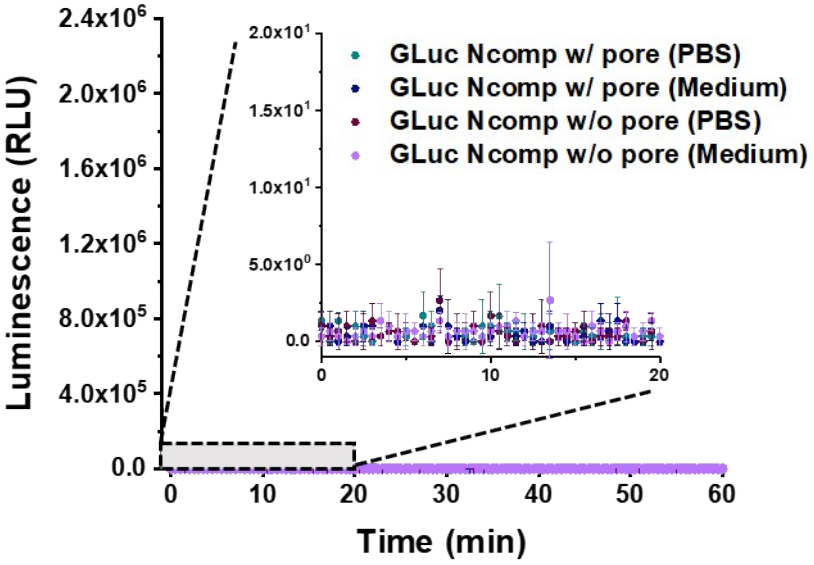

Fig S5. Luminescence production in the absence of coelenterazine substrate: GLuc Ncomp with OmpF in PBS (blue), GLuc Ncomp with OmpF in cell medium (dark blue), GLuc Ncomp without OmpF in PBS (purple), GLuc Ncomp without OmpF in cell medium (light purple).

