Electronic Supplementary Material (ESI) for Nanoscale.


# Triggering the nanophase separation of albumin through multivalent binding to glycogen for drug delivery in 2D and 3D multicellular constructs

Agata Radziwon,[a] Sukhvir K. Bhangu,[b] Soraia Fernandes,[c] Christina Cortez-Jugo[a], Robert De Rose[a], Brendan Dyett,[b] Marcin Wojnilowicz,[a] Petra Laznickova,[c,] Jan Fric,[c] Giancarlo Forte,[c] Frank Caruso,*[,a] and Francesca Cavalieri*[,b,d]

[a]Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia.

[b]School of Science, RMIT University, Melbourne, Victoria 3000, Australia.

[c]International Clinical Research Center (ICRC), St Anne's University Hospital, CZ-65691 Brno, Czech Republic

[d]Dipartimento di Scienze e Tecnologie Chimiche, Università degli Studi di Roma Tor Vergata, via della ricerca scientifica 1, 00133, Rome, Italy

**Corresponding Authors**

*E-mail: fcaruso@unimelb.edu.au; francesca.cavalieri@rmit.edu.au

**Keywords:** glycogen, albumin, nanodrug, cancer therapy, 3D cell culture

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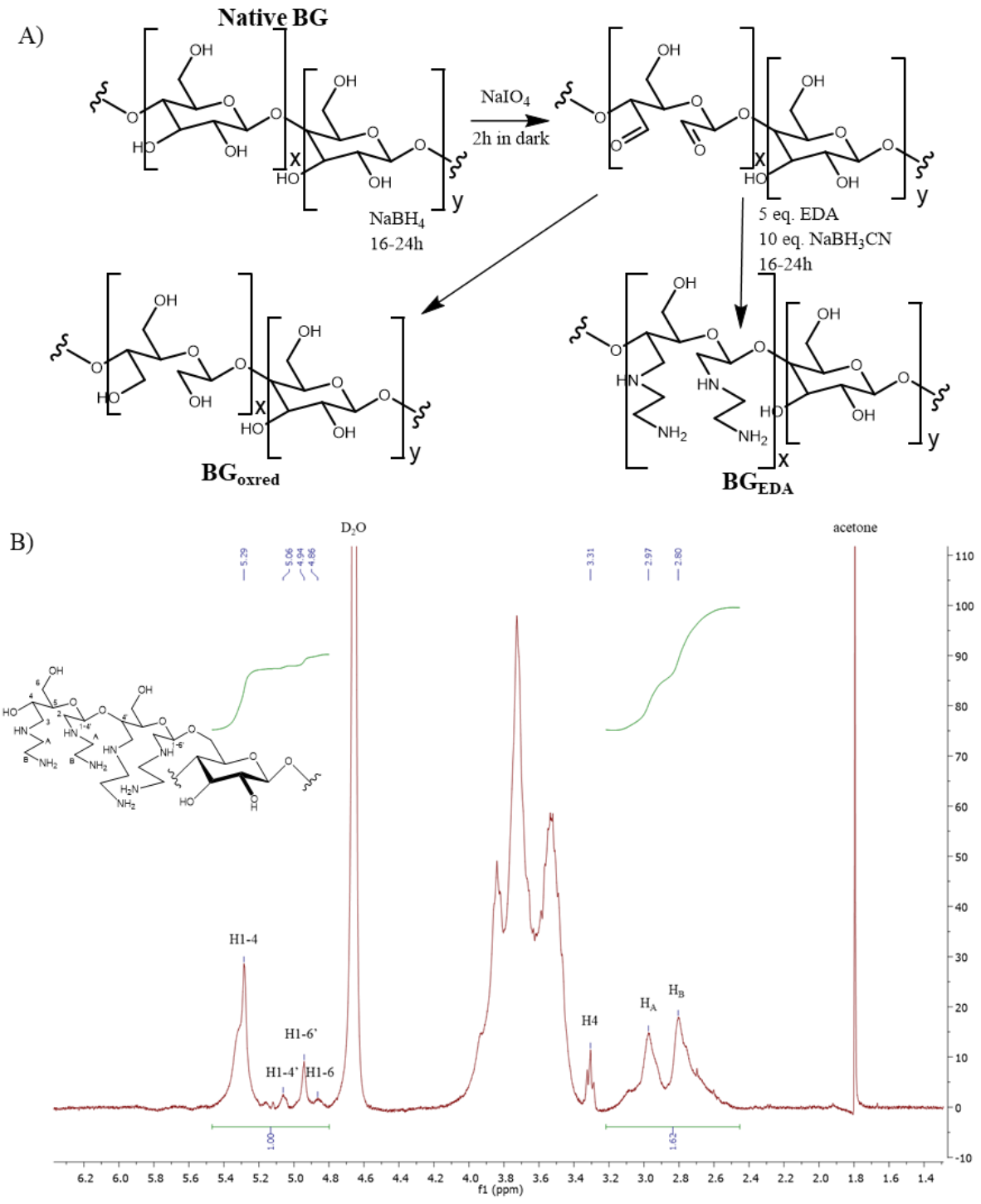


**Figure S1 A)** Reaction scheme for the synthesis of $BG_{EDA}$ and $BG_{oxred}$. **B)** $^{1}$H-NMR spectrum of $BG_{EDA}$ measured on a Bruker 400 MHz NMR instrument at 50 ºC in $D_2O$.

**Table S1.** Properties of $BG_{oxred}$ glycogen nanoparticles with various substitution degrees (DS, as determined by NMR), size, charge (determined by DLS) and degradability by α-amylase. Glycogen from bovine liver (BG) was used as substrate in the ox-red reactions and was modified in 6, 19 and 26%.

| **DS** | **Size (nm)** | **Charge (mV)** | **Degradability (%)** |
|---|---|---|---|
| 0% | 19 ± 4 | -11 ± 7 | 30 ± 8 |
| 6 % | 22 ± 9 | -7 ± 16 | 35 ± 7 |
| 19 % | 26 ± 13 | -13 ± 12 | 34 ± 3 |
| 26% | 17 ± 6 | -17 ± 8 | 51 ± 5 |

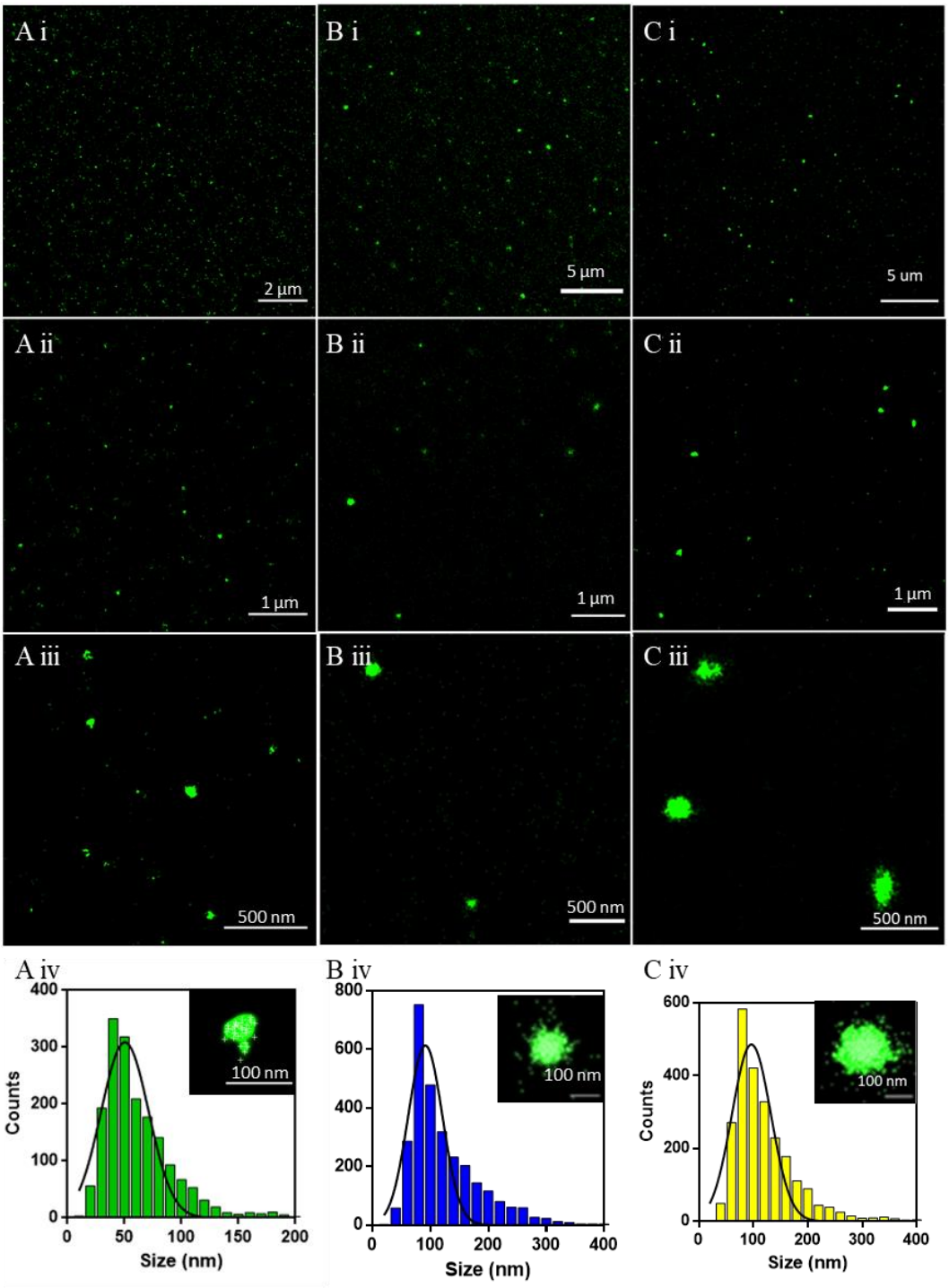


**Figure S2** Representative STORM images of **A)** $BG_{EDA}$; **B)** $NC_{BGEDA/BSA}$ and **C**) $NC_{BGEDA/BSA}$ incubated in 10% FBS for 5 h. Images i – iii show the same sample at different magnifications; images iv present the corresponding histogram plots of the size distribution. Insets show magnified $BG_{EDA}$ particles or $NC_{BGEDA/BSA}$ ($n \geq 1800$).

