# Electronic Supplementary Information

## Controlling the Semi-Permeability of Protein Nanocapsules Influences the Cellular Response to Macromolecular Payloads

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### Materials

All chemicals were used as received, except for polyglycerol polyricinoleate (PGPR). PGPR was purified by dissolution in cyclohexane and centrifugation at 5845 rcf for 4 minutes to precipitate solid impurities. Then the supernatant was recovered and dried to yield the purified PGPR. Ovalbumin (OVA, lyophilized powder, ≥98%, A2512), Proteinase K (from *Tritirachium album*, lyophilized powder, ≥30 units/mg), Hydroxyl-PEG-NHS ester (MW 5 000), resiquimod (≥98% (HPLC), R848) and rhodamine B-isothiocyanate (RITC) were purchased from Sigma Aldrich. Lutensol AT50 was purchased from BASF, polyglycerol polyricinoleate (PGPR) from Danisco, 2,4-toluene diisocyanate (TDI) from TCI Chemicals, bicinchoninic acid (BCA) and fluorescamine from Alfa Aesar. Human blood buffy coats was taken at the Department of Transfusion Medicine Mainz after obtaining informed consent in accordance with the Declaration of Helsinki. Sartorius™ Vivaspin™ 20 centrifugal filter devices were purchased from Thermo Fisher Scientific (0.2 µm molecular weight cut-off).

### Methods

#### Determination of the protein nanocapsule concentration

The concentration of protein nanocapsules was determined with a protein assay using bicinchoninic acid (BCA) as the substrate. First, 100 mg of BCA, 200 mg of sodium carbonate, 16 mg of sodium tartrate and 95 mg of sodium hydrogen carbonate were dissolved in 10 mL of deionized water and the pH value was adjusted to 11.3 using 3.0 M NaOH. To this solution, 200 µL of a copper sulfate solution (50 mg of $CuSO_4 \cdot 5\ H_2O$ in 1 mL of deionized water) were

added. Then, 200 µL of the resulting solution were mixed with 10 µL of either protein standard solution or the protein nanocapsule dispersion of unknown concentration. The solution was incubated at 60°C for 30 min. The absorbance at 565 nm was recorded, and the protein concentration was determined by comparison to the standard curve prepared with ovalbumin (OVA).

**Determination of the encapsulation efficiency**

The encapsulation efficiency of the PEG payload was determined after every step of the nanocapsules purification. Initially, the payload (PEG) was dissolved in the nanodroplets containing the protein solution and was encapsulated in situ by the crosslinking reaction occurring. Prior to the transfer of the nanocapsules to water, when the NCs were washed in toluene, the encapsulation efficiency appeared to be quantitative. However, after the transfer of the NCs to water, unencapsulated, or poorly encapsulated, PEG could be washed away. To quantify the encapsulation efficiency, the concentration of PEG was measured using fluorescence spectroscopy to record the emission spectra of the Rhodamine B tag attached to the PEG ($\lambda_{ex}$=553 nm, $\lambda_{em}$=565-600 nm). First, the concentration of PEG in the unwashed aqueous suspension was measured from the total fluorescence intensity ($I_t$) of the PEG in solution and the PEG trapped in the NCs. Then, the samples were separated by centrifugal filtration, and the concentration of unencapsulated PEG, i.e. the concentration of PEG in the solution recovered from the centrifugal filtration, was measured ($I_w$). Finally, the NCs were redispersed following the centrifugal filtration, and the concentration of PEG in the resuspended samples was measured ($I_{NC}$). Systematically, $I_{NC} + I_w = I_t$, and the encapsulation efficiency was defined as $100(I_t-I_w)/I_t$.

**Fluorescent labeling of ovalbumin**

OVA (100 mg) was dissolved in 3 mL PBS buffer (pH 7.4) and 3 mg of Cy-5 NHS ester was added. The reaction was stirred overnight and then dialyzed against water for 3 days. The blue product was recover after lyophilization.

**Synthesis of PEG-R848**

PEG-NHS (160 mg) was dissolved in 4 mL of dry DCM and resquimod (R848) (10 mg) was added. Then, 6 µL trimethylamine was added, and the reaction was stirred overnight. The solvent was evaporated under reduced pressure and water was added to the remaining solid powder. The dissolved product was dialyzed against water for three days and freeze-dried.