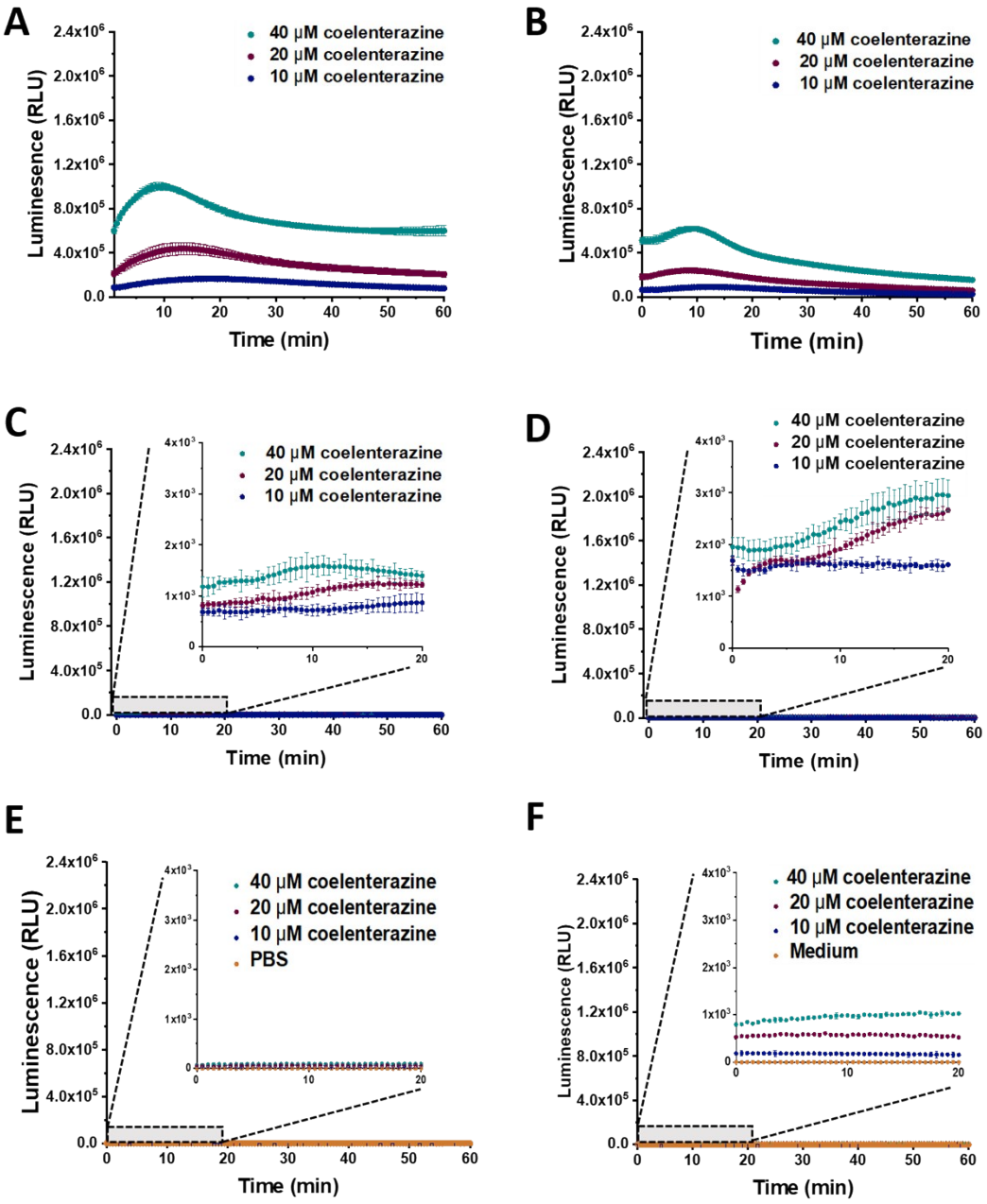

Figure S6. Activity of GLuc Ncomp with and without OmpF in presence of different coelenterazine concentrations in PBS and cell medium. (A) Activity of GLuc Ncomp with OmpF in PBS. (B) Activity of GLuc Ncomp with Ompf in cell medium. C) Activity of GLuc Ncomp without OmpF in PBS. (D) Activity of GLuc Ncomp without OmpF in cell medium. (E) Luminescence background in PBS alone in presence of different concentrations of coelenterazine. (F) Luminescence background in cell medium alone in presence of different concentrations of coelenterazine.

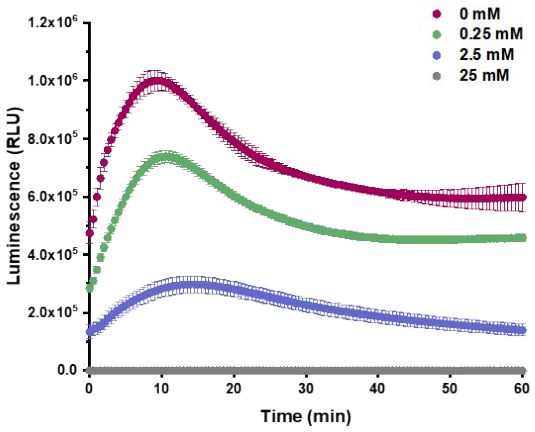

Figure S7. Activity of GLuc Ncomp in PBS in presence of (0, 0.25, 2.5, 25 mM) ascorbic acid. Ascorbic acid reduce the production of light via prevention of the coelenterazine oxidation in a dose dependent manner.

