Supporting Information

Multicomponent Encapsulation into Fully Degradable Protein Nanocarriers *via* Interfacial Azide-Alkyne Click Reaction Allows the Co-Delivery of Immunotherapeutics

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Materials

All chemicals and materials were used as received. Human serum albumin (> 99% purity) was purchased from Sigma Aldrich as well as 1,6-hexanediol (99%), propiolic acid, sulfurylchloride and imidazole. Cyclohexane (HPLC grade) was purchased from VWR. Fluorescamine was purchased from Alfa Aesar. The block copolymer poly((ethylene-co-butylene)-*b*-(ethylene oxide)) P((E/B)-*b*-EO) used as the oil soluble surfactant was synthesized as described in literature and consists of a poly((ethylene-co-butylene) block (NMR: M_n = 3900 g/mol) and a poly(ethylene oxide) block (NMR: M_n = 2700 g/mol). The anionic surfactant sodium dodecyl sulfate (SDS) was purchased from Sigma Aldrich. Cy5-Oligo was purchased from IBA Lifesciences. Proteinase K from tritirachium album (≥ 30 units/mg) and peroxidase from horseradish (≥ 50 units/mg) was purchased from Sigma Aldrich. Resiquimod (R848), muramyl dipeptide (MDP) and polyinosinic-polycytidylic acid (Poly(I:C) LMW) was purchased from Invivogen. Amicon Ultra-2 centrifugal filter devices were purchased from Merck Millipore (100 kDa) nominal molecular weight limit (NMWL). The magnesium and calcium free phosphate-buffered saline, was purchased from Life Technologies. Demineralized water was used for all experiments.

Experiments

1. Synthesis of 1,6-hexandiol dipropiolate (HDDP)

The dialkyne crosslinker was synthesized by esterification following the literature. Briefly, hexandiole (4 g, 33.85 mmol), propiolic acid (9.48 g, 135.39 mmol) and *p*-TsOH (333.33 mg, 5 mol%) were dissolved in 120 mL toluene and stirred at 135 °C under reflux for 3 days using a dean-stark apparatus. Afterwards, the reaction solution was washed twice with 100 mL saturated $NaHCO_3$ solution and twice with 100 mL water. The solvent was removed and the product purified by column chromatography (PE:EtOAc 10:1). The product was obtained as colorless crystals. Yield: 3.37 g, 45%.

^{1}H NMR (300 MHz, CD_2Cl_2) δ 4.10 (t, 4H, O-C$\underline{H}_2$), 2.87 (s, 2H, $\underline{H}$C ≡ C), 1.61 (m, 4H, C$\underline{H}_2$-CH_2-O), 1.32 (m, 4H, CH_2-C$\underline{H}_2$-CH_2) ppm. ^{13}C NMR (300 MHz, CD_2Cl_2) δ 153, 74.6, 66.6, 28.5, 25.8 ppm.

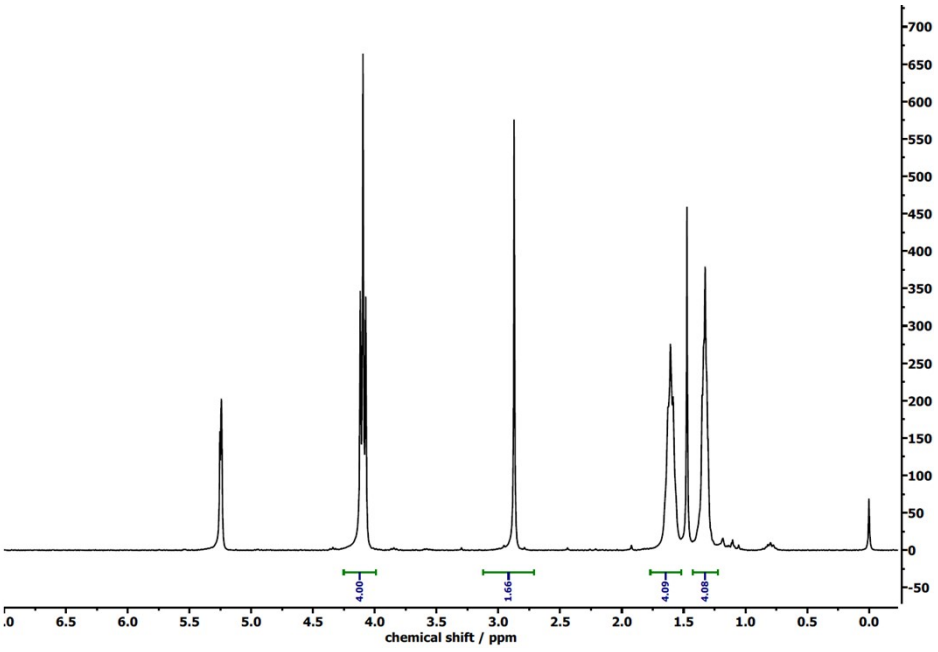


Figure S1. ^{1}H NMR spectrum of 1,6-hexandiol dipropiolate in CD_2Cl_2.

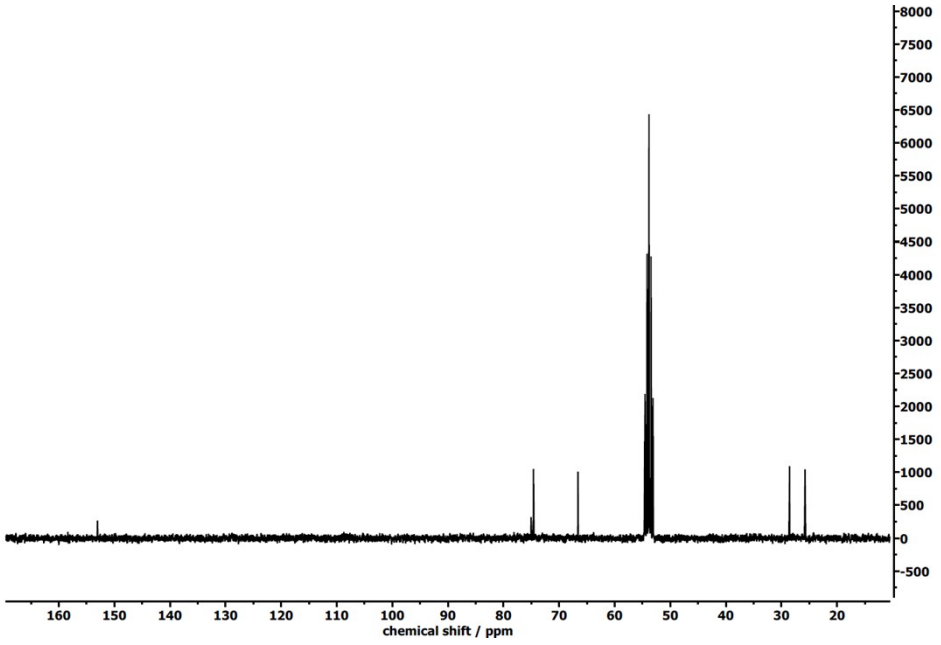


Figure S2. ^{13}C NMR spectrum of 1,6-hexandiol dipropiolate in CD_2Cl_2.

2. Synthesis of disulfide 1,6-hexanediol dipropiolate (HDDP-SS)

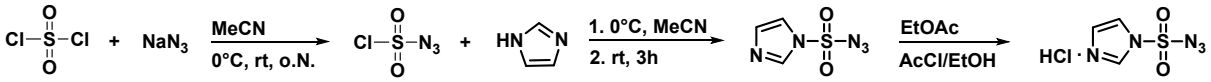
The dialkyne crosslinker was synthesized by esterification following the literature. Briefly, bis(2-hydroxyethyl) disulfide (3.0 g, 19.5 mmol), propiolic acid (5.4 g, 78.0 mmol) and *p*-TsOH (360 mg, 2 mmol) were dissolved in 150 mL benzene and stirred under reflux for 3 days using a dean-stark apparatus. Afterwards, 100 mL saturated $NaHCO_3$ solution was added to the reaction solution and the organic phase separated. The aqueous phase was washed twice with 100 mL diethylether. The organic phases are combined and dried over Na_2SO_4. The solvent was removed and the product purified by column chromatography (n-hexane:EtOAc = 3:1). The product was obtained as colorless crystals. Yield: 2.3 g, 46%.