**Rhodamine labeling of PEG-R848**

Polymer functionalized R848 (50 mg) was dissolved in 4 mL DMSO and Rhodamine B isothiocyanate (2 mg) was added. The reaction was stirred overnight and dialyzed against water for 5 days. The product was recovered after lyophilisation.

**Isolation of dendritic cells**

Leukocytes were obtained from leukapheresis products collected from healthy donors after informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation at 1200 *g* for 20 min and 21°C using SepMate tubes (StemCell Technologies) and Histopaque-1077 (Sigma-Aldrich). Subsequently, the PBMC fraction was extracted and primary dendritic cells were isolated using the Blood Dendritic Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions.

**Intracellular uptake and toxicity evaluation**

Dendritic cells (2 x $10^5$ / well) were cultured at 37°C and 5% CO2 in 96-well U-bottom plates (Greiner Bio-One) in serum-free X-VIVO 15 medium (Lonza). Cells were incubated with different concentrations of Cy5-NCs for 20h due to the decreased viability of the DCs after more prolonged periods. After incubation, cell supernatant was removed and dendritic cells were detached by adding 200 µL of PBS buffer (containing 0.5% BSA and 2 mM EDTA) and incubating for 20min on ice. Afterward, the cells were transferred to FACS round-bottom tubes, washed with 1 mL FACS buffer (PBS containing 2% of fetal calf serum) and centrifuged (400 *g*, 10 min, 4°C). The supernatant was discarded, and cells were stained with 5 µl 7-AAD (BD Pharmingen) for 5 minutes. Afterward, the cells were characterized by flow cytometry (BD LSR II). Data analysis was conducted with the FlowJo software 10.6.1.

**Cellular activation**

For characterization of cellular activation, dendritic cells were incubated and stimulated as mentioned above. After transferring the cells to FACS round-bottom tubes and washing them, the cells were stained for 30 min at 4°C with the following fluorochrome-conjugated antibodies: CD83 (FITC), CD123 (PE-Cy7) and HLA-DR (APC) all from BD Pharmingen, CD86 (V450) and CD80 (BV510) both from BD Horizon and CD1c (PerCP-eFluor 710) from eBioscience. After staining, the cells were washed and characterized by flow cytometry (BD LSR II). Data analysis was conducted with the FlowJo software 10.6.1.

**Material characterization**

Scanning electron microscopy (SEM) was performed by a 1530 Gemini LEO (Zeiss) microscope. For the measurement, 8.0 µL of the purified sample in toluene was drop-casted

on a silica wafer and allowed to dry under ambient temperature. Transmission electron microscopy (TEM) was performed by a Jeol 1400 transmission microscope with a voltage of 120 kv.

The CD measurement was carried out on a J-1500 JASCO circular dichroism spectrophotometer using a cuvette with a path length of 0.1 cm. The spectra were measured between 260 nm to 190 nm at a protein nanocapsule concentration of 0.02 mg/mL.

Zeta potential measurements were performed by diluting the nanocapsule dispersion in potassium chloride solution and measured with a Malvern ZetaSizer (Malvern Instruments, UK).

The size distribution of the nanocapsules was measured by dynamic light scattering (DLS) at 20 °C using a Malvern NanoS90 device at 90° angle.

Fluorescence intensity measurements were performed in 96-well plates on an Infinite M1000 plate reader from Tecan, Switzerland.

**Additional figures**

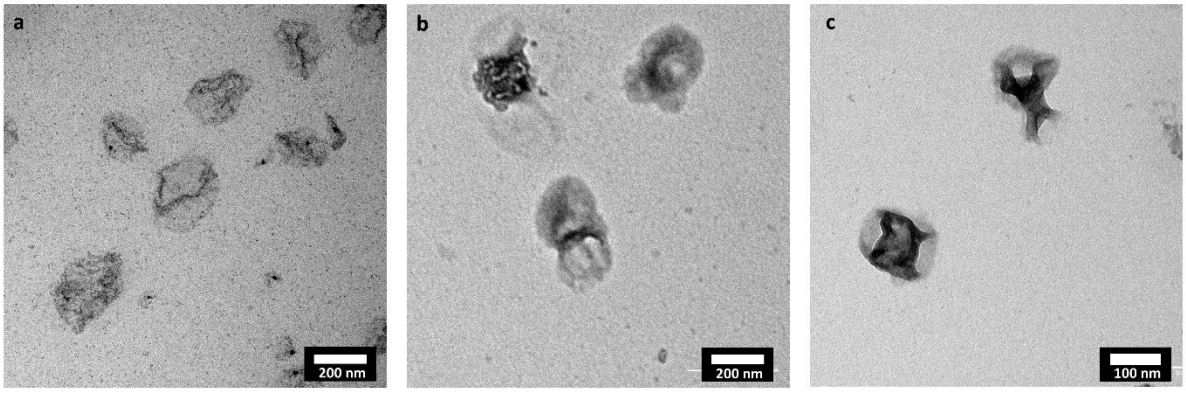


Figure S1. TEM analysis of the protein nanocapsules with a varying crosslinking degree a) low CL, b) medium CL, c) high CL.