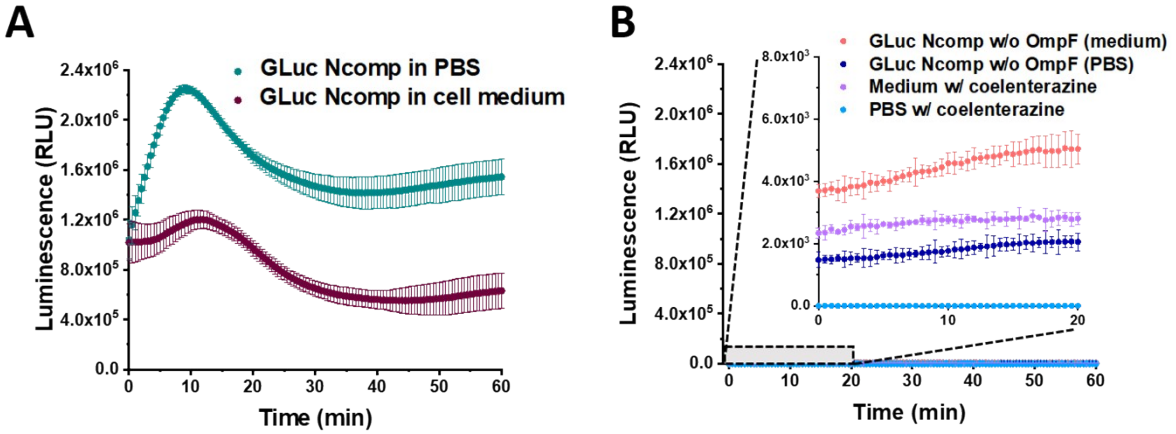

Figure S8. Luminescence production at higher concentration of GLuc Ncomp (50 µL of 2 mg/mL, final concentration = 1 mg/mL). (A) Activity of concentrated GLuc Ncomp in PBS and cell medium. (B) Luminescence background of control samples: GLuc Ncomp without OmpF in PBS and cell medium and coelenterazine in PBS and cell medium. The ratio of the reactants is held constant (50 µL of 160 µM coelenterazine).