^1H NMR (300 MHz, $CDCl_3$) δ 4.41 (t, 4H, O-C$\underline{H}_2$), 2.96-2.92 (m, 6H, S-CH_2 and $\underline{H}C \equiv C$) ppm. ^{13}C NMR (300 MHz, CD_2Cl_2) δ 152.3, 75.5, 74.3, 74.3, 63.9, 36.7 ppm.

3. Synthesis of 1-imidazole-sulfonyl azide hydrochloride

The azide transfer agent was synthesized according to Goddard-Borger *et al.*[1] Briefly, sodium azide (1.63 g, 25 mmol) was suspended in 25 mL MeCN and cooled down to 0 °C. Under vigorous stirring sulfurylchloride (3.34 g, 25 mmol) was added drop-wisely. The reaction solution was slowly brought to room temperature and the reaction carried out at room

temperature overnight. Again, the reaction solution is cooled down to 0 °C and imidazole (3.23 g, 47.5 mmol) added in small portions. The reaction is stirred at room temperature for 3 h. Afterwards, 50 mL EtOAc is added and the reaction solution is washed twice with 50 mL saturated $NaHCO_3$ solution and twice with 50 mL water. The organic phase is dried over $MgSO_4$ and filtered. A mixture of AcCl/EtOH is slowly added to the reaction solution at 0 °C. The product is filtered, washed with EtOAc and dried. Yield: 3.13 g.



^{1}H NMR (300 MHz, D_2O) δ 7.44 (dd, 1H, N-CH=CH), 7.85 (dd, 1H, HC=CH-N), 9.17 (dd, 1H, N=CH-N) ppm. ^{13}C NMR (300 MHz, CD_2Cl_2) δ 152.3, 75.5, 74.3, 74.3, 63.9, 36.7 ppm.

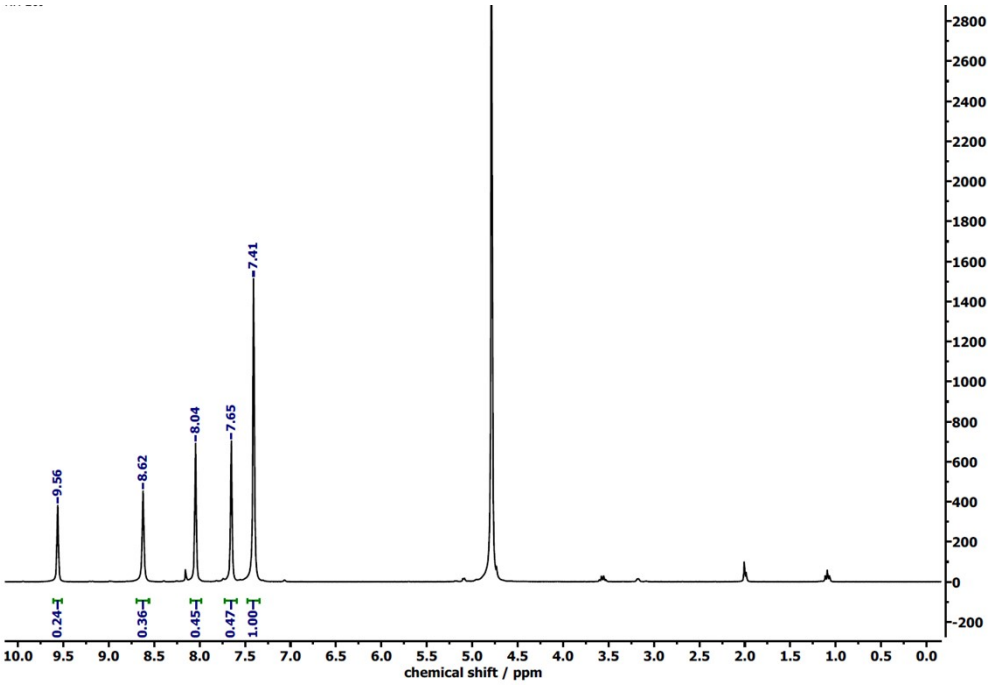


Figure S3. ^{1}H NMR spectrum of 1-imidazole-sulfonyl azide hydrochloride in D_2O.

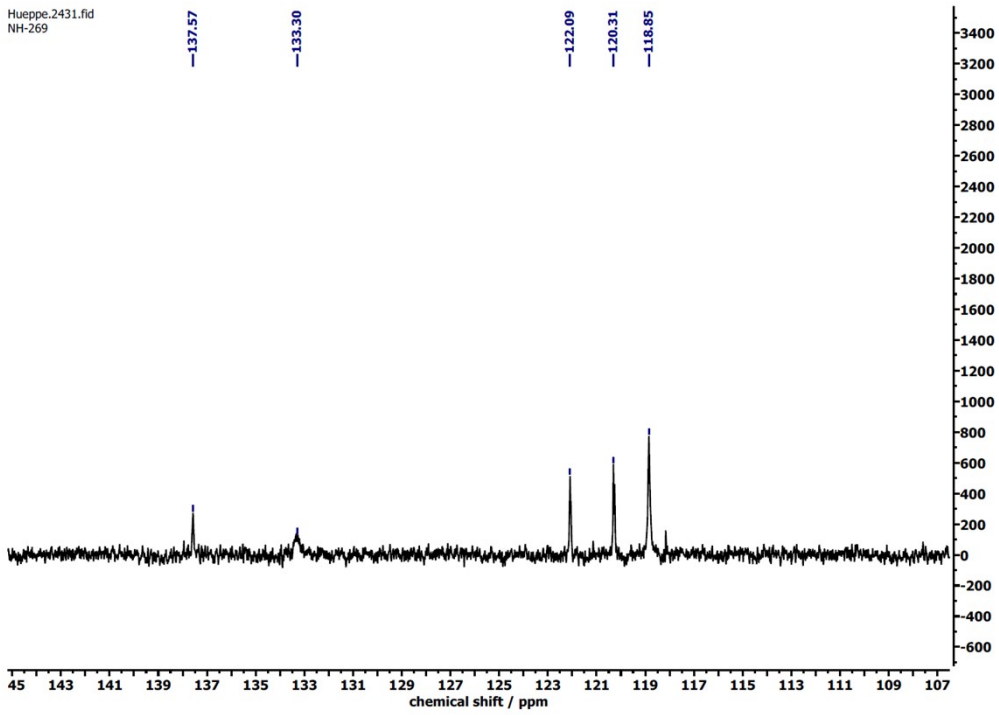


Figure S4. ^{13}C NMR spectrum of 1-imidazole-sulfonyl azide hydrochloride in D_2O.

4. Azidation of proteins with 1-imidazole-sulfonyl azide hydrochloride

The protein (1g) was dissolved in 20 mL K_2CO_3 solution of pH 11. The azide transfer agent (276 mg) was dissolved in 2 mL water and added dropwisely to the protein solution. The pH value of the reaction solution was adjusted to pH 11 with 1 M NaOH. The reaction solution was stirred at room temperature for 48 h. The product was purified by dialysis (MWCO 1K) and lyophilized. Yield: 0.93 g. The amount of azide moieties was determined using the fluorescamine assay.

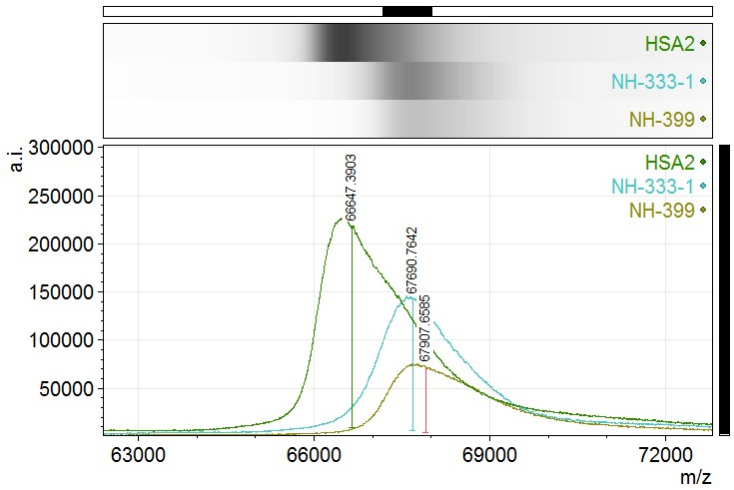


Figure S5. MALDI-TOF of human serum albumin (HSA, green), HSA-N_3 via copper-catalyzed azide-functionalization (blue) and BSA-N_3 via copper-free azide-functionalization.