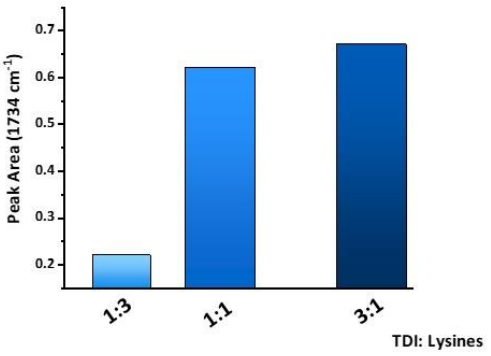


Figure S2. Increase in urea linkages present in the OVA NC with the increasing amount of crosslinker (TDI), measured from the area of the peak at 1734 $cm^{-1}$ in the IR-spectrum for OVA NCs with 3:1, 1:1 and 1:3 ratio of TDI:Lysines.

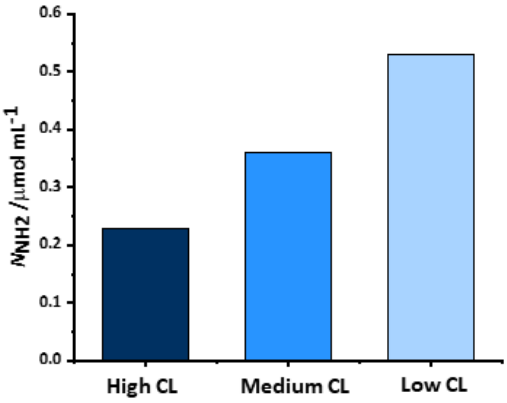


Figure S3. Concentration of free amino groups in the protein nanocapsules with low, medium and high crosslinking densities (1 mg/mL protein nanocapsule concentration).

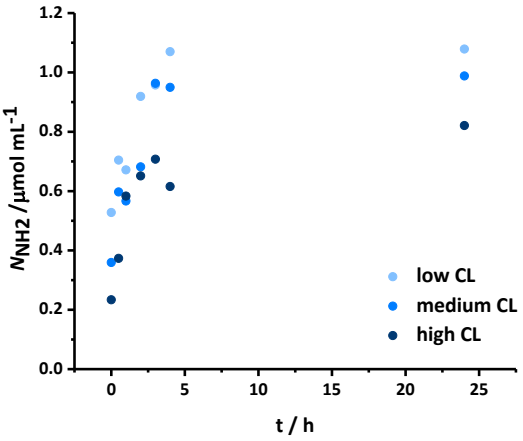


Figure S4. Variation in the absolute number of amine groups in the NCs suspension during the degradation of the OVA NCs by proteinase K (1 u per mg of protein NCs).

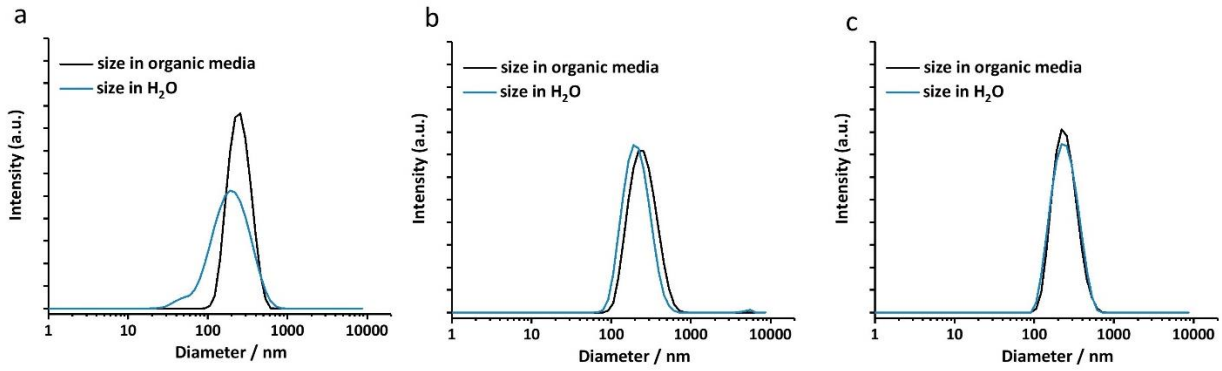


Figure S5. Size distribution of the OVA NCs measured by DLS for a) low CL, b) medium CL and c) high CL as prepared in toluene (black) and after transfer to water (blue).