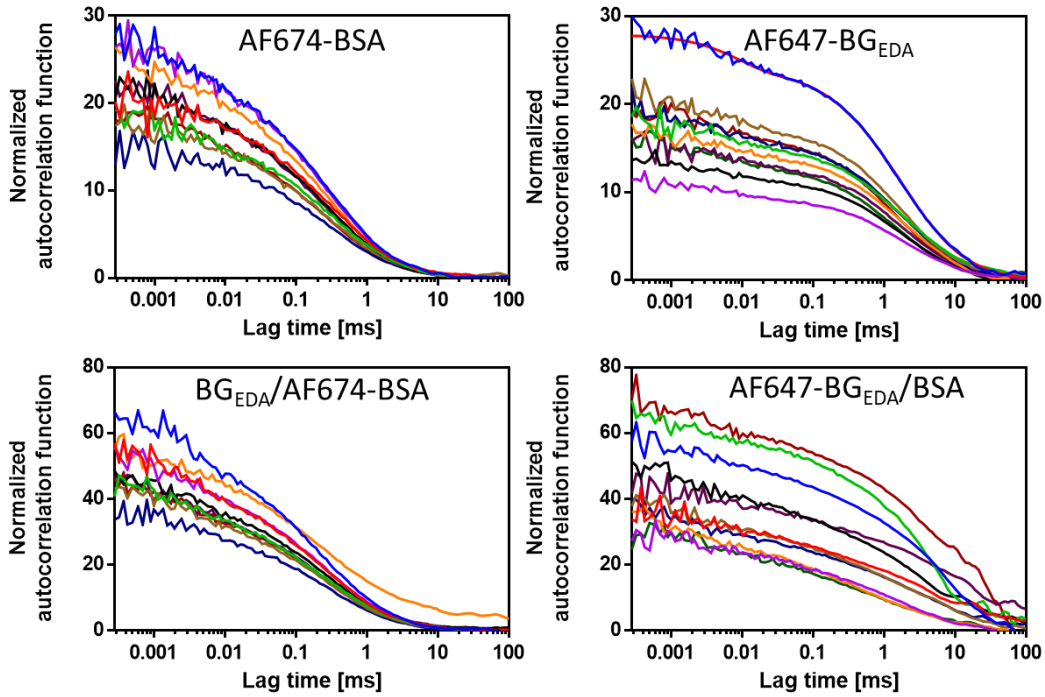


**Figure S3** Figures showing representative autocorrelation functions obtained by FCS for AF647-BSA, AF647-BG$_{EDA}$ and NC$_{BGEDA/BSA}$ based on the diffusion of AF647-BSA or AF647-BG$_{EDA}$.

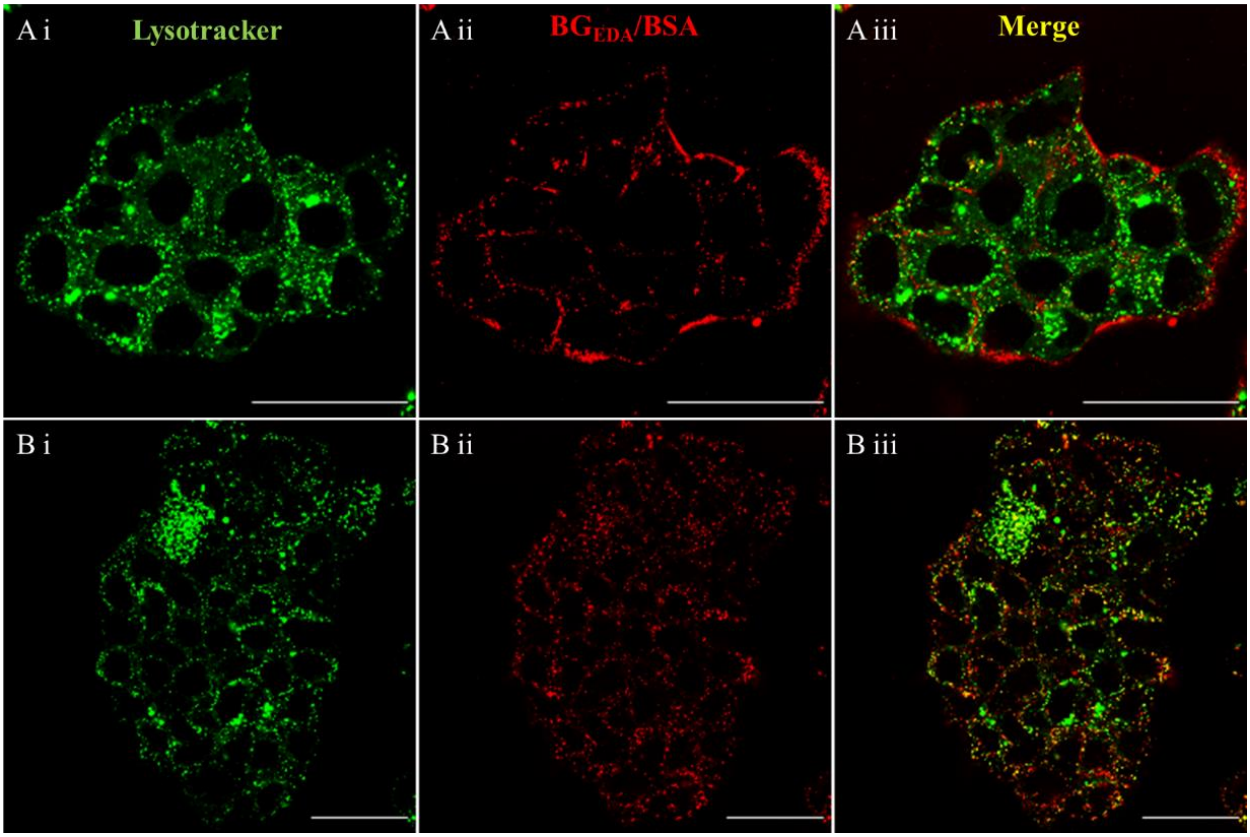


**Figure S4** Representative CLSM images of BT474 cells after A) 4 h and B) 24 h incubation with $NC_{AF647\text{-}BGEDA/BSA}$ (ii, red). Endo/lysosomal vesicles are stained with LysoTracker Green (I; green). Scale bar is 50 µm.

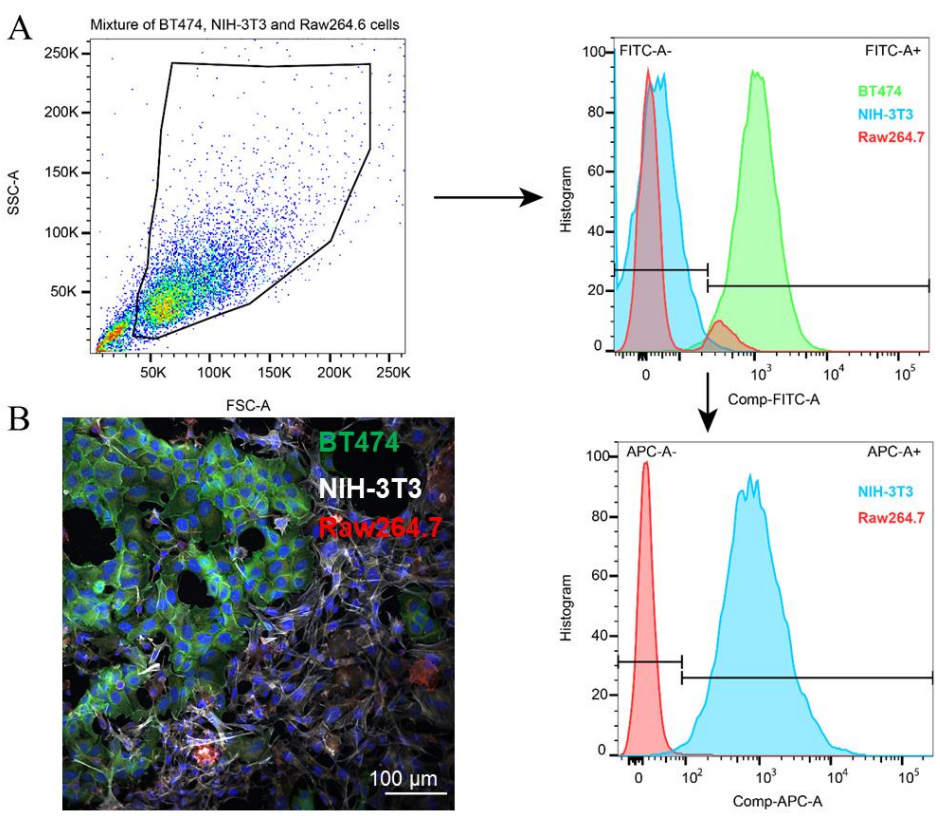


**Figure S5 A)** Gating strategy for discrimination of specific cell lines based on the labelling with spectrally distinct dyes. **B)** Representative confocal microscopy image of the co-cultured BT474, 3T3 and CD-11 cells. BT474 cells are stained with anti E-cadherin antibody (green), Raw264.7 cells with anti CD-11 antibody (red), actin is stained in white. Cell nuclei were counterstained with Hoechst (blue).