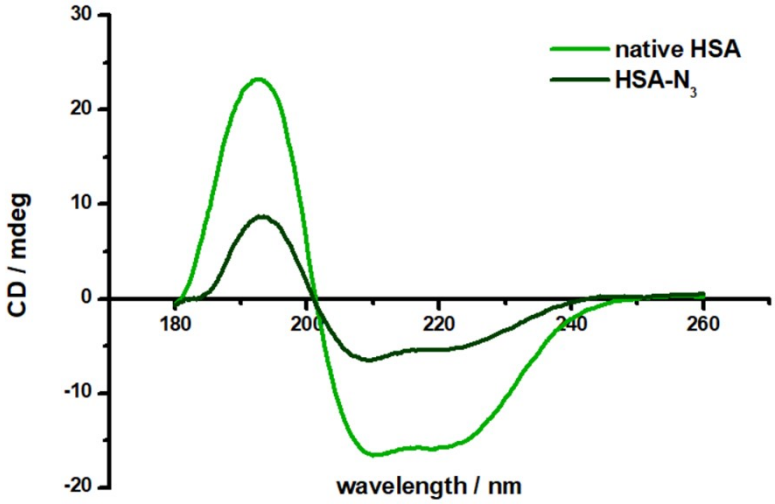
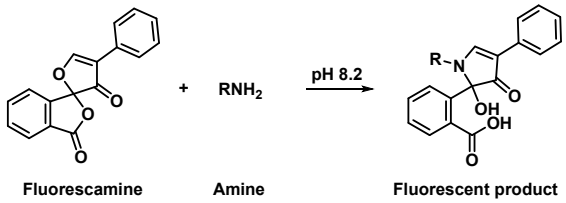


Figure S6. Circular dichroism (CD) spectra of native human serum albumin (HSA) and azide-functionalized HSA.

5. Fluorescamine assay

The quantitative amount of azide groups was determined with the fluorescamine assay in borate buffer at pH 8.2.



Scheme S1. Reaction of fluorescamine with amines to a fluorescent product at pH 8.2.

Glycine was used for the standard calibration curve and lysozyme was used as a reference. A decreased amount of amine groups was determined for the protein after azide-functionalization, indicating a successful reaction.

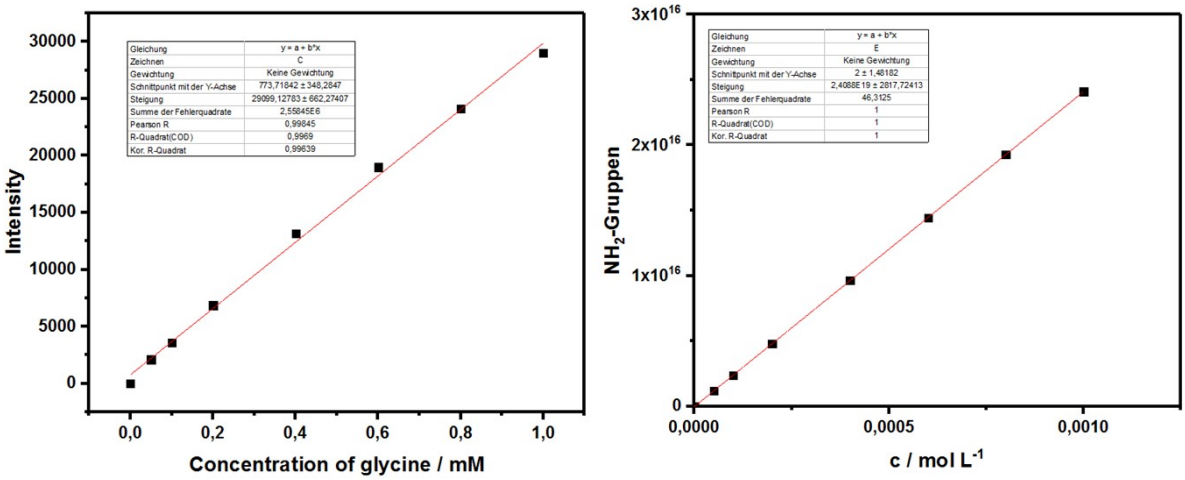


Figure S7. Fluorescamine assay, standard calibration with glycine.

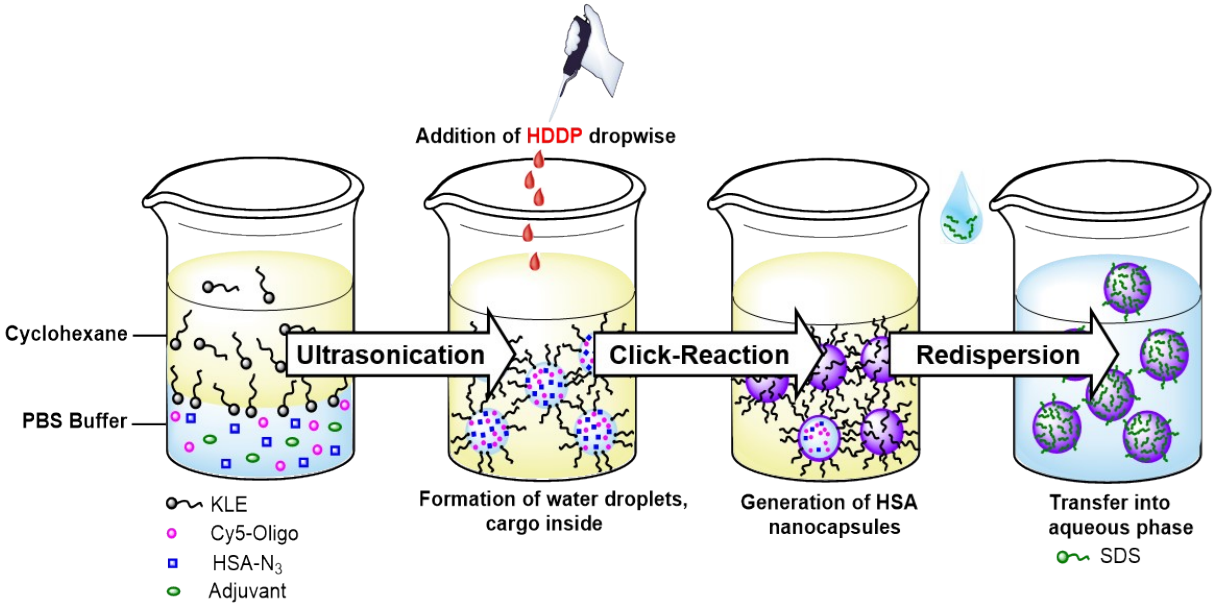
Table 1. Azide-functionalization of human serum albumin at different pH value and time. Number of azide groups ($N_{ex}(N_3)$) quantified by the theoretical ($N_{theo}(NH_2)$) and experimental ($N_{ex}(NH_2)$) number of amines, measured with the fluorescamine assay.

	pH	*t*/h	$N_{theo}(NH_2)$	$N_{ex}(NH_2)$	$N_{ex}(N_3)$
HSA, nat.	-	-	30-35	35	-
HSA-N_3		2	-	37	-
HSA-N_3		4	-	33	2
HSA-N_3	8.2	8	-	30	5
HSA-N_3		16	-	29	6
HSA-N_3		24	-	29	6

HSA-N_3		48	-	21	14
HSA-N_3		2	-	28	7
HSA-N_3		4	-	22	13
HSA-N_3	9.5	8	-	13	22
HSA-N_3		16	-	6	29
HSA-N_3		24	-	5	30
HSA-N_3		48	-	2	33
HSA-N_3		2	-	10	16
HSA-N_3		4	-	3	32
HSA-N_3	11	8	-	2	33
HSA-N_3		16	-	3	32
HSA-N_3		24	-	3	32
HSA-N_3		48	-	2	33