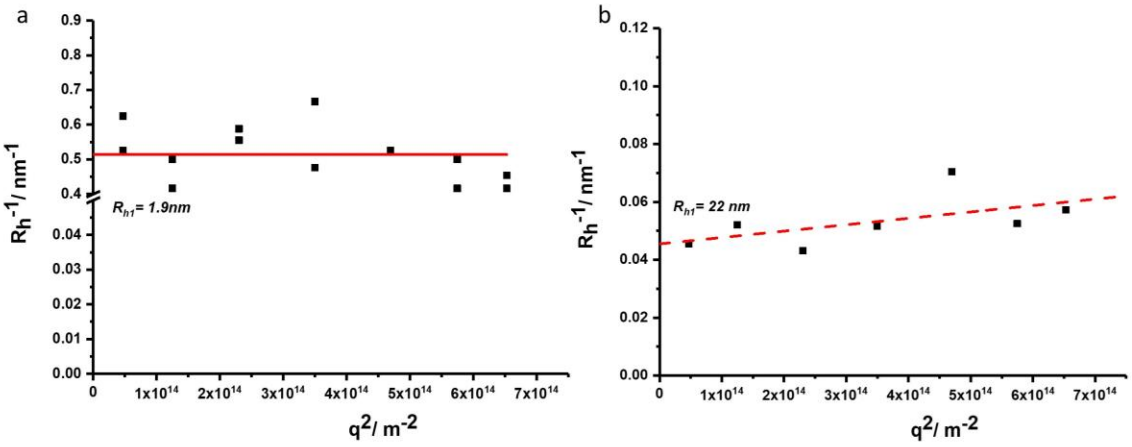


Figure S6. Hydrodynamic radius of PEG-Rhodamine prepared with (a) PEG 5k and (b) PEG 600k.

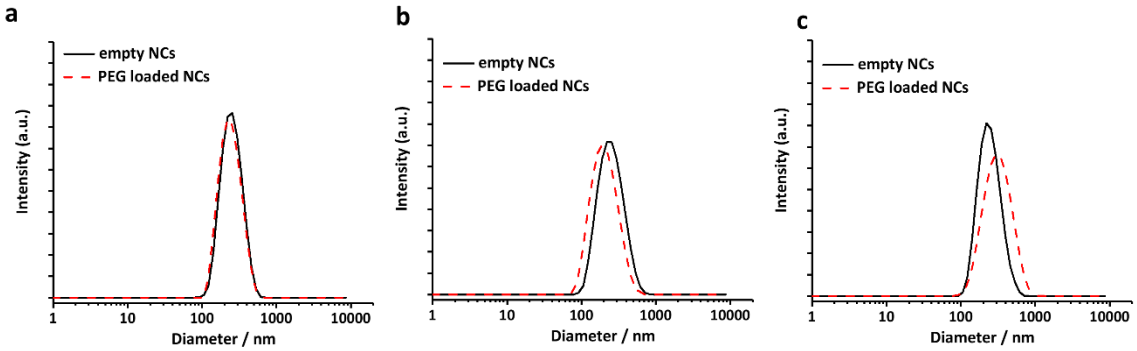


Figure S7. Size distribution of the OVA NCs measured by DLS for a) low CL, b) medium CL and c) high CL in toluene without (black) and with encapsulation of labeled PEG (red).