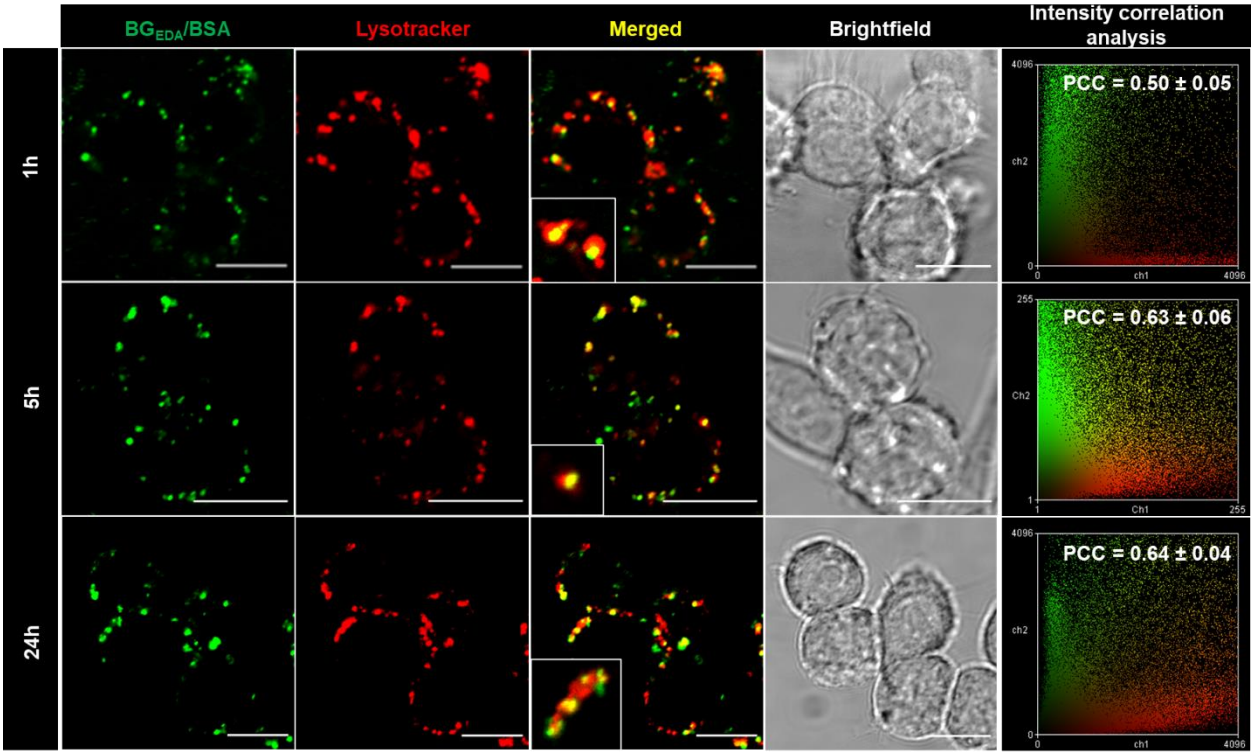


**Figure S6.** Representative CLSM images of $NC_{BGEDA/BSA}$ and Lysotracker™ in Raw 264.7 cells taken after 1, 5 and 24 h incubation. Scale bars are 10 µm.

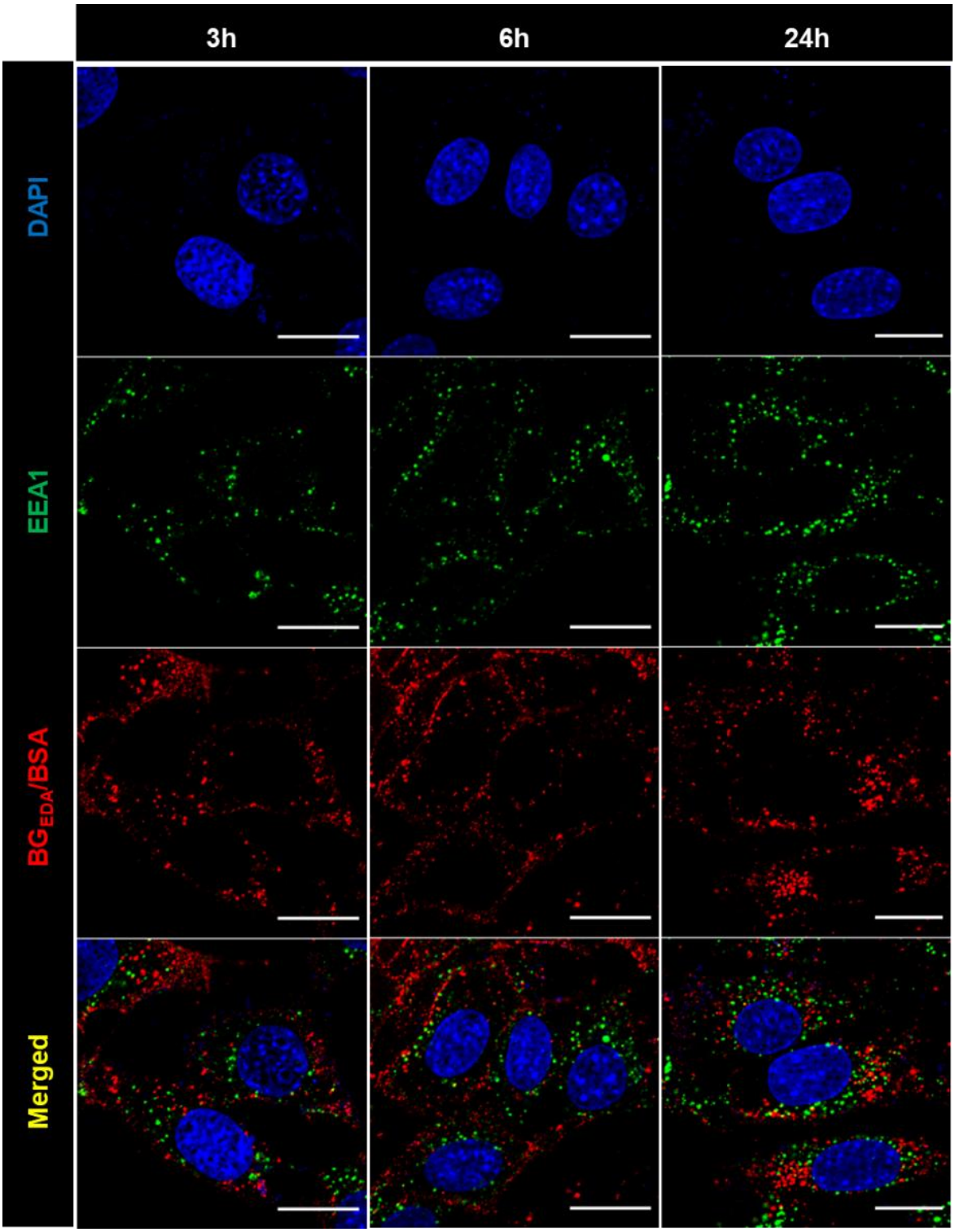


**Figure S7.** Representative CLSM images of colocalization studies of $NC_{BGEDA/BSA}$ (red) and early endosomes (green; labelled with anti-EEA1 antibody) in NIH-3T3 cells taken after 3 h, 6 h, and 24 h post particle addition. Scale bars are 20 µm.