6. Formation of protein nanocarrier

First, the azide-functionalized protein (50 mg) is dissolved in 0.4 mL NaCl solution (c = 14.4 mg/mL) and 100 µL Cy5-Oligo (c = 0.1 nmol/µL). 35.7 mg of surfactant poly((ethylene/butylene)-*block*-(ethylene oxide)) (P((E/B)-*b*-EO)) were dissolved in 7.5 g of cyclohexane and the mixture was added to the aqueous solution. The two phases were homogenized by ultrasound under ice-cooling (70% amplitude, 3 min, 20 s pulse, 10 s pause). A third solution containing of 10.7 mg P((E/B)-*b*-EO), 35.7 mg crosslinker in 5 g of cyclohexane and was then added dropwise to the stirred miniemulsion at 40 °C. The reaction was carried out at 40 °C for 24 h. Afterwards, the protein nanocarriers were purified by repetitive centrifugation (1500 g, 20 °C) and redispersion in cyclohexane to remove excess of surfactant and crosslinker. For the transfer of the nanocarriers into aqueous media, 500 µL of concentrated nanocarrier dispersion in cyclohexane is added dropwise to 5 mL 0.1 wt% SDS solution under shaking in an ultrasonication bath for 3 min. Then, the emulsion was stirred open over night to evaporate the organic solvent. The protein nanocarriers in water were purified by repetitive centrifugation and washing in Amicon Ultra-2 centrifugal filters (MWCO 100 kDa).



Scheme S2. Preparation of protein nanocarriers crosslinked with HDDP in inverse miniemulsion and transfer into water.

Table S2. Analytical data of human serum albumin and horse radish peroxidase nanocarriers.

Protein	N_{theo} (NH_2)	N_{ex} (NH_2)	N_{ex} (N_3)	Linker	$d_{i,CH}$ / nm	PDI_{CH}	$d_{i,water}$ / nm	PDI_{water}	ζ /mV
HSA	30-35	11	19-24	HDDP	302	0.13	264	0.23	-30
HSA	30-35	11	19-24	HDDP-SS	327	0.14	254	0.17	-34
HRP	6	2	4	HDDP	424	0.13	231	0.34	-28

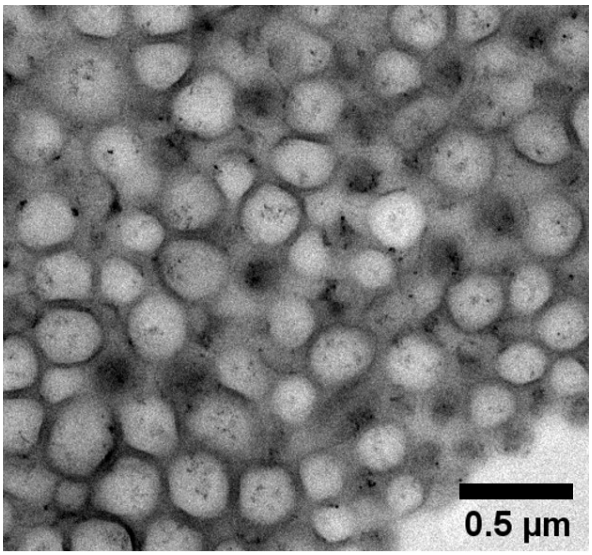


Figure S8. Transmission electron micrograph of human serum albumin nanocarrier.

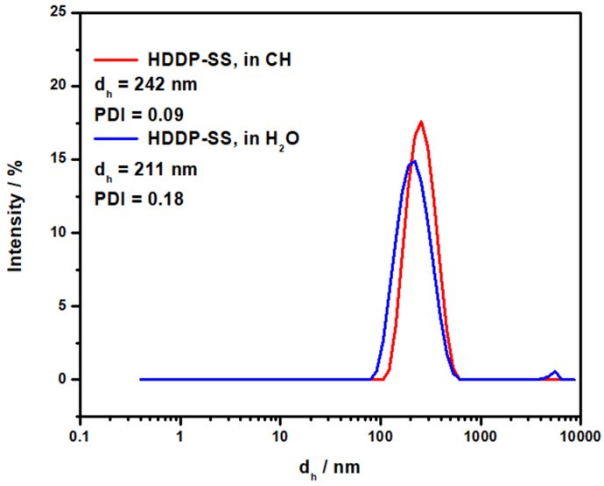


Figure S9. DLS measurement of HSA-HDDP-SS nanocarriers in cyclohexane (red) and water (blue).

7. Characterization of protein nanocarriers

Dynamic light scattering (DLS) was used to determine the average size and size distribution of the nanocarriers. A diluted dispersion (10 µL sample diluted in 1 mL cyclohexane or 50 µL sample diluted in 200 µL H_2O) was measured on a Malvern Zetasizer Nano S (Malvern Panalytical) equipped with a detector at 90° scattering mode at 20 °C. The zeta potential of the nanocarriers were measured in 10^{-3} M potassium chloride solution with a ZetaNanosizer (Malvern Panalytical) at 20 °C. Scanning electron microscopy (SEM) studies were done on a field emission microscope (LEO (Zeiss) 1530 Gemini, Oberkochen, Germany) working at an

accelerating voltage of 170 V. The silica wafers are cleaned in the plasma oven prior to use. Then, 2 µL of a diluted nanocarrier dispersion in cyclohexane or distilled water (concentration similar to samples for DLS) were dropped onto the wafers and dried under ambient conditions for 15 min. No additional contrast agent was applied. The solid content of the nanocarrier dispersion was measured gravimetrically. The fluorescence intensities for all mentioned experiments were measured using a microplate reader (Infinite M1000, Tecan, Switzerland).

8. Determination of encapsulation efficiency and permeability

The encapsulation efficiency is determined by measuring the fluorescence intensity of using a microplate reader (Infinite M1000 Tecan, Switzerland). The unpurified nanocarrier dispersion after transfer into 0.1 wt% SDS are concentrated in an Amicon centrifuge filter 100 K for 30 min at 500 g. The amount of non-encapsulated dye was measured in the supernatant and the encapsulation efficiency determined in proportion to the fluorescence intensity of the un-purified dispersion. After washing the aqueous dispersion, the permeability of the nanocarrier was measured using the same method. The dispersion was concentrated in an Amicon Centrifuge filter at a certain time point and the amount of leaked dye measured in the supernatant.

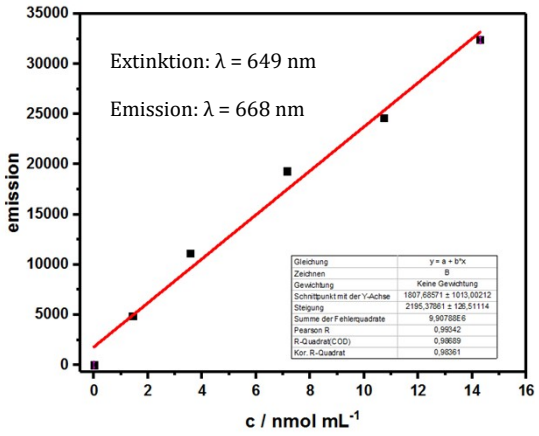


Figure S10. Standard calibration curve of Cy5-Oligo fluorescence.

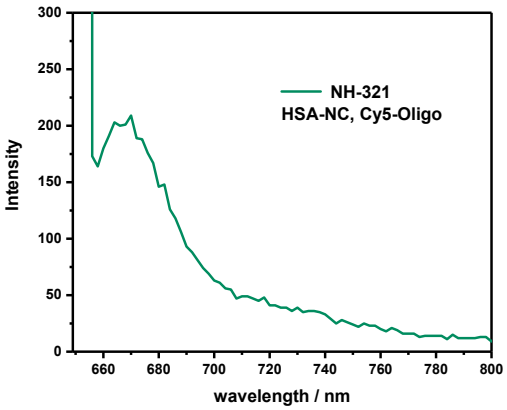


Figure S11. Fluorescence curve of HSA nanocarriers with encapsulated Cy5-oligo.