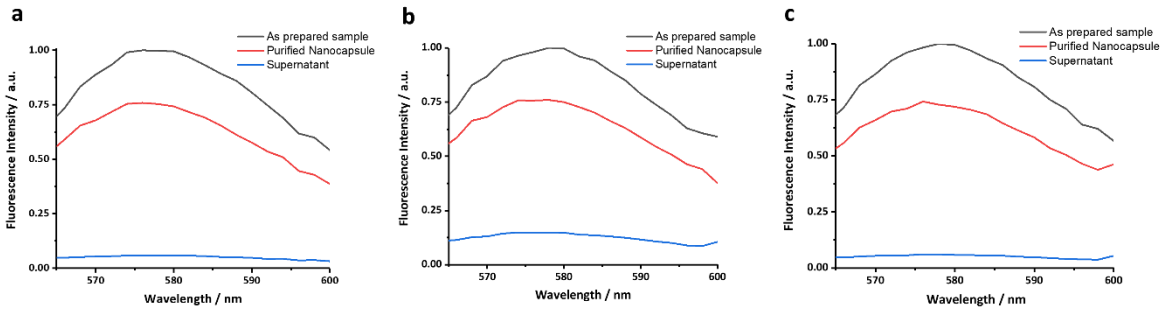


Figure S8. Encapsulation efficiency in the OVA NCs. Fluorescence emission spectra of PEG-Rhodamine B MW 5 kDa in the suspension of NCs following their transfer to water (black), of the water separated from the NCs by centrifugal filtration (blue) and of the NCs redispersed in PBS buffer (red).

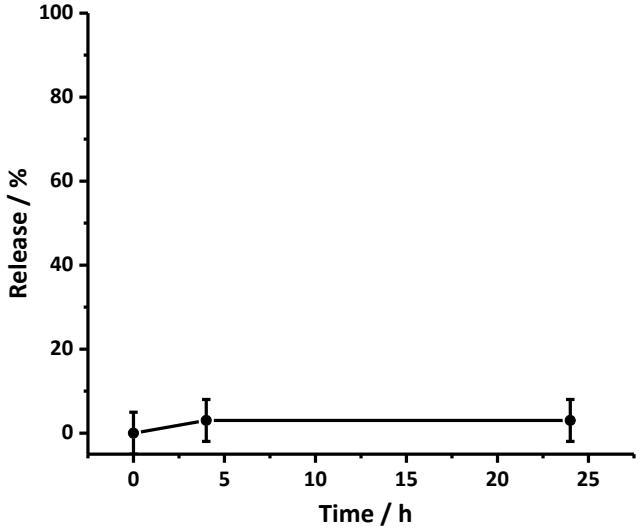


Figure S9. Release of PEG-Rhodamine (MW 5kDa) from crosslinked OVA nanocapsules in the absence of enzymatic degradation by Proteinase K.

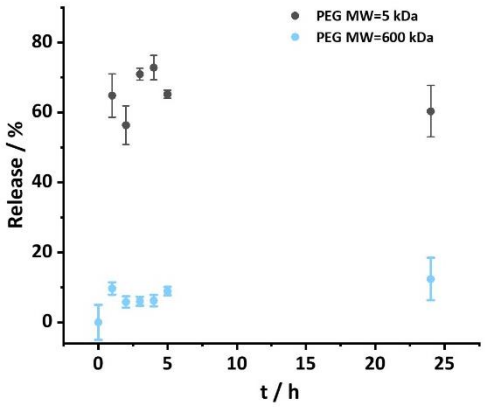


Figure S10. Release kinetics of PEG of different MW from low CL NCs following incubation of the NCs with Proteinase K at a concentration 0.025 u/ mg of NCs.

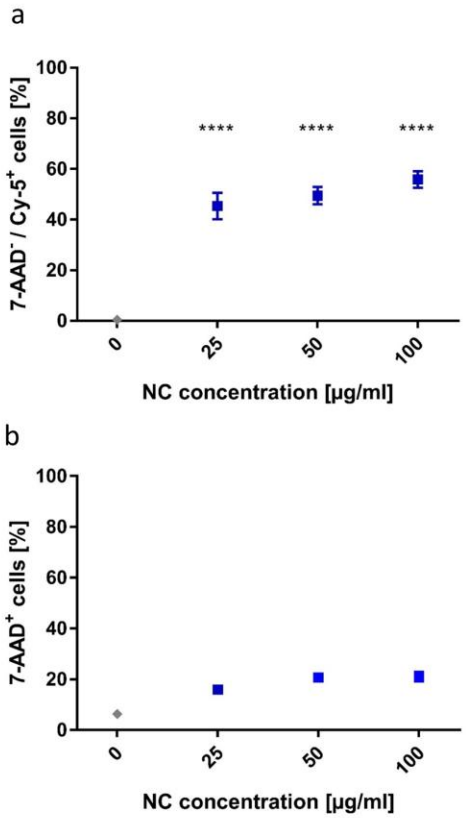


Figure S11. Uptake (a) and toxicity (b) of increasing concentrations of highly CL OVA-Cy-5 NCs. The DCs were incubated with different concentrations of NCs for 20h and subsequently analyzed via flow cytometry.