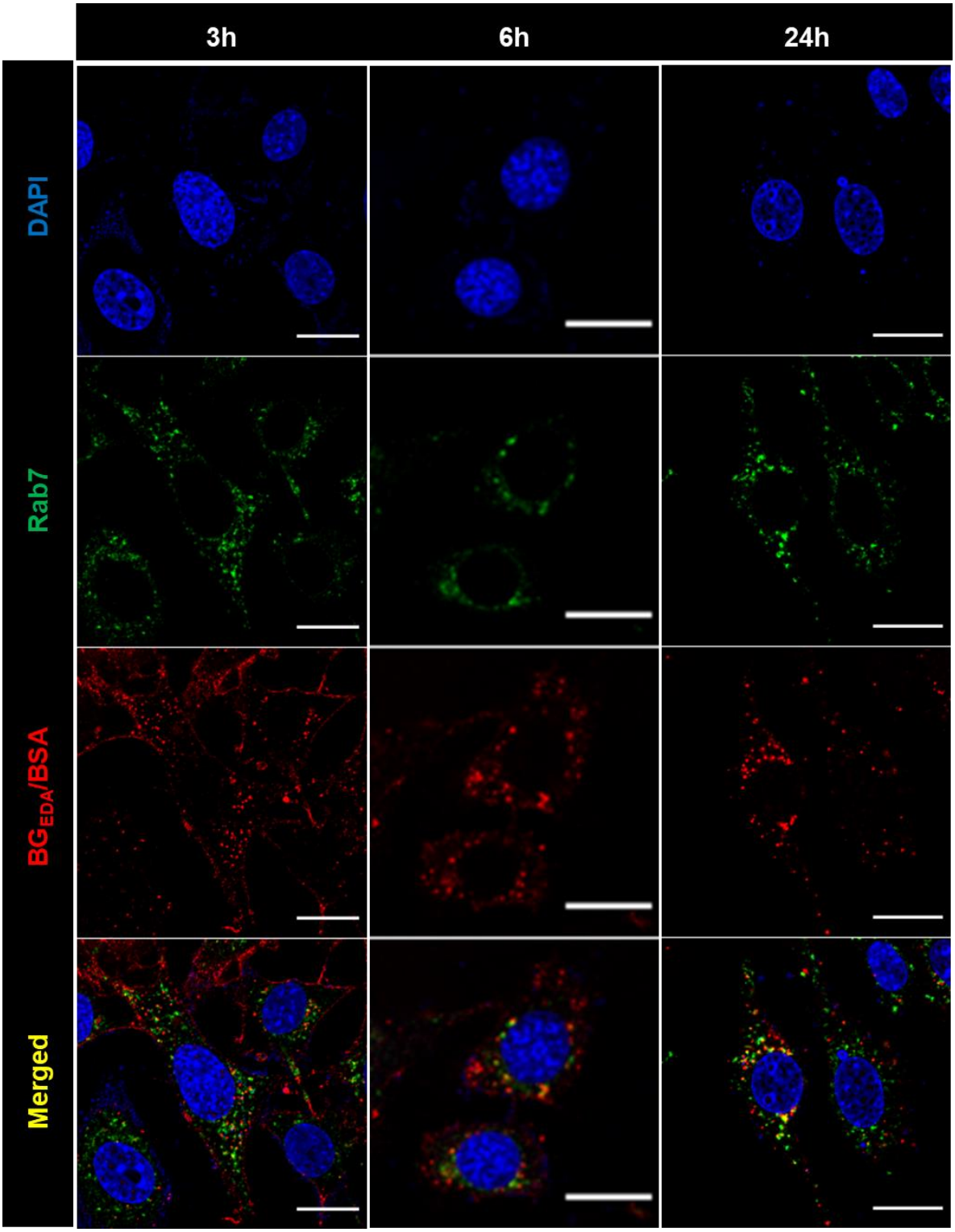


**Figure S8** Representative CLSM images of colocalization studies of $NC_{BGEDA/BSA}$ (red) and late endosomes (green; labelled with anti-Rab7 antibody) in NIH-3T3 cells taken after 3, 6 and 24 h transfection. Scale bar = 20 µm.

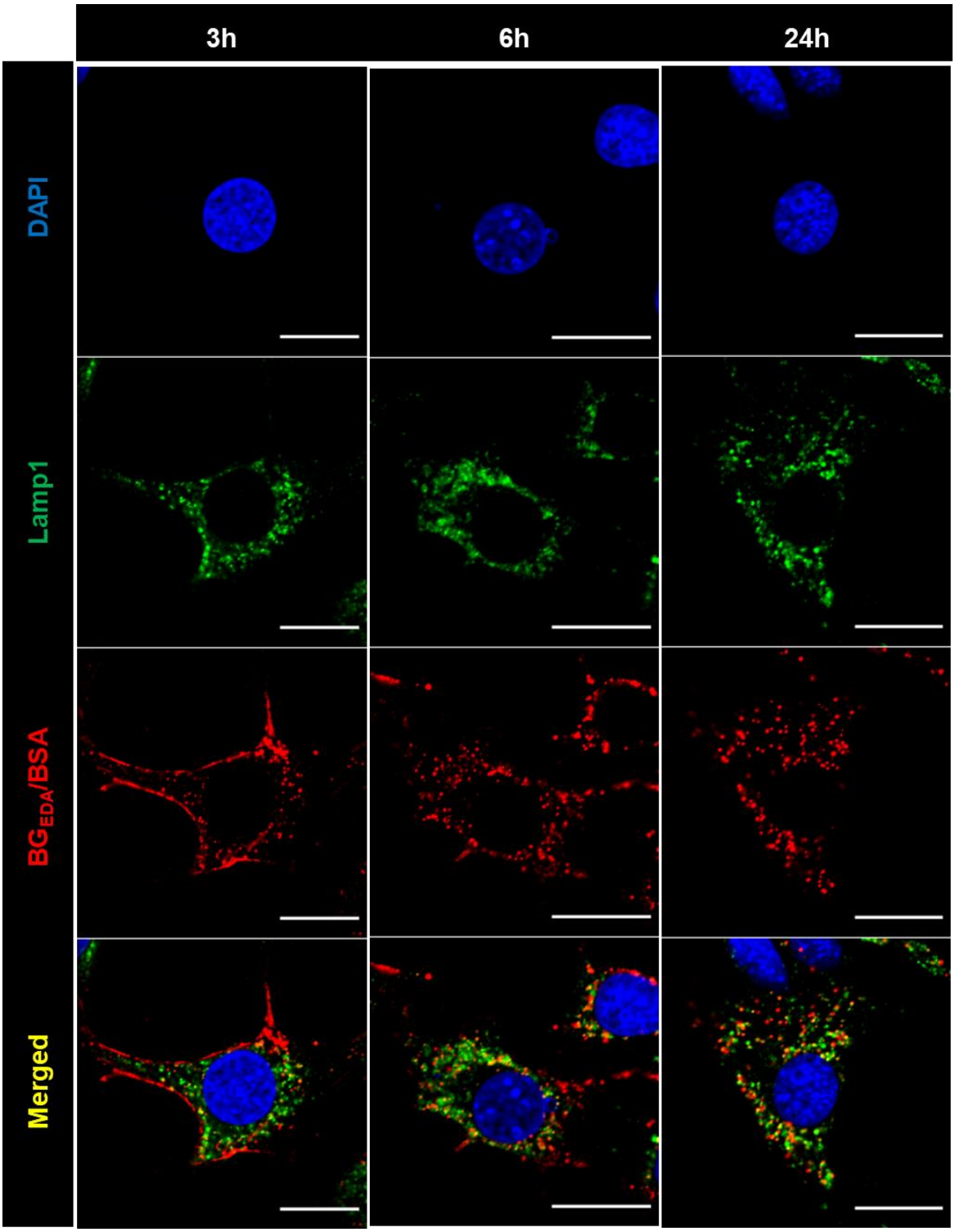


**Figure S9** Representative CLSM images of colocalization studies of $NC_{BGEDA/BSA}$ (red) and lysosomes (green; labelled with anti-Lamp1 antibody) in NIH-3T3 cells taken after 3, 6 and 24 h transfection. Scale bar = 20 µm.