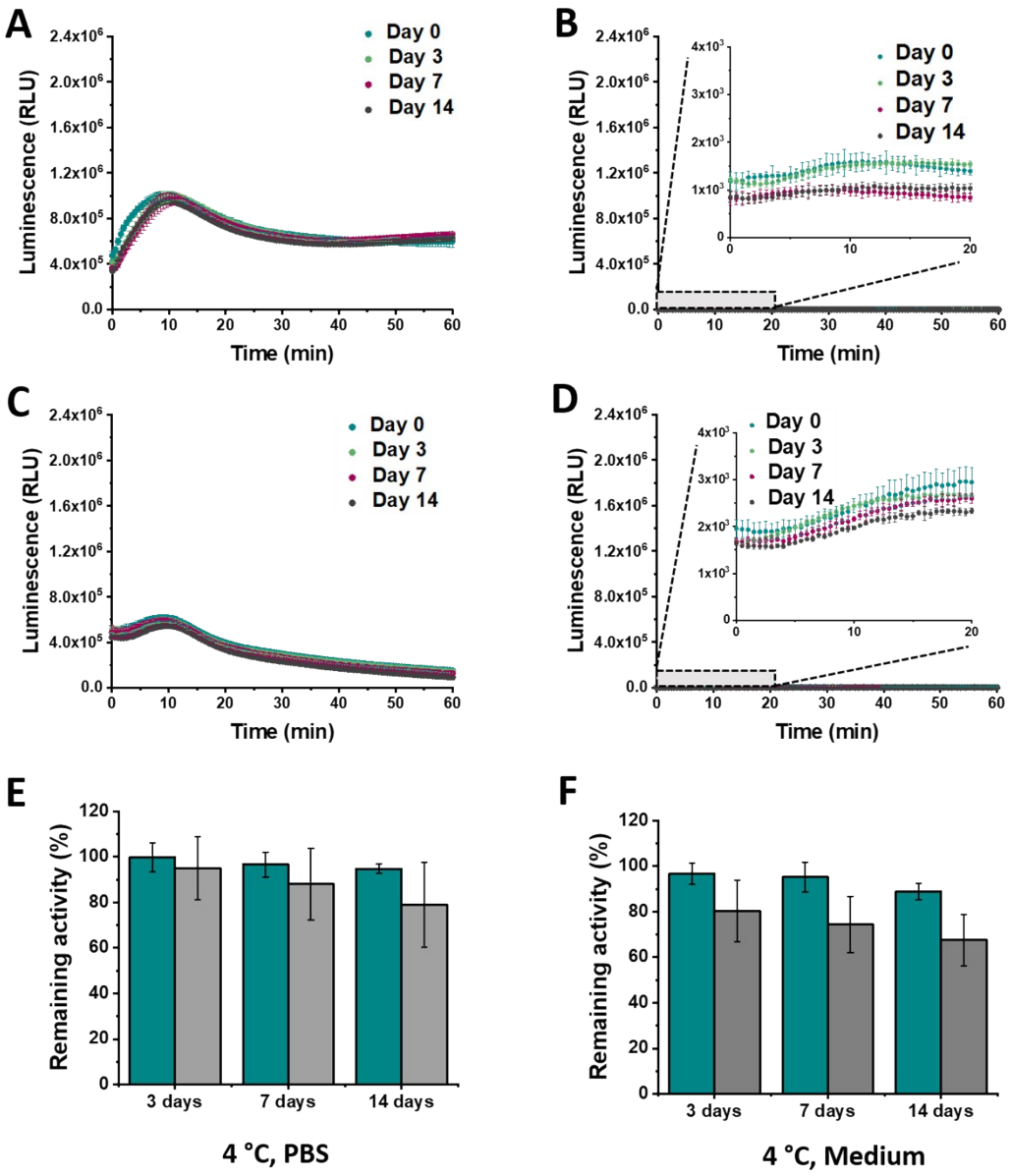

Figure S9. Activity of GLuc Ncomp with and without OmpF after incubation at 4 °C for 3, 7 and 14 days. (A) Activity of GLuc Ncomp with OmpF in PBS. (B) Activity of GLuc Ncomp without OmpF in PBS. C) Activity of GLuc Ncomp with OmpF in cell medium. (D) Activity of GLuc Ncomp without OmpF in cell medium. (E) Remaining percentages of activity of free (grey) and encapsulated GLuc (cyan) in culture medium, upon storage at 4 °C in PBS. (F) Remaining percentages of activity of free (grey) and encapsulated GLuc (cyan) in culture medium, upon storage at 4 °C in culture medium.