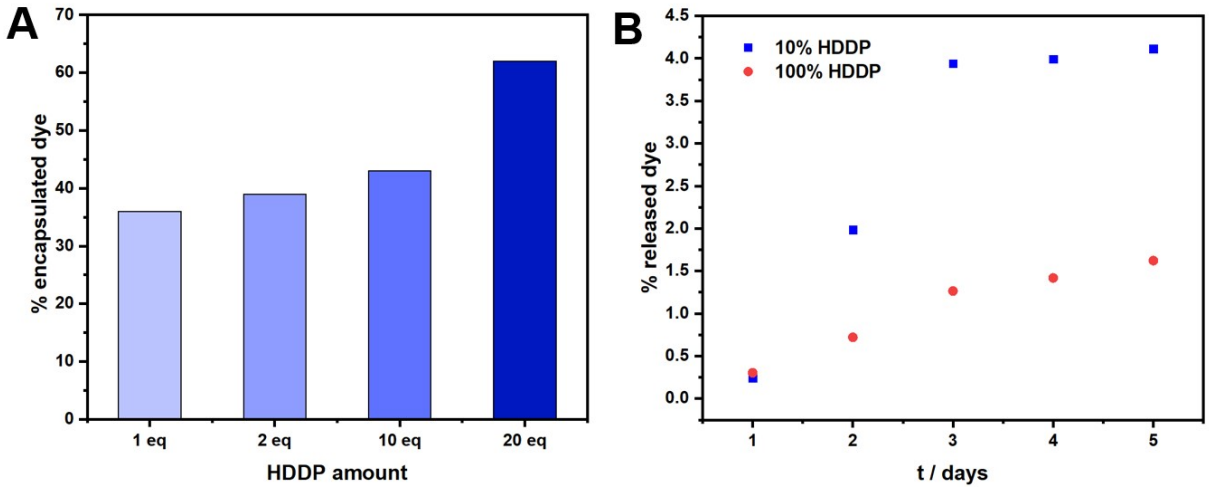


Figure S12. A) Amount of encapsulated dextran-sulforhodamine B (M = 10 kDa) after transfer of protein nanocarriers crosslinked with different amount of HDDP to water. Measured by fluorescence. B) Amount of released sulforhodamine 101 upon storage at room temperature measured by fluorescence.

9. Degradability of protein nanocarrier

The enzymatic degradation of the protein nanocapsules were performed with proteinase K and determined by release of Cy5-Oligo. A 0.1 wt% nanocarrier dispersion in PBS buffer was treated with a proteinase K solution (30 U/mL) at 37 °C. After the enzymatic degradation the dispersion is filtered by centrifugation in an Amicon centrifuge filter 3K at 500 g for 30 min and the amount of released dye in the supernatant measured by fluorescence. The degradation of the nanocarriers by proteinase K is also monitored by DLS measurements every 5 min over

10 h. The reduction-responsive properties of HDDP-SS-crosslinked protein nanocarriers were investigated with dithiothreitol (DTT) by release of Cy5-Oligo. A 0.1 wt% nanocarrier dispersion in PBS was treated with a DTT solution (25 mM) at 20 °C. After the reductive degradation, the dispersion is filtered by centrifugation in an Amicon centrifuge filter 3K at 500 g for 30 min and the amount of released dye in the supernatant measured by fluorescence. The degradation of the nanocarriers by DTT is also monitored by DLS measurements every 5 min over 10 h. In both cases, enzymatic and reductive degradation, a sample treated with PBS buffer serves as a control sample and every experiment was performed in triplets.

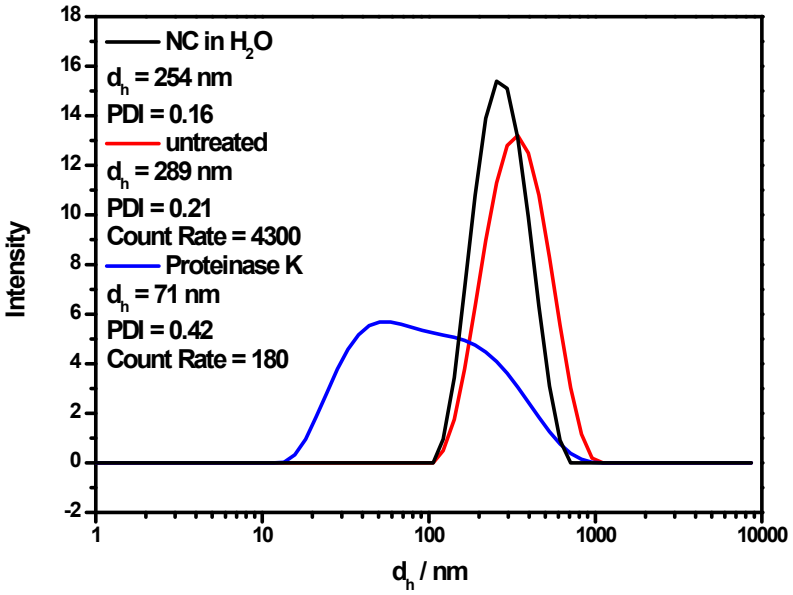


Figure S13. DLS curves of protein nanocarrier before and after treatment with proteinase K.

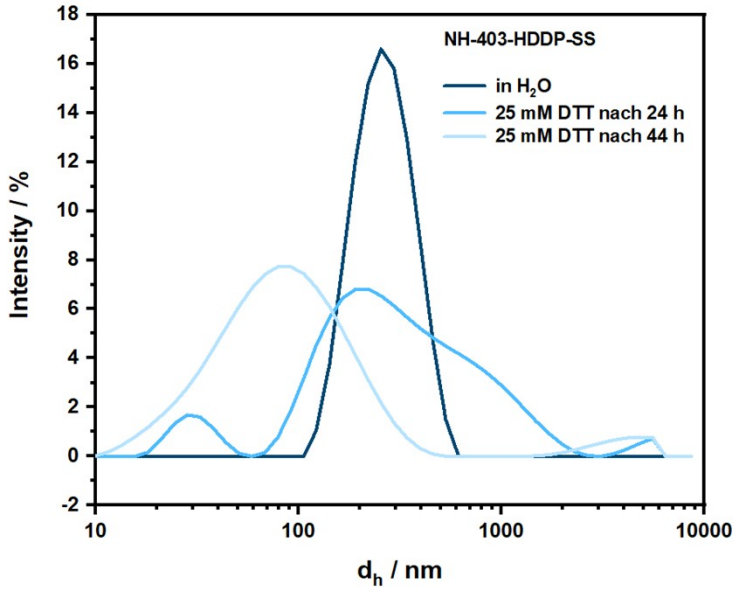


Figure S14. DLS measurement of protein nanocarriers before and after treatment with DTT.

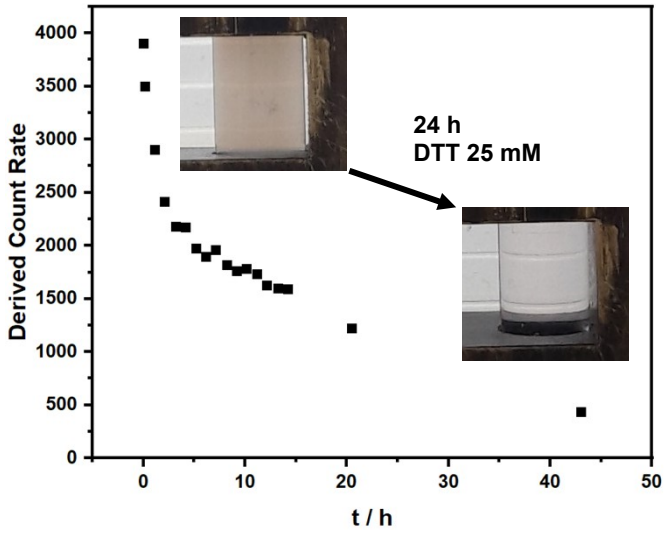


Figure S15. Influence of the derived count rate of human serum albumin nanocarriers crosslinked with HDDP upon treatment of DTT measured by DLS.