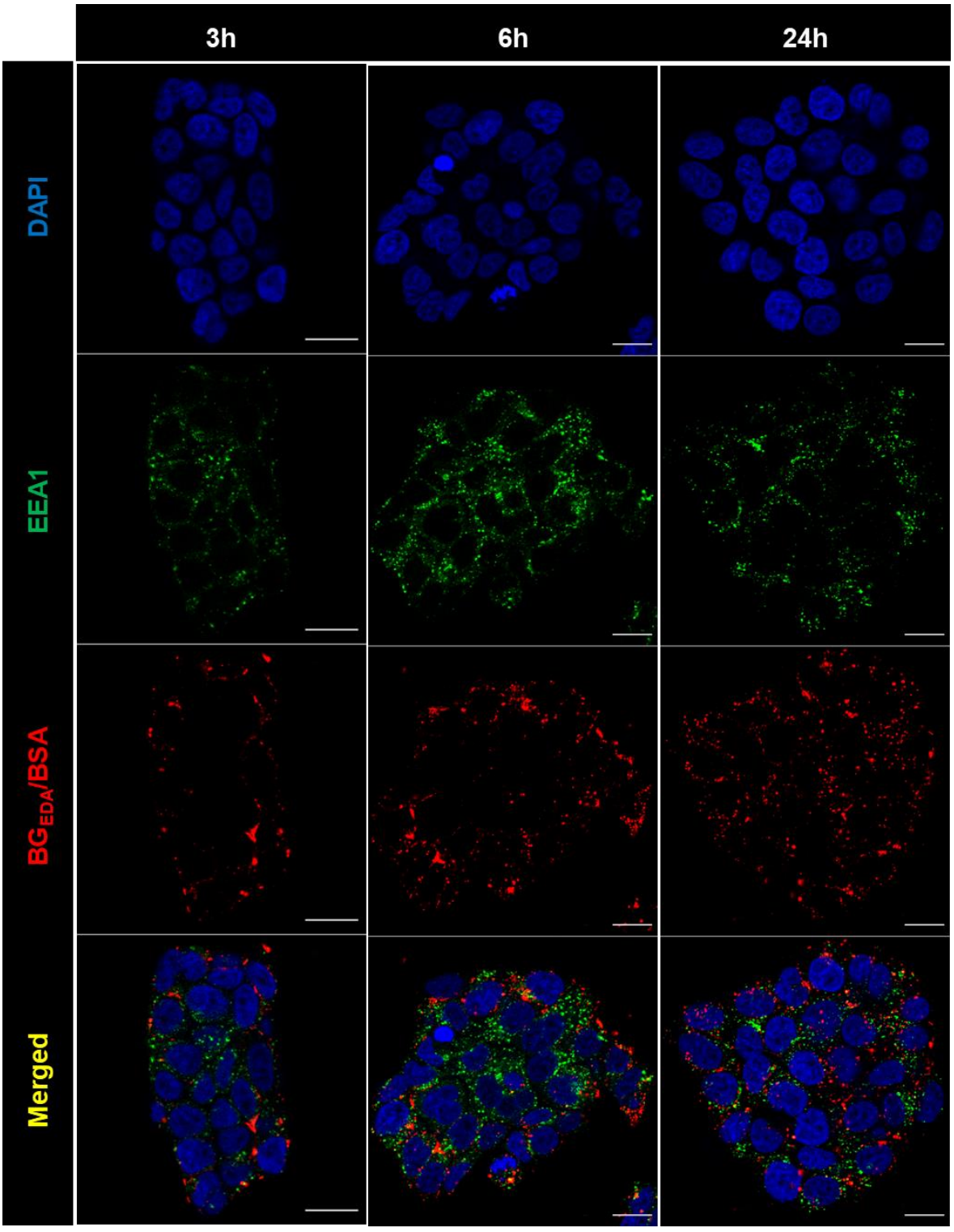


**Figure S10.** Representative CLSM images of colocalization studies of $NC_{BGEDA/BSA}$ (red) and early endosomes (green; labelled with anti-EEA1 antibody) in BT474 cells taken after 3, 6 and 24 h transfection. Scale bar = 20 µm.

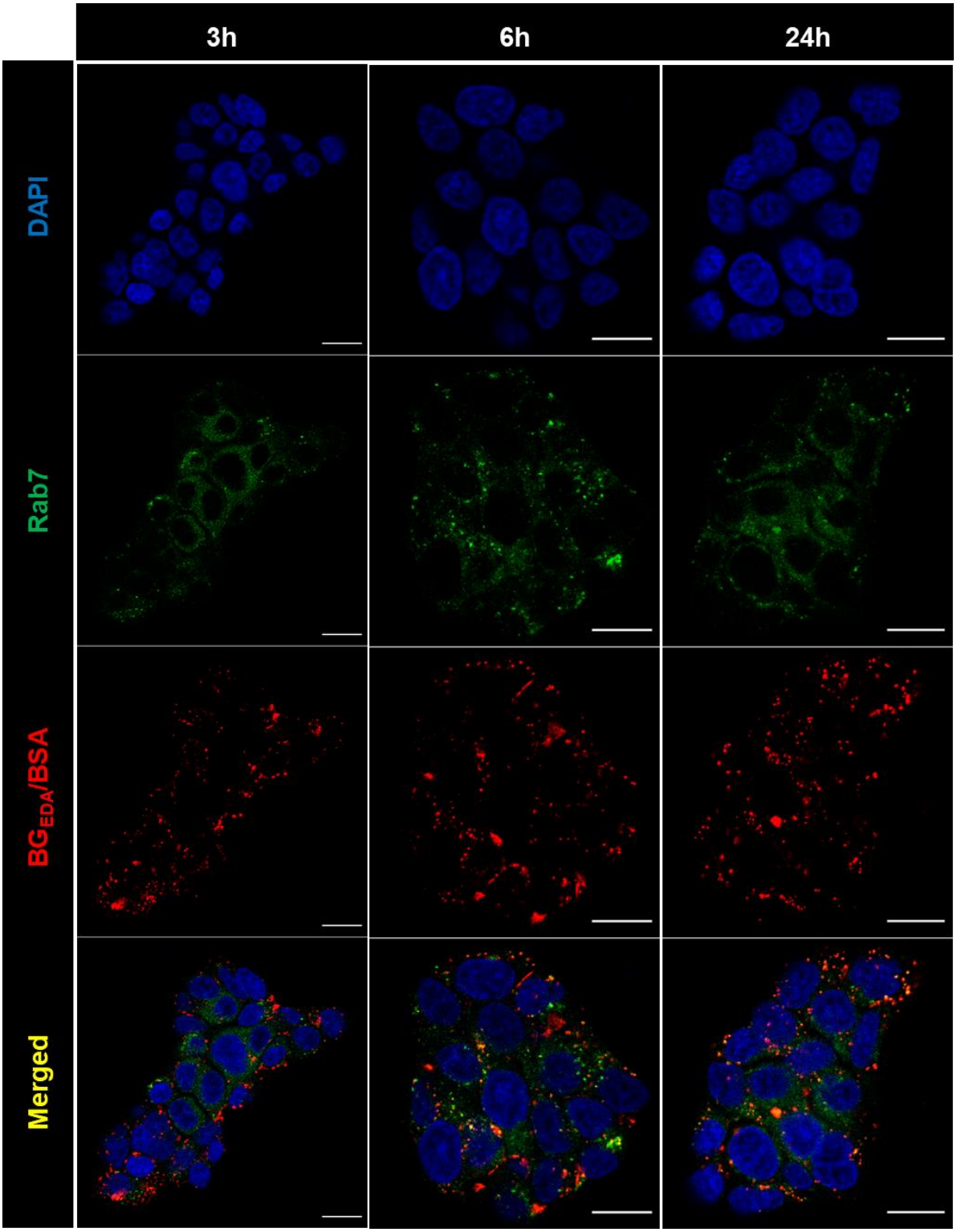


**Figure S11** Representative CLSM images of colocalization studies of $NC_{BGEDA/BSA}$ (red) and late endosomes (green; labelled with anti-Rab7 antibody) in BT474 cells taken after 3, 6 and 24 h transfection. Scale bar = 20 µm.

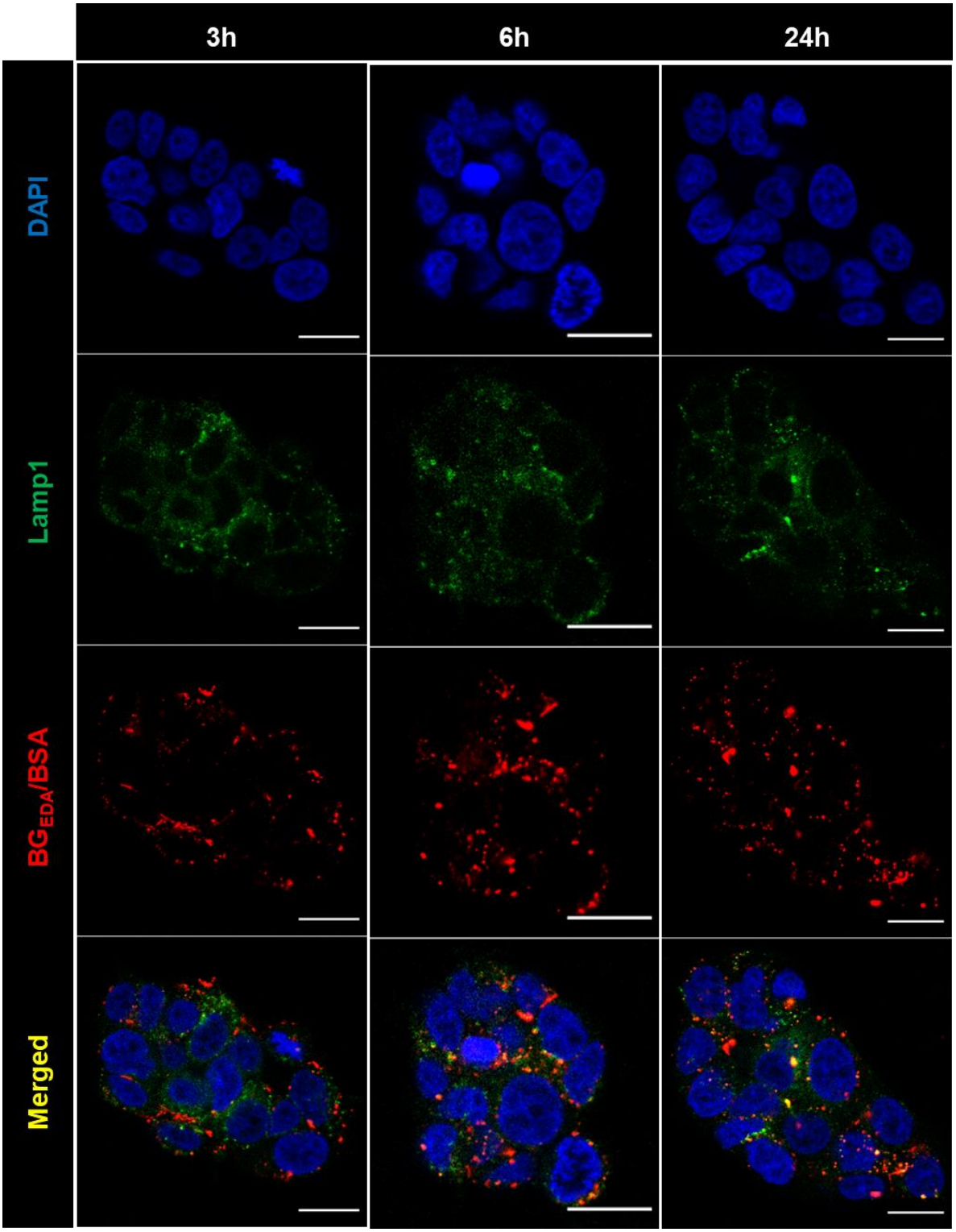


**Figure S12** Representative CLSM images of colocalization studies of $NC_{BGEDA/BSA}$ (red) and lysosomes (green; labelled with anti-Lamp1 antibody) in BT474 cells taken after 3, 6 and 24 h transfection. Scale bar = 20 µm.