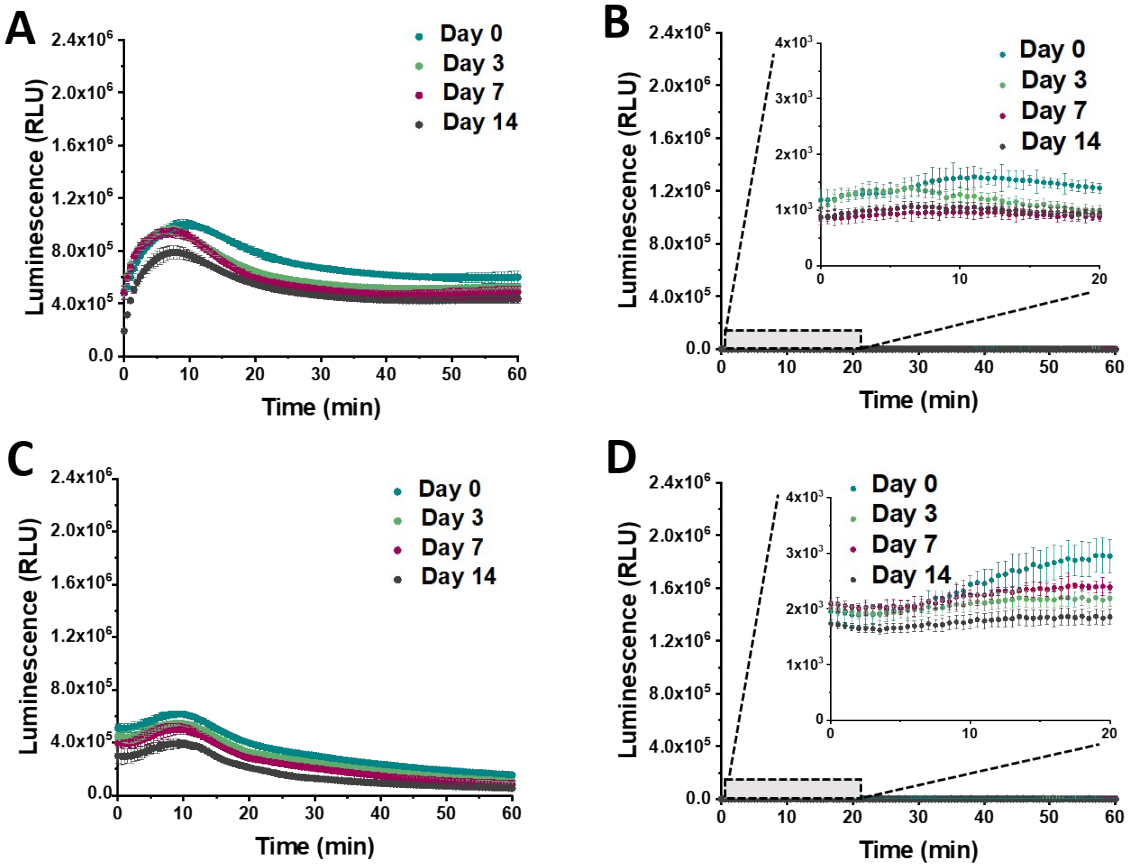

Figure S10. Activity of GLuc Ncomp with and without OmpF after incubation at 37 °C for 3, 7 and 14 days. (A) Activity of GLuc Ncomp with OmpF in PBS. (B) Activity of GLuc Ncomp without OmpF in PBS. C) Activity of GLuc Ncomp with OmpF in cell medium. (D) Activity of GLuc Ncomp without OmpF in cell medium.