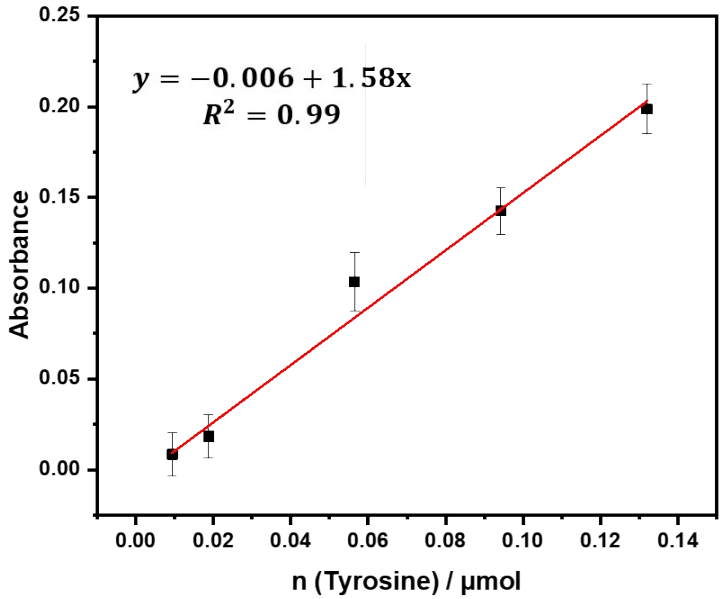


Figure S16. Tyrosine calibration curve for the determination of th enzyme activity of proteinase K.

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Table S3. Enzymatic activity assay of proteinase K under the influence of DTT with hemoglobin as substrate. Proteinase K solution incubated with DTT at 37 °C for 10 min, 30 min and 2 h.

Sample	Prot K / UmL^{-1}	DTT /$mmolL^{-1}$	Activity /Umg^{-1}	Error /Umg^{-1}	Activity /Umg^{-1}	Error /Umg^{-1}	Activity /Umg^{-1}	Error /Umg^{-1}
1	0.1	-	62	10	110	27	138	61
2	0.1	1	39	10	110	27	135	75
3	0.1	3	47	11	125	29	138	72
4	0.1	5	45	9	124	6	139	73
5	0.1	25	102	9	161	4	212	84
6	1	-	62	6	51	2	62	6
7	1	1	61	6	52	4	61	6
8	1	3	63	9	52	3	63	9
9	1	5	63	6	52	3	63	6
10	1	25	67	7	53	3	67	7

10. Determination of enzyme concentration in protein nanocarrier

A protein assay with bicinchoninic acid (BCA) was used as the substrate to determine the concentration of the enzyme in the nanocarrier dispersion. Briefly, BCA (100 mg), sodium carbonate (200 mg), sodium hydrogen carbonate (95 mg) and sodium tartrate (16 mg) were dissolved in 10 mL of distilled water and the pH of the solution was adjusted to 11.3 by using 3.0 M NaOH. A solution of $CuSO_4$ x 5 H_2O (50 mg/mL, 200 µL) in distilled water was added to the substrate solution. 10 µL of protein or nanocarrier dispersion was mixed with 200 µL of metal-containing substrate solution and incubated at 60 °C for 30 min. The absorbance of the sample was measured at 565 nm and the enzyme concentration determined by a standard calibration with native enzyme.

11. Enzymatic activity

The enzymatic activity of HRP and HRP-HDDP nanocarriers were determined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as substrate. Briefly, the substrate was dissolved in 100 mM potassium phosphate buffer (pH 5.0) to a concentration of 5 mg/mL. The enzyme in different stages of the nanocarrier preparation were diluted to a concentration of 0.002 mg/mL protein in a solution of 0.5% Triton X-100 in 40 mM PBS buffer (pH 6.8). The ABTS solution (190 µL) was mixed with the enzyme solution (3.3 µL) in a 96-well plate and the reaction started with addition of 0.3% (w/w) hydrogen peroxide solution (6.6 µL). The absorbance at 405 nm was monitored by a UV/Vis spectrophotometer and the enzyme activity determined by a standard calibration with native HRP.

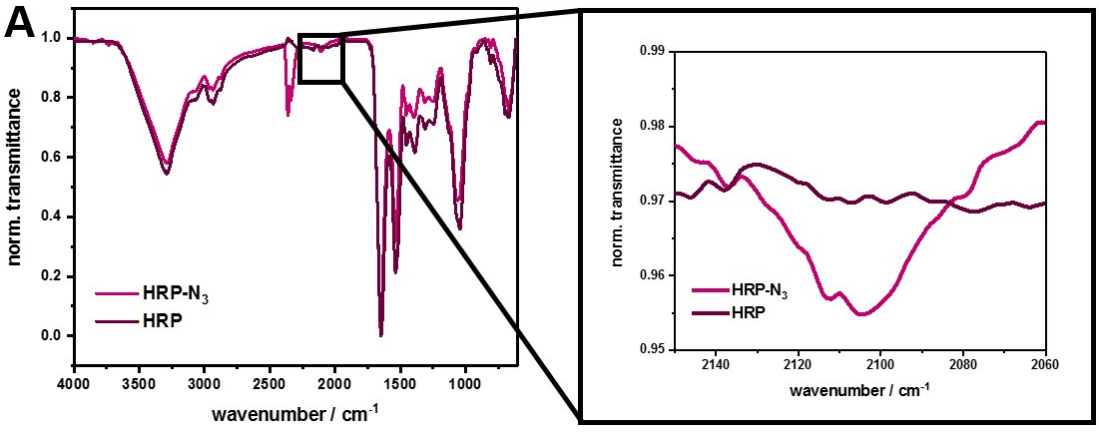


Figure S17. IR spectra of horse radish peroxidase and azide-functionalized HRP.

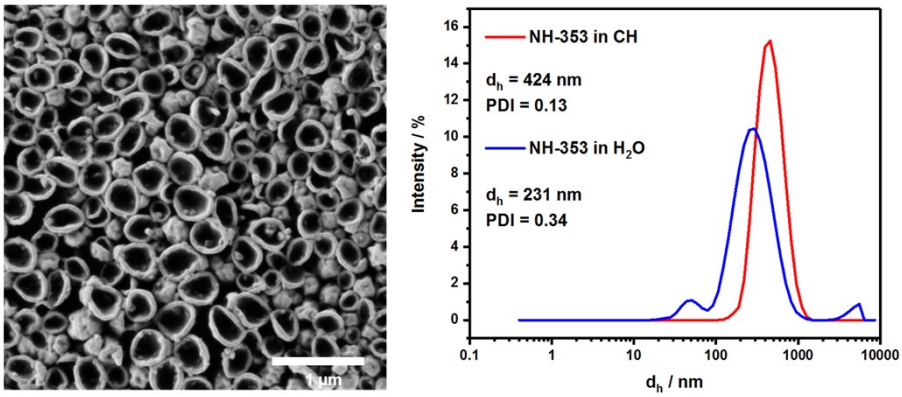


Figure S18. Scanning electron micrograph and DLS measurements of HRP-HDDP nanocarriers in cyclohexane.

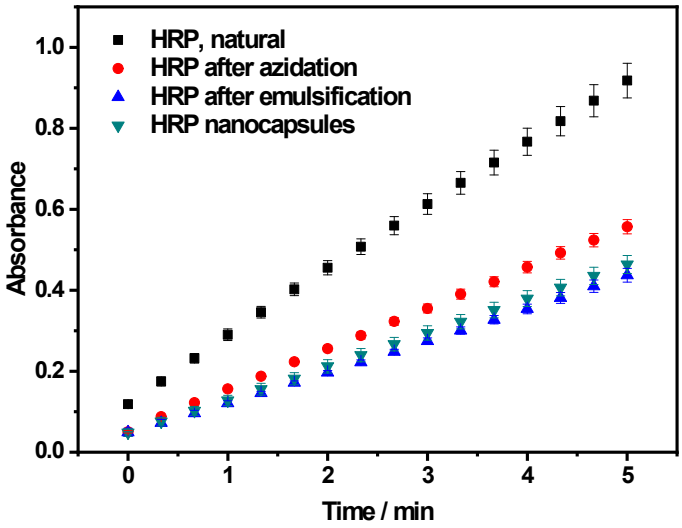


Figure S19. Enzymatic activity of horse radish peroxidase during the preparation of HRP nanocarriers.