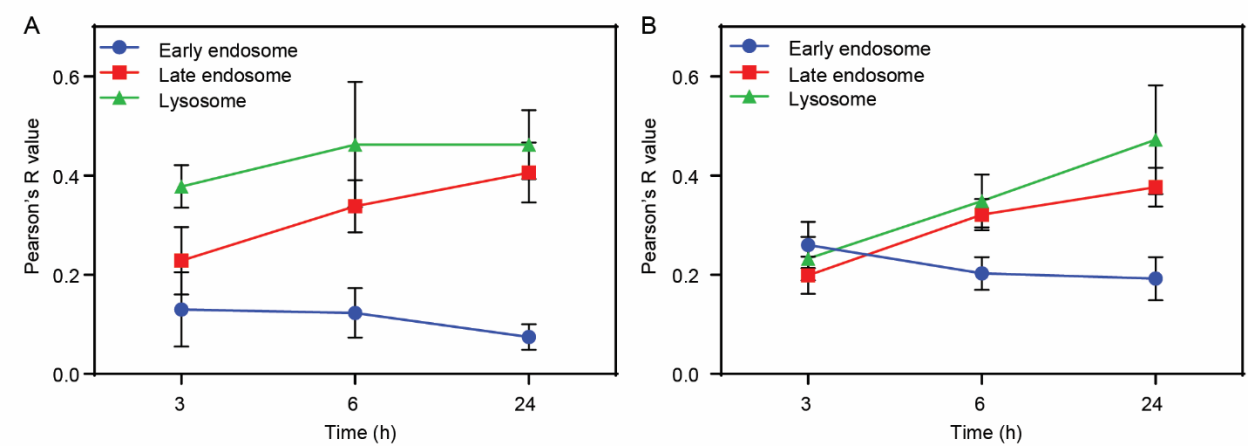


**Figure S13. A)** The Pearson's R value for colocalization of $NC_{BGEDA/BSA}$ with endocytosis markers after 3, 6 and 24 h incubation of NIH-3T3 cells and **B)** BT-474 cells.

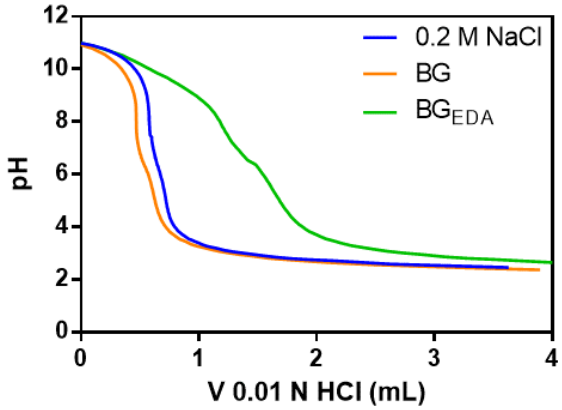


**Figure S14** Potentiometric titration of native BG and $BG_{EDA}$ by HCl performed in 200 mM NaCl solution.

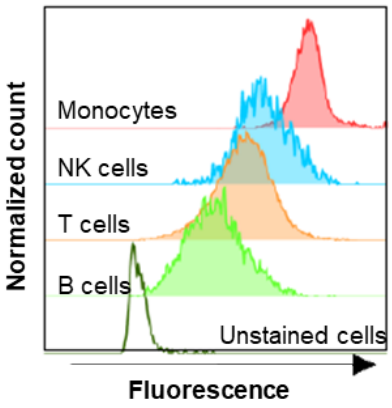


**Figure S15.** Flow cytometry histograms showing the shift in mean fluorescence intensity of different PBMCs subsets: $CD14^+$ monocytes, $CD3^+$ T cells, $CD19^+CD20^+$ B cells and $CD57^+$ NK cells after 2 h incubation with $NC_{AF488\text{-}BGEDA/BSA}$ compared with unstained PBMCs.

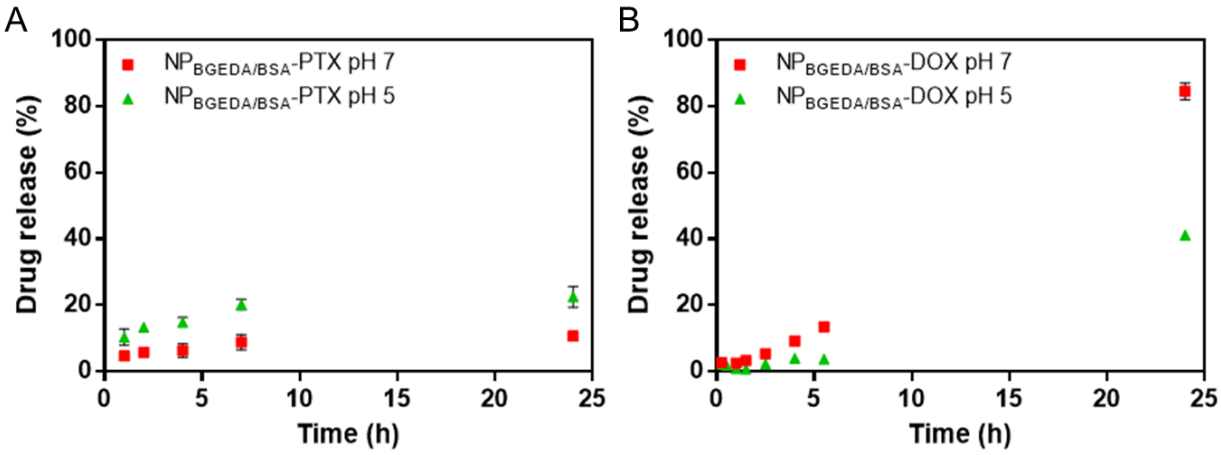


**Figure S16 A)** Drug release profile of $NC_{BGEDA/BSA}$ loaded with PTX as a function of time in D-PBS at pH 7 and 5. **B)** Drug release profile of $NC_{BGEDA/BSA}$ loaded with DOX as a function of time in D-PBS at pH 7 and 5.

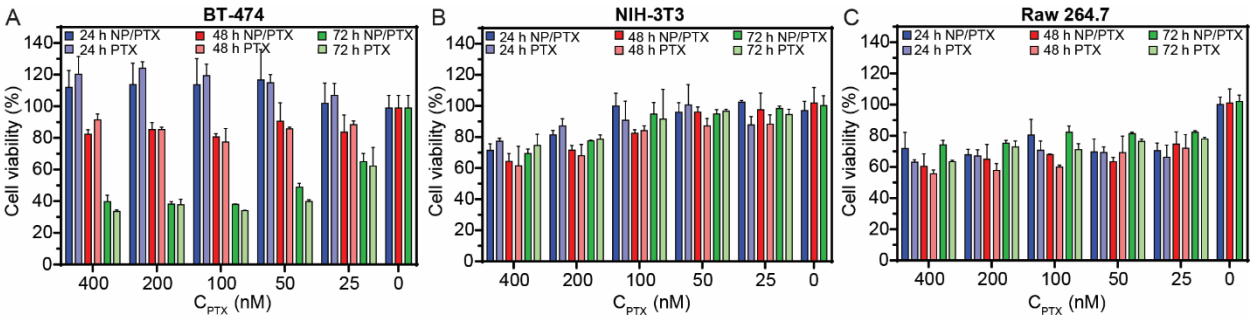


**Figure S17.** Cytotoxicity of $NC_{BGEDA/BSA}$-PTX in **A)** BT-474 cells, **B)** NIH-3T3 cells and **C)** Raw 264.7 cells after 24, 48 and 72 h incubation at different concentrations. The data are shown as the mean ± standard deviation (SD) ($n$ = 3).