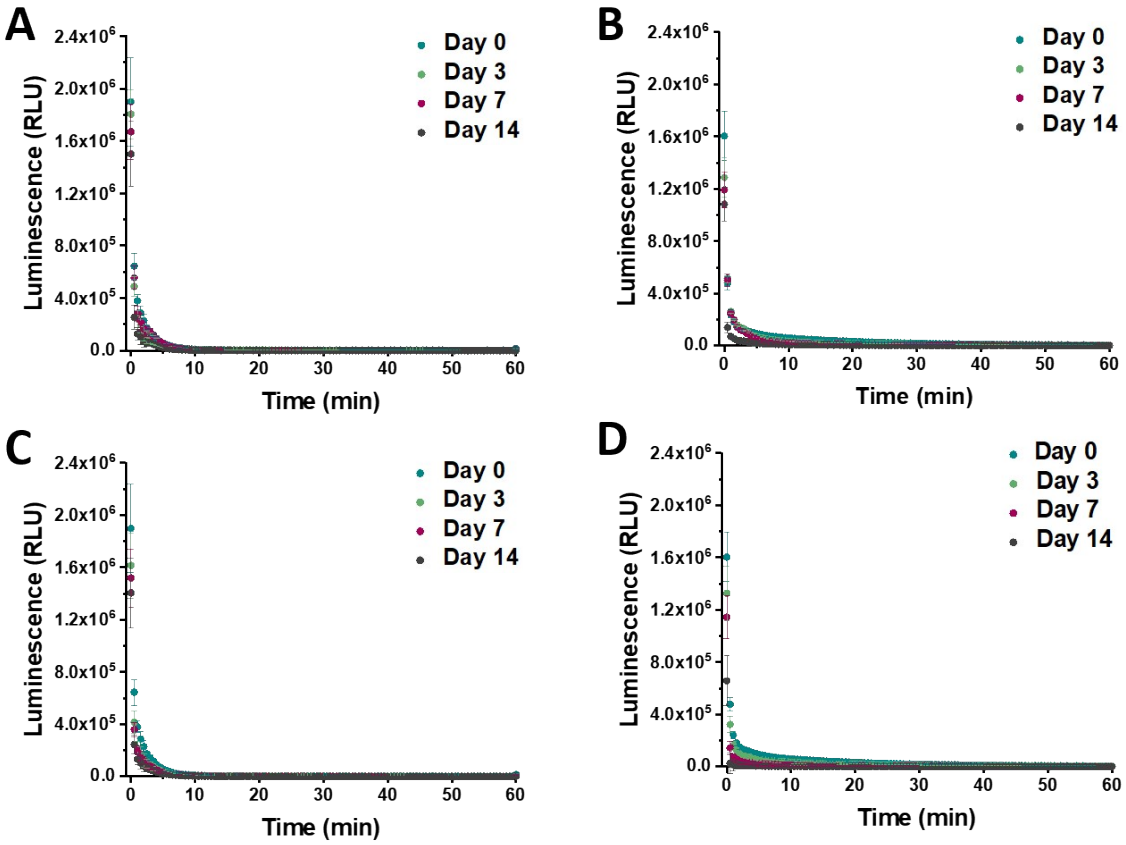

Figure S11. Activity of non-encapsulated (free) GLuc after incubation at 4 °C or 37 °C for 3, 7 and 14 days. (A) Activity of free GLuc at 4 °C PBS. (B) Activity of free GLuc at 4 °C in cell medium. (C) Activity of free GLuc at 37 °C in PBS. (D) Activity of free GLuc at 37 °C in cell medium.

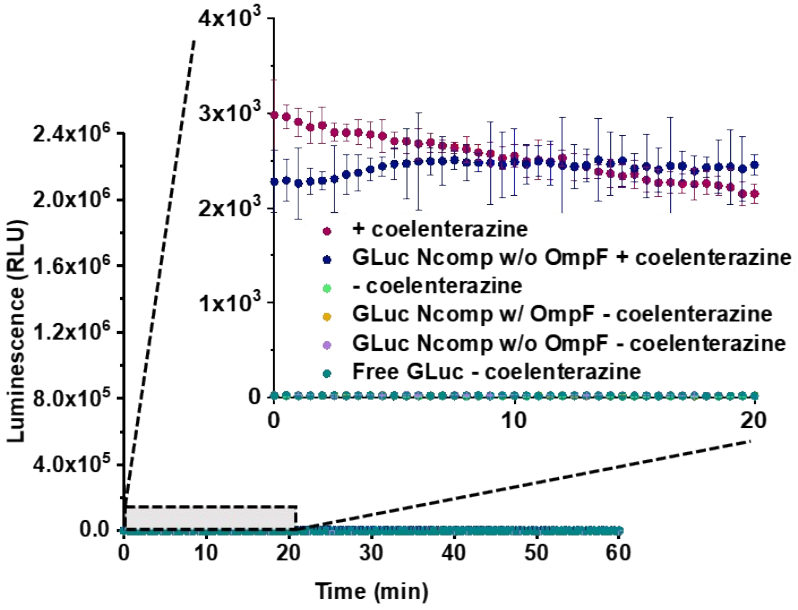

Figure S12. Controls for luminescence production in cell supernatant: Cell supernatant with coelenterazine (pink), GLuc Ncomp without OmpF with coelenterazine (dark blue), cell supernatant without coelenterazine (green), GLuc Ncomp with OmpF without coelenterazine (orange), GLuc Ncomp without OmpF without coelenterazine (light purple), non-encapsulated (free) GLuc without coelenterazine (light blue). In the absence of coelenterazine, the signals remain below 30 RLU.