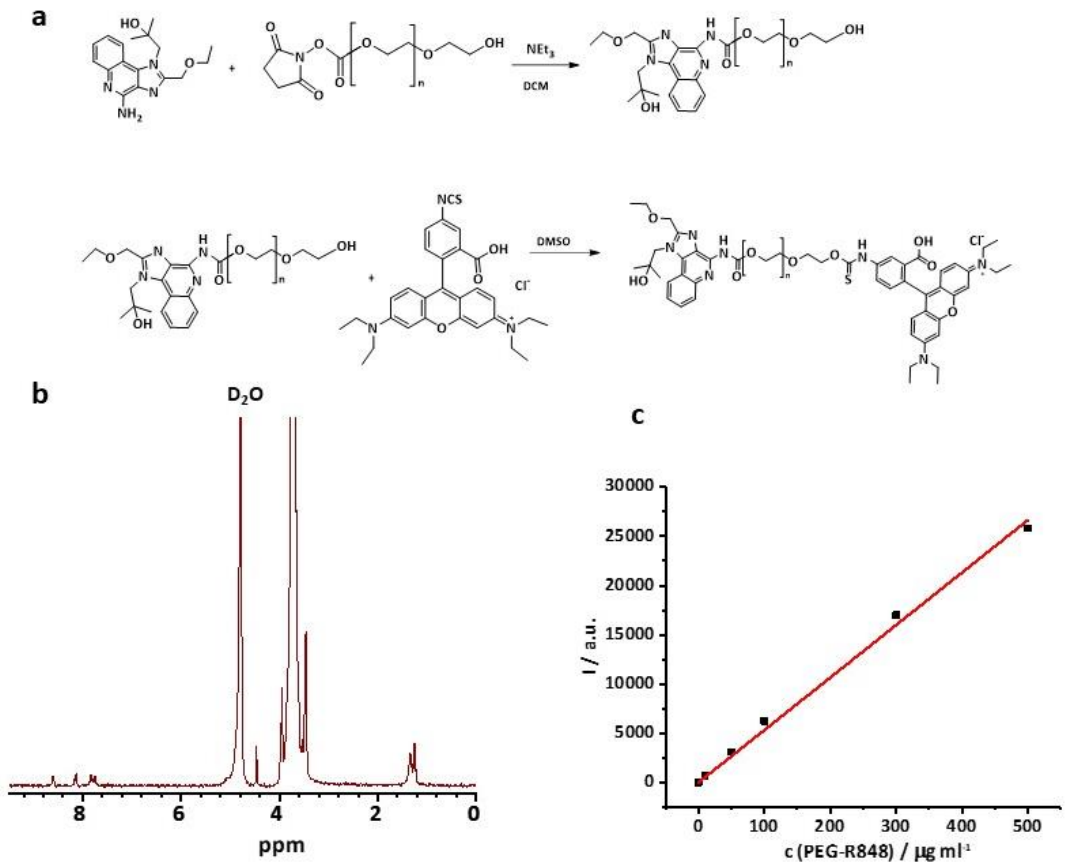


Figure S12. a) synthesis of PEG-R848, b) $^{1}$H-NMR spectrum of PEG-R848, c) calibration curve of Rhodamine B-PEG-R848.

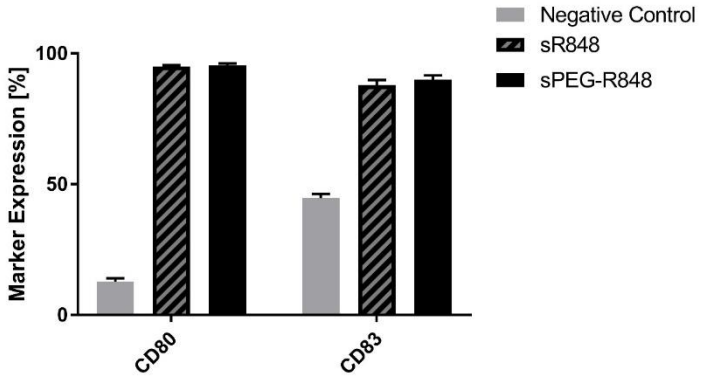


Figure S13. Activation of DCs after incubation with soluble R848 and PEG-functionalized R848. The activation of cells was assessed by measuring CD80 and CD83 expression levels via flow cytometry.