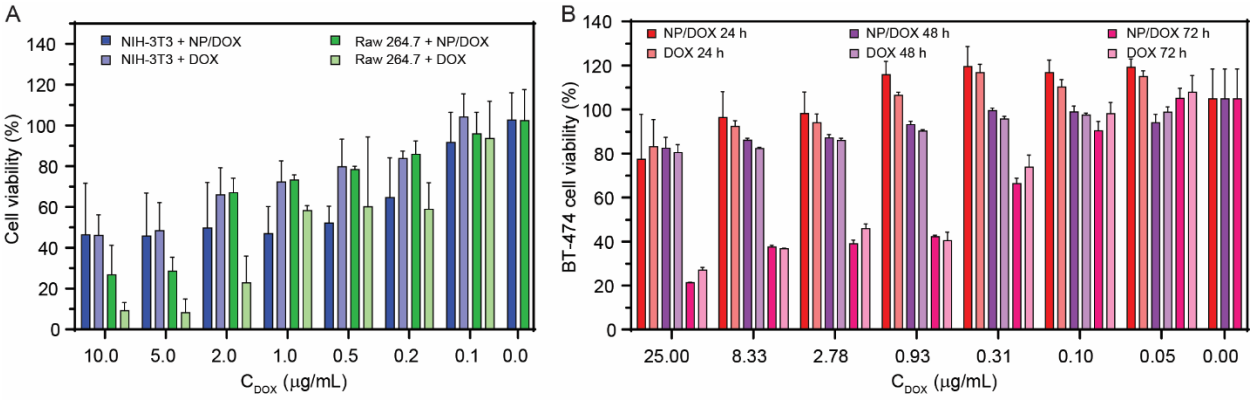


**Figure S18. A)** Cytotoxicity of $NC_{BGEDA/BSA}$-DOX nanocomplexes and free DOX in NIH-3T3 cells and Raw 264.7 cells after 24 h incubation at different concentrations and **B)** BT-474 cells after 24, 48 and 72 h incubation. The data are shown as the mean ± standard deviation (SD) ($n$ = 3). Cytotoxicity was measured using the alamar blue assay.

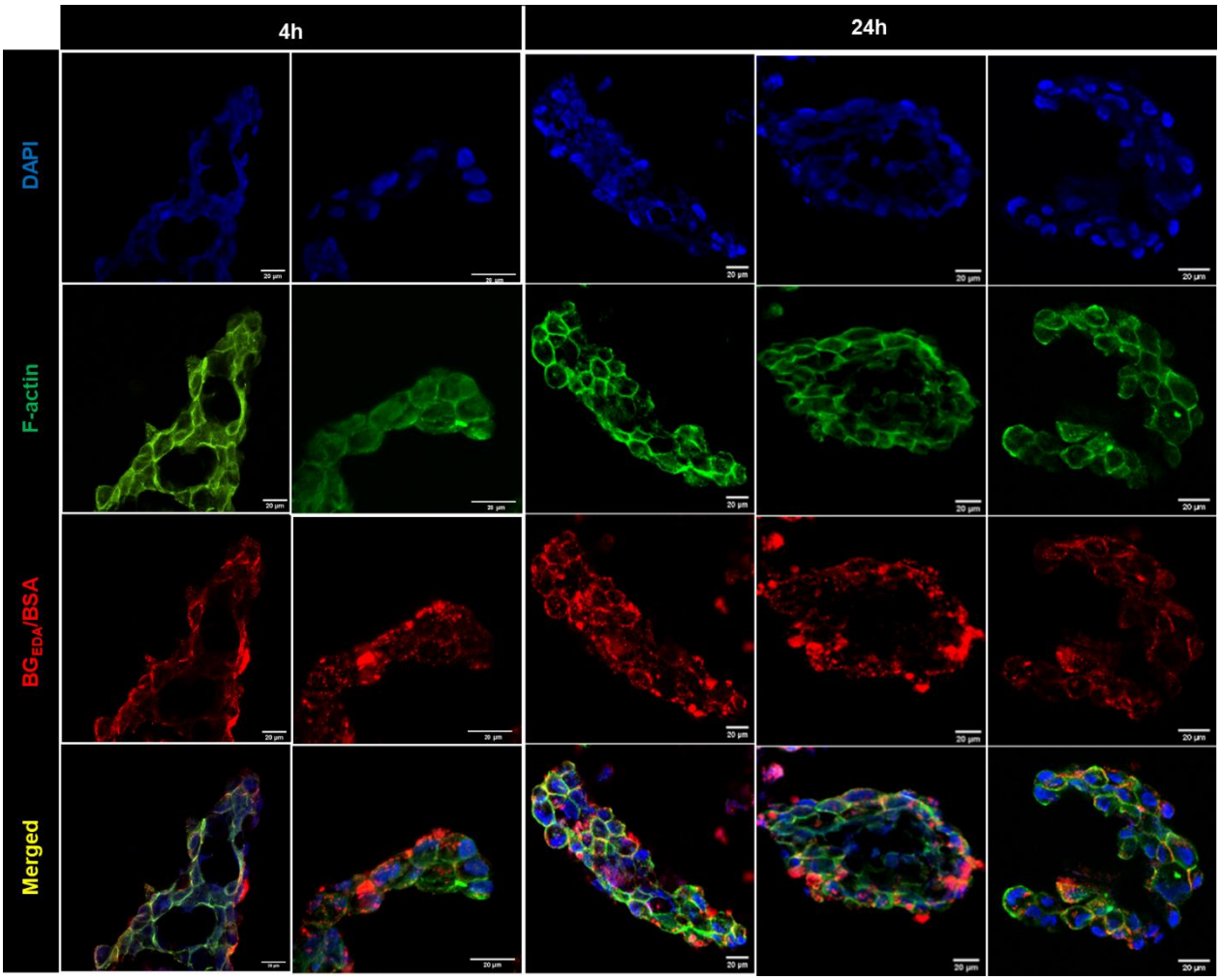


**Figure S19** Representative CLSM images of collagen scaffold co-cultured with NIH-3T3, BT474 and Raw264.7 cells and stained for F-Actin (phalloidin) and nucleus (DAPI) after incubation with NC$_{BGEDA/BSA}$ (red) for 4 and 24 h. Scale bars are 20 µm.

## Experimental Section

### Synthesis of $BG_{oxred}$ nanoparticles

25 mg (0.15 mmol of glucose units) of glycogen from bovine liver were dissolved in 0.5 M acetic buffer (pH = 5.5). Then, 6.6 mg (0.03 mmol), 3.3 mg (0.015 mmol) or 1.65 mg (0.0075 mmol) of sodium periodate was added to obtain 20 %, 10 % and 5 % degree of substitution, respectively, and reaction was performed for 2 h protected from the light. Afterwards, 3x excess of $NaBH_4$ was added (0.09 mmol, 0.045 mmol and 0.0025 mmol, respectively), the reactions were degassed, and stirred overnight. The product was purified by dialysis (14 kDa cut off) against Milli-Q water for 3 days (9 times water change) and subsequently freeze dried. Yield: 85%. Degree of substitution of modified glycogen was determined by $^1$H NMR. $^1$H NMR spectra were recorded on a Varian INOVA 400 instrument, operating at 400 MHz after dissolution in deuterated water ($D_2O$).

### $BG_{EDA}$ degradability studies

The rate of glycogen particle hydrolysis by α- and β-amylase was determined using a Somogyi-Nelson assay adjusted for a microtiter plate.[1] For the assay, 25 µL of 2 mg $mL^{-1}$ solutions of BG, $BG_{EDA}$ in 16 mM sodium acetate buffer, pH 4.8 were treated with 25 µL of α- or β-amylase solution in $mqH_2O$ (1 U $mg^{-1}$) for 3 h in 96-well plate in triplicate. After the incubation working solution was added (50 µL), the plate was covered and heated in 95ºC in water bath for 20 min. Then, plate was cooled down to the room temperature, the arsenomolybdate color reagent was added (50 µL) into each well followed by the 1.5 h incubation for the complete color development. The absorbance was measured at 750 nm with an Infinite M200 microplate reader (Tecan, Switzerland). The working solution was prepared by mixing four parts of Solution I and one part

of Solution II immediately before experiment. Solution I was prepared by dissolving sodium potassium tartrate tetrahydrate (1.2 g), sodium carbonate (2.4 g), sodium bicarbonate (1.6 g), and sodium sulfate (14.4 g) in 80 mL of water. Solution II was prepared by dissolving copper sulfate pentahydrate (0.4 g) and sodium sulfate (3.6 g) in 20 mL of water. Two solutions were stored separately to prevent copper oxidation.[2] The color reagent was prepared by dissolving ammonium molybdate (2.5 g) in water (45 mL) and concentrated sulfuric acid (2.1 mL), followed by addition of sodium arsenate dibasic pentahydrate (0.3 g) dissolved in 2.5 mL of deionized water and mixed with the ammonium molybdate solution. The reagent was incubated at 37 °C for 24–48 h and stored in a brown bottle.[3]

**Potentiometric titration of $BG_{EDA}$ and $NC_{BGEDA/BSA}$**

The buffering capacity of $BG_{EDA}$ nanoparticles was measured by the dissolving 10 mg of sample in 5 mL solution of 200 mM NaCl. The pH was adjusted to 11 with 0.1 N NaOH and the suspension was titrated with 0.01 HCl to pH 2.

**Immunofluorescent analysis of BT474, NIH-3T3 and Raw264.7 co-culture**

For confocal analysis, BT474, NIH3T3 and Raw264.7 cells were seeded at the density of 20,000 cells/well, 10,000 cells/well and 5,000 cells/well, respectively, in 8-well Lab-Tek chamber and incubated at 37 °C overnight. Then, the cells were washed with D-PBS, fixed with 4 % paraformaldehyde for 15 min, washed and permeabilized with 0.1% Triton X-100 solution in PBS for 5 min, washed again 3 x with D-PBS and immunostained for 2 h with anti-E-cadherin for BT474 cells (1:200 dilution in 1 % BSA/PBS), anti-CD11b/ITGAM antibody for Raw264.7 cells (dilution 1:200 in 1 % BSA/PBS), followed by incubation with goat-anti rabbit AF488 conjugate and mouse anti-rat AF555 conjugates for 1 h at 2 µg/mL concentration in D-PBS. Afterwards, all

cells were stained with AF647-phalloidin for actin and with Hoechst for nuclei. The cells were imaged with a Nikon A1R confocal microscope with a 60× 1.4 NA oil immersion objective.