12. Quantification of adjuvants in protein nanocarriers

For the quantification of adjuvants in the protein nanocapsules (PNCs), the PNCs were degraded by proteinase K (30 U/mL) at 37 °C overnight. The PNCs remains and the enzyme were separated from the released adjuvants through a centrifuge filter (MWCO 3K, 30 min, 1500 g). The amount of Resiquimod (R848) was determined by fluorescence (λ_{ex} = 260 nm, λ_{em} = 360 nm) using a standard calibration curve (Figure S20). Muramyl dipeptide (MDP) was

determined from the supernatant using the Morgan-Elson Reaction (Scheme S3). The supernatant (50 µL) was mixed with borate buffer (50 µL, pH 9) and incubated at 100 °C for 3 min. The mixture is cooled to room temperature and DMAB (500 µL) was added to the mixture. The mixture was incubated again at 37 °C for 15 min and afterwards the absorbance measured at 585 nm. The MDP was quantified by a standard calibration (Figure S21). Poly(I:C) was quantified from the full mixture after degradation as it has a too high molecular weight to be separated from the proteins. The full mixture of PNCs after degradation with proteinase K was eluted through a reverse phase HPLC column using a mixture of Acetonitrile, 0.01% formic acid and 0.02 mol/L ammoniumacetate. The Poly(I:C) signal was quantified using a standard calibration curve (Figure S22).

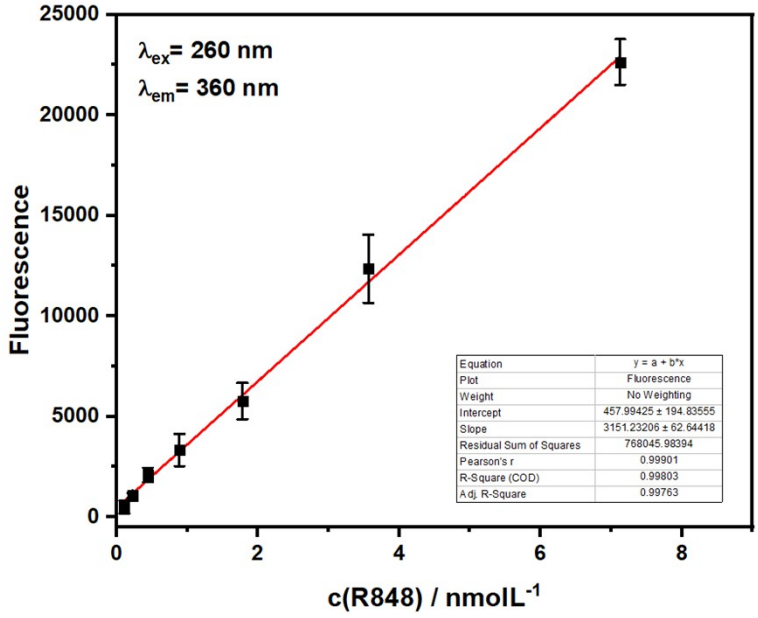
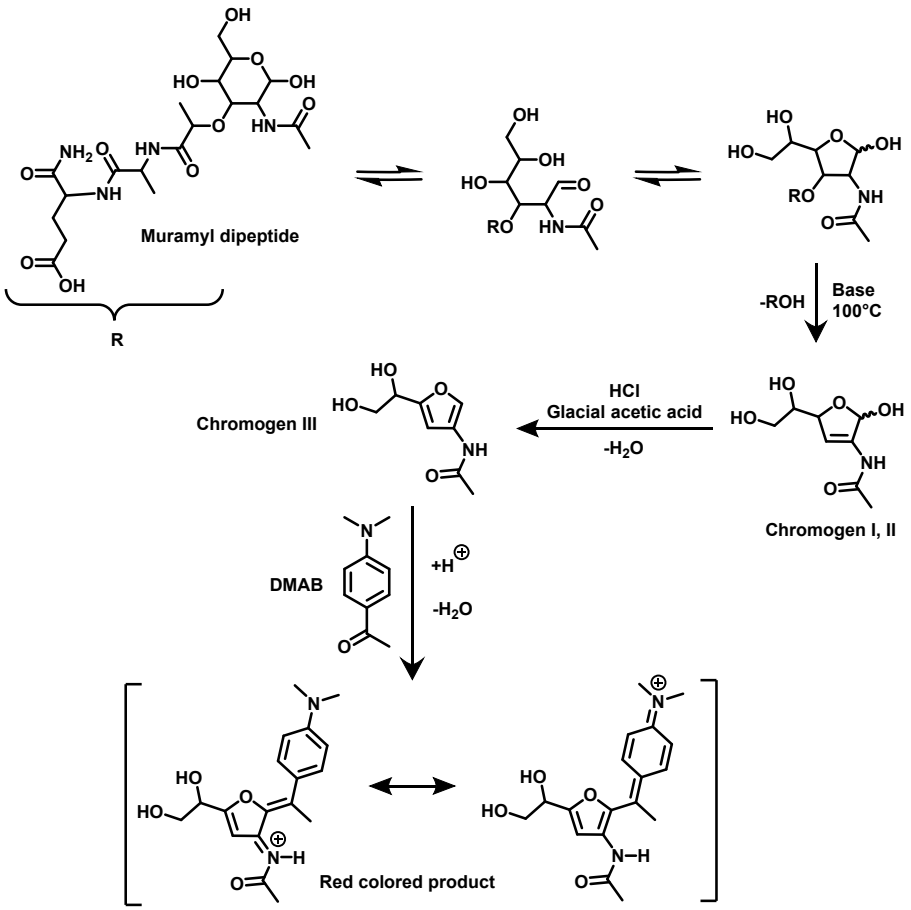


Figure S20. Standard calibration curve of R848 by fluorescence measured at 360 nm.

Scheme S3. Quantification of muramyl dipeptide with Morgan-Elson reaction.



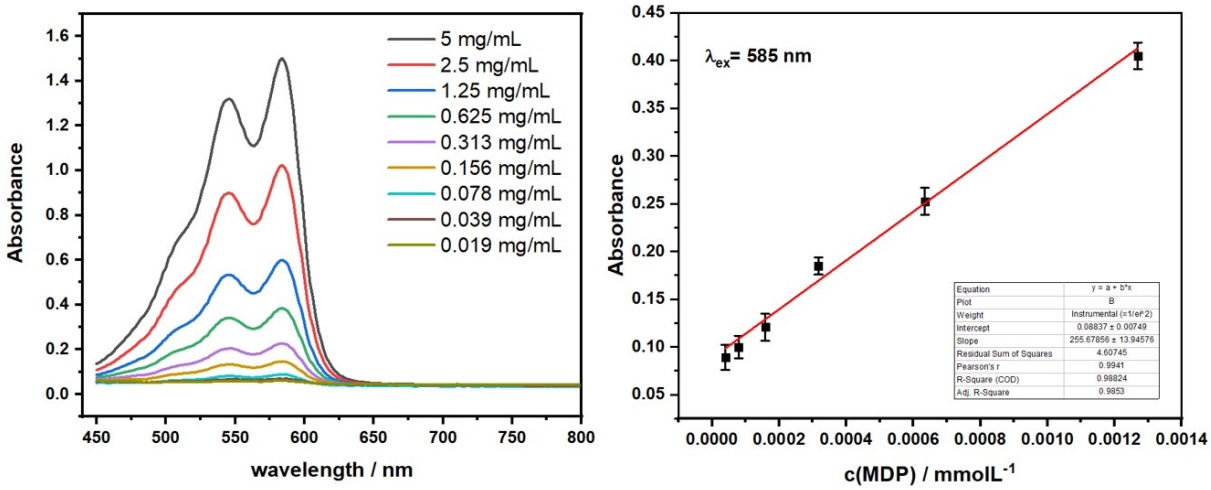


Figure S21. Absorbance measurements of MDP assay via Morgan-Elson reaction with DMAB.