## Additional equations

The obstruction model stipulates that the coefficient of diffusion (D) or a probe of radius (R) in a network composed of fibers of thickness ($r_f$) creating a mesh of size ($\xi$) is given by:

$$D = D_0 \exp\left(-\pi\left(\frac{R+r_f}{\xi+2r_f}\right)^2\right) \qquad \text{eq S1}$$

Thus the average mesh size is:

$$\xi = \frac{R+r_f}{\sqrt{\frac{ln\left(\frac{D}{D_0}\right)}{-\pi}}} - 2r_f \qquad \text{eq S2}$$

Where $D_0$ is the coefficient of diffusion of the probe in a pure solvent of viscosity $\eta_0$, as defined by the Stoke-Einstein relation:

$$D_0 = \frac{k_b T}{6\pi\eta_0 R} \qquad \text{eq S3}$$

The coefficient of diffusion, using the Einstein–Smoluchowski formalism is defined by a characteristic time (t) and a characteristic distance diffused (r):

$$D = \frac{r^2}{2t} \qquad \text{eq S4}$$

For a molecule to be release, the characteristic distance diffused must be at least the thickness of the nanocapsule shell (ca. 10 nm).

In absence of protease, no diffusion (release) of $PEG_{5K}$ was observed even after 24 hours. These value can be used to approximate the maximal D of $PEG_{5K}$ in such system using equation S4 and the unperturbed average mesh size ($\xi_0$)

$$\xi_0 = \frac{R_{PEG\ 5k}+r_f}{\sqrt{\frac{\ln\left(\frac{D(t)}{D_{0,PEG\ 5k}}\right)}{-\pi}}} - 2r_f = \frac{R_{PEG\ 5k}+r_f}{\sqrt{\frac{\ln\left(\frac{\frac{h^2}{2t_1}}{D_{0,PEG\ 5k}}\right)}{-\pi}}} - 2r_f \qquad \text{eq S5}$$

After the addition of protease, the average mesh size increases and this increase in size led to the release of the payload. The diffusing molecules were able to go through the shell of the nanocapsule in less than 30 min.

The release of PEG5k can be observed when the average mesh size is larger than $\xi_{PEG5K}$:

$$\xi_{PEG_{5K}} = \frac{R_{PEG\ 5k}+r_f}{\sqrt{\frac{\ln\left(\frac{\frac{h^2}{2t_2}}{D_{0,PEG\ 5k}}\right)}{-\pi}}} - 2r_f \approx 1.2\xi_0 \qquad \text{eq S6}$$

Similarly, the release of PEG5k can be observed when the average mesh size is larger than $\xi_{PEG600K}$:

$$\xi_{PEG_{600K}} = \frac{R_{PEG\,600K} + r_f}{\sqrt{\frac{\ln\left(\frac{\frac{h^2}{2t_2}}{D_{0,PEG\,600K}}\right)}{-\pi}}} - 2r_f \approx 26\xi_0 \qquad \text{eq S7}$$

Assuming that the number of "pores" on a nanocapsule of size $r_{NC}$ is:

$$N = \frac{4\pi {r_{NC}}^2}{\xi^2} \qquad \text{eq S8}$$

Since the number of pores decreases as the nanocapsule is degraded by protease, the average mesh size after a degradation time t can be expressed as

$$\xi(t) = \sqrt{\frac{4\pi {r_{NC}}^2}{\frac{4\pi {r_{NC}}^2}{{\xi_0}^2} - N_{NH_2}(t)}} \qquad \text{eq S9}$$

The number of $NH_2$ per NC produced ($N_{NH2}(t)$) by the degradation after time t:

$$N_{NH_2}(t) = \frac{4\pi {r_{NC}}^2 \left({\xi_0}^2 - \xi^2(t)\right)}{{\xi_0}^2(i) \cdot \xi^2(t)} \qquad \text{eq S10}$$

And the concentration of amine produced during degradation in the nanocapsule suspension will be

$$C_{NH_2}(t) = \frac{N_{NH_2}(t) \times C_{NC}}{6.022 \cdot 10^{23}\ \mathrm{mol}^{-1}} \qquad \text{eq S11}$$