**Cell viability in monocultures**

For the viability measurements, BT474, NIH-3T3 and Raw264.7 cells were seeded at a seeding density $1 \times 10^4$ cells/well on a 96-well plate in 100 µL DMEM medium supplemented with 10% fetal bovine serum (FBS) and grown overnight. Then, the media was discarded, and cells were treated with $NC_{BGEDA/BSA}$-PTX or $NC_{BGEDA/BSA}$-DOX. After incubation, the transfection medium was discarded and replaced with fresh, prewarmed 10% Alamar Blue/DMEM or 10% PrestoBlue/DMEM solution, followed by further incubation for 3 h. The viability results were recorded on UV spectrophotometer at 570 nm, using 600 nm as a reference wavelength.

**Minimum Information Reporting in Bio–Nano Experimental Literature (MIRIBEL)**

The studies conducted herein, including material characterization, biological characterization, and experimental details, conform to the MIRIBEL reporting standard for bio–nano research.[4] We include a companion checklist of these components in the Tables 1-3-

**Supplementary Table 1. Material characterization***

| **Question** | **Yes** | **No** |
|---|---|---|
| 1.1 Are "**best reporting practices**" **available** for the nanomaterial used? For examples, see *Chem. Mater.* **28** (2016) 3535; http://doi.org/10.1021/acs.chemmater.6b01854 and *Chem. Mater.* **29** (2017) 1; http://doi.org/10.1021/acs.chemmater.6b05235 | | √ |
| 1.2 If they are available, **are they used**? If not available, ignore this question and proceed to the next one. | | |

| | | |
|---|---|---|
| 1.3 Are extensive and clear instructions reported detailing all steps of **synthesis** and the resulting **composition** of the nanomaterial? For examples, see *Chem. Mater.* **26** (2014) 1765; http://doi.org/10.1021/cm500632c, and *Chem. Mater.* **26** (2014) 2211; http://doi.org/10.1021/cm5010449. Extensive use of photos, images, and videos are strongly encouraged. For example, see *Chem. Mater.* **28** (2016) 8441; http://doi.org/10.1021/acs.chemmater.6b04639 | √ | |
| 1.4 Is the **size** (or **dimensions**, if non-spherical) and **shape of** the nanomaterial reported? | √ | |
| 1.5 Is the **size dispersity** or **aggregation** of the nanomaterial reported? | √ | |
| 1.6 Is the **zeta potential** of the nanomaterial reported? | √ | |
| 1.7 Is the **density (mass/volume)** of the nanomaterial reported? | | √ |
| 1.8 Is the amount of any **drug loaded** reported? 'Drug' here broadly refers to functional cargos (e.g., proteins, small molecules, nucleic acids). | √ | |
| 1.9 Is the **targeting performance** of the nanomaterial reported, including **amount** of ligand bound to the nanomaterial if the material has been functionalised through addition of targeting ligands? | √ | |
| 1.10 Is the **label signal** per nanomaterial/particle reported? For example, fluorescence signal per particle for fluorescently labelled nanomaterials. | | √ |
| 1.11 If a material property not listed here is varied, has it been **quantified**? | NA | |
| 1.12 Were characterizations performed in a **fluid mimicking biological conditions**? | √ | |
| 1.13 Are details of how these parameters were **measured/estimated** provided? | √ | |
| Explanation for **No** (if needed): | | |

*****Ideally, material characterization should be performed in the same biological environment as that in which the study will be conducted. For example, for cell culture studies with nanoparticles, characterization steps would ideally be performed on nanoparticles dispersed in cell culture media.

If this is not possible, then characteristics of the dispersant used (e.g., pH, ionic strength) should mimic as much as possible the biological environment being studied.

**Supplementary Table 2. Biological characterization***

| Question | Yes | No |
|---|---|---|
| 2.1 Are **cell seeding details**, including **number of cells plated, confluency at start of experiment,** and **time between seeding and experiment** reported? | √ | |
| 2.2 If a standardised cell line is used, are the **designation and source** provided? | √ | |
| 2.3 Is the **passage number** (total number of times a cell culture has been subcultured) known and reported? | √ | |
| 2.4 Is the last instance of **verification of cell line** reported? If no verification has been performed, is the time passed and passage number since acquisition from trusted source (e.g., ATCC or ECACC) reported? For information, see *Science* **347** (2015) 938; http://doi.org/10.1126/science.347.6225.938 | | √ |
| 2.5 Are the results from **mycoplasma testing** of cell cultures reported? | √ | |
| 2.6 Is the **background signal of cells**/**tissue** reported? (E.g., the fluorescence signal of cells without particles in the case of a flow cytometry experiment.) | √ | |
| 2.7 Are **toxicity studies** provided to demonstrate that the material has the expected toxicity, and that the experimental protocol followed does not? | √ | |
| 2.8 Are details of media preparation (**type of media, serum,** any **added antibiotics**) provided? | √ | |
| 2.9 Is a **justification of the biological model** used provided? For examples for cancer models, see *Cancer Res.* **75** (2015) 4016; http://doi.org/10.1158/0008-5472.CAN-15-1558, and *Mol. Ther.* **20** (2012) 882; http://doi.org/10.1038/mt.2012.73, and *ACS Nano* **11** (2017) 9594; http://doi.org/10.1021/acsnano.7b04855 | √ | |
| 2.10 Is characterization of the **biological fluid** (*ex vivo*/*in vitro*) reported? For example, when investigating protein adsorption onto nanoparticles dispersed in | √ | |

| | | |
|---|---|---|
| blood serum, pertinent aspects of the blood serum should be characterised (e.g., protein concentrations and differences between donors used in study). | | |
| 2.11 For **animal experiments**, are the ARRIVE guidelines followed? For details, see *PLOS Biol.* **8** (2010) e1000412; http://doi.org/10.1371/journal.pbio.1000412 | NA | |
| Explanation for **No** (if needed):<br><br>2.4: Cells were purchased from the American Type Culture Collection. The passage number was reported and standard mycoplasma test was conducted. | | |

*For *in vitro* experiments (e.g., cell culture), *ex vivo* experiments (e.g., in blood samples), and *in vivo* experiments (e.g., animal models). The questions above that are appropriate depend on the type of experiment conducted.

**Supplementary Table 3. Experimental details***

| Question | Yes | No |
|---|---|---|
| 3.1 For cell culture experiments: are **cell culture dimensions** including **type of well**, **volume of added media**, reported? Are cell types (i.e.; adherent vs suspension) and **orientation** (if non-standard) reported? | √ | |
| 3.2 Is the **dose of material administered** reported? This is typically provided in nanomaterial mass, volume, number, or surface area added. Is sufficient information reported so that regardless of which one is provided, the other dosage metrics can be calculated (i.e. using the dimensions and density of the nanomaterial)? | √ | |
| 3.3 For each type of imaging performed, are details of how **imaging** was performed provided, including details of **shielding, non-uniform image processing**, and any **contrast agents** added? | √ | |
| 3.4 Are details of how the dose was administered provided, including **method of administration, injection location, rate of administration,** and details of **multiple injections**? | NA | |
| 3.5 Is the methodology used to **equalise dosage** provided? | NA | |
| 3.6 Is the **delivered dose** to tissues and/or organs (in vivo) reported, as % injected dose per gram of tissue (%ID $g^{-1}$)? | NA | |
| 3.7 Is **mass of each organ/tissue measured** and **mass of material** reported? | NA | |
| 3.8 Are the **signals of cells/tissues with nanomaterials** reported? For instance, for fluorescently labelled nanoparticles, the total number of particles per cell or the fluorescence intensity of particles + cells, at each assessed timepoint. | √ | |
| 3.9 Are **data analysis details**, including **code used** for analysis provided? | √ | |
| 3.10 Is the **raw data** or **distribution of values** underlying the reported results provided? For examples, see *R. Soc. Open Sci.* **3** (2016) 150547; http://doi.org/10.1098/rsos.150547, https://opennessinitiative.org/making-your-data-public/, | | √ |

| | | |
|---|---|---|
| http://journals.plos.org/plosone/s/data-availability, and https://www.nature.com/sdata/policies/repositories | | |
| Explanation for **No** (if needed): | | |

* The use of protocol repositories (e.g., *Protocol Exchange* http://www.nature.com/protocolexchange/) and published standard methods and protocols (e.g., *Chem. Mater.* **29** (2017) 1; http://doi.org/10.1021/acs.chemmater.6b05235, and *Chem. Mater.* **29** (2017) 475; http://doi.org/10.1021/acs.chemmater.6b05481) are encouraged.

**Supplementary references**

1. Shao, Y. J.; Lin, A. H. M., Improvement in the quantification of reducing sugars by miniaturizing the Somogyi-Nelson assay using a microtiter plate. *Food Chem* **2018,** *240*, 898-903.
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