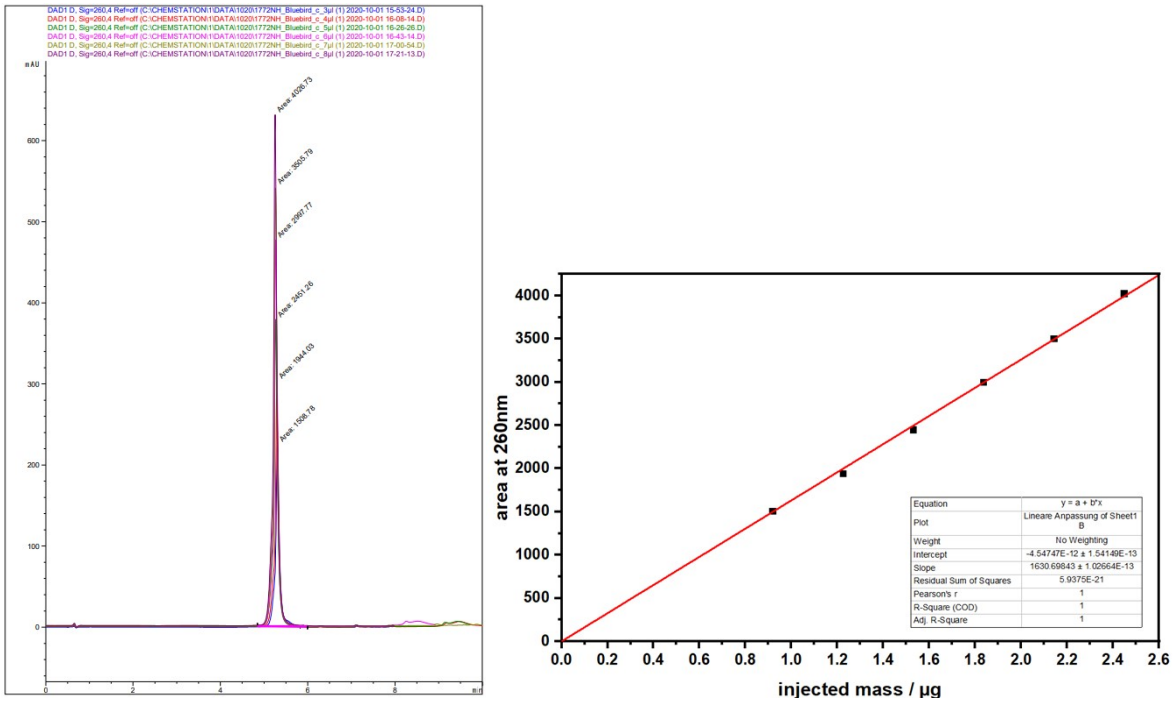


Figure S22. Standard calibration curve of Poly(I:C) measured by HPLC.

Table S4. Encapsulation efficiencies of dye and adjuvants into human serum albumin nanocarriers crosslinked with HDDP or HDDP-SS. Variation of the concentration of R848, MDP and Poly(I:C) in the nanocarriers.

No.	Linker	Adj.	$EE_{Cy5\ Oligo}$ / %	$c_{R848,theo}$ / molmL^{-1}	c_{R848} / molmL^{-1}	c_{R848} / %	$c_{MDP,theo}$ / molmL^{-1}	c_{MDP} / molmL^{-1}	c_{MDP} / %	$c_{PolyI:C,theo}$ / molmL^{-1}	$c_{PolyI:C}$ / molmL^{-1}	$c_{PolyI:C}$ / %	$d_{h,H2O}$ / nm	PDI	ζ / mV
1	HDDP	-	86	-	-	-	-	-	-	-	-	-	203	0.16	-32
2		R848	80	$4.50*10^{-8}$	$2.88*10^{-8}$	64	-	-	-	-	-	-	245	0.17	-40
3		MDP	84	-	-	-	$1.02*10^{-7}$	$5.92*10^{-8}$	58	-	-	-	212	0.22	-38
4		PolyI:C	85	-	-	-	-	-	-	$2.00*10^{-10}$	$1.84*10^{-10}$	92	215	0.20	-42
5		All 3	85	$8.91*10^{-8}$	$3.15*10^{-8}$	35	$1.02*10^{-7}$	$3.73*10^{-8}$	37	$2.00*10^{-10}$	$5.20*10^{-11}$	26	302	0.25	-34
6		All 3	87	$4.50*10^{-8}$	$2.84*10^{-8}$	63	$1.02*10^{-7}$	$5.55*10^{-8}$	53	$2.00*10^{-10}$	$1.30*10^{-10}$	65	218	0.21	-38
7		All 3	73	$2.23*10^{-8}$	$1.77*10^{-8}$	79	$1.02*10^{-7}$	$5.68*10^{-8}$	56	$2.00*10^{-10}$	$1.30*10^{-11}$	65	356	0.35	-28
8		All 3	90	$4.50*10^{-8}$	$2.84*10^{-8}$	64	$5.08*10^{-8}$	$3.59*10^{-8}$	71	$2.00*10^{-10}$	$1.46*10^{-11}$	73	366	0.18	-29
9		All 3	91	$4.50*10^{-8}$	$3.05*10^{-8}$	69	$2.54*10^{-8}$	$1.86*10^{-8}$	73	$2.00*10^{-10}$	$1.60*10^{-11}$	80	252	0.07	-32
10		All 3	52	$4.50*10^{-8}$	$3.23*10^{-8}$	74	$1.02*10^{-7}$	$7.06*10^{-8}$	69	$5.00*10^{-11}$	$4.65*10^{-11}$	93	194	0.27	-31
11	HDDP-SS	-	57	-	-	-	-	-	-	-	-	-	258	0.16	-34
12		R848	57	$4.50*10^{-8}$	$3.34*10^{-8}$	75	-	-	-	-	-	-	254	0.14	-33
13		All 3	73	$4.50*10^{-8}$	$2.93*10^{-8}$	65	$1.02*10^{-7}$	$6.43*10^{-8}$	63	$2.00*10^{-10}$	$1.22*10^{-10}$	61	304	0.19	-32

13. *In vitro* experiments with BMDCs

Bone marrow-derived dendritic cells (BMDC) were differentiated from bone marrow progenitors (BM cells) of 8- to 10-week-old C57BL/6mice. Briefly, the bone marrow was

obtained by flushing the femur, tibia, and hip bone with Iscove's Modified Dulbecco's Medium (IMDM) containing 5% FCS (Sigma-Al-drich) and 50 µMβ-mercaptoethanol (Roth, Karlsruhe, Germany). For the analysis of DC maturation and nanocarrier uptake/binding and degradation via flow cytometry the BM cells (2 × 105cells/1.25 mL) were seeded in 12 well suspension culture plates (Greiner Bio-One,Frickenhausen, Germany) with culture medium (IMDM with 5% FCS, 2 mL-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin [all from Sigma-Aldrich], and 50 µM β-mercaptoethanol), supplemented with 5% of GM-CSF containing cell culture supernatant derived from X63.Ag8–653 myeloma cells stably transfected with a murine GM-CSFexpression construct. On day 3, 500 µL of the same medium was added into each well. On day 6, 1 mL of the old medium was replaced with 1 mL fresh medium per well. For other DC based assays the BMcells (2 × 106cells/10 mL) were seeded on bacterial dishes (Ø 94 mm; Greiner Bio-One). On day 3 and 6, an additional 5 mL of culture medium was added into these dishes. Before usage, all nanoparticle solutions were checked for endotoxin contaminations by limulus amebocyte lysate (LAL) assay (Thermo Fisher Scientific) according to the manufacturer's instructions.

Confocal imaging

Uptake of PNCs containing fluorescent Cy5-Oligo (red) was monitored by Confocal Laser Scanning Microscopy (cLSM). To this end, BMDC (3 × 105cells) on day 7 of culture were seeded in chamber slides(Thermo Fisher Scientific) and treated with 150 µg/mL PNCs for 3 h at 37 °C. After that, the chamber slides were washed, and the samples were incubated with DAPI (Sigma-Aldrich) to stain the cell nuclei (blue). Unbound dye was washed off. Samples were assayed using a Zeiss LSM710 (Carl Zeiss) and analyzed using ImageJ (NIH, Bethesda, USA) and ZEN