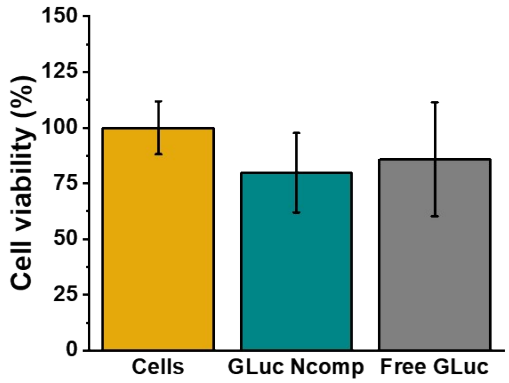

Figure S13. Cell viability as percentage of control MCF-7 cells incubated with PBS (yellow), GLuc Ncomp (cyan) and free GLuc (grey). No significant decrease in viability is observed as compared to control cells, indicating the GLuc Ncomp and free GLuc are nontoxic.

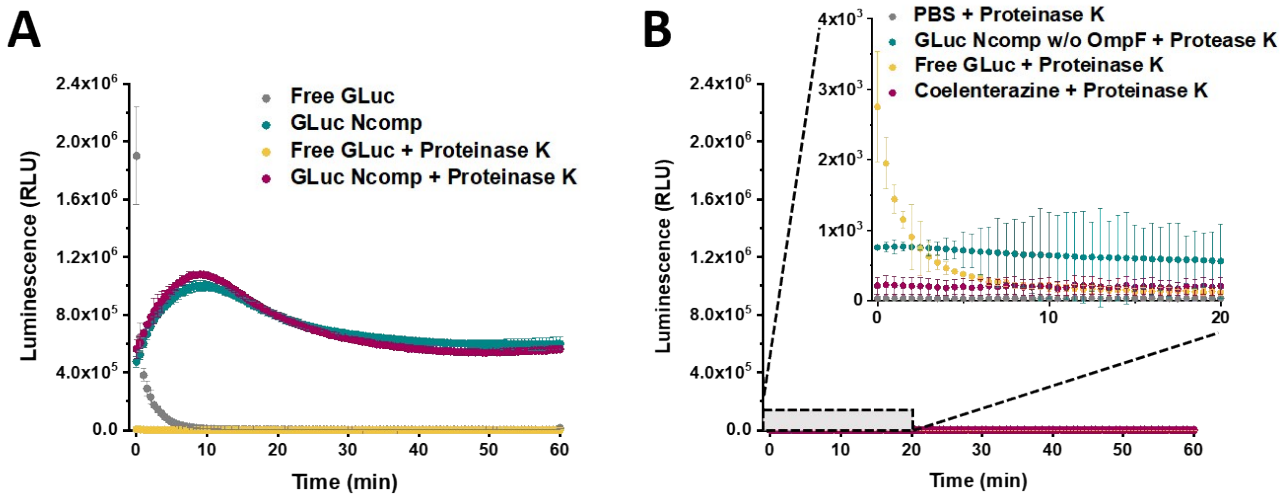

Figure S14. Activity of free and encapsulated GLuc in presence of Proteinase K. (A) Activity of Free GLuc in absence (grey) and in presence (yellow) of Protease K, as compared to encapsulated GLuc in absence (blue) and in presence (purple) of Proteinase K. (B) Activity of free GLuc as compared to controls in presence of Proteinase K: PBS (grey), GLuc Ncomp without OmpF (blue), Free GLuc (yellow) and coelenterazine (purple). The samples have been incubated for 24 h at 37 °C with 0.1 mg/mL of Proteinase K.

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2. D. Daubian, J. Gaitzsch and W. Meier, *Polym. Chem.*, 2020, **11**, 1237–1248.

3. Q. Chen, H. Schönherr and G. J. Vancso, *Small*, 2009, **5**, 1436–1445.

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5. D. R. Arifin and A. F. Palmer, *Biomacromolecules*, 2005, **6**, 2172–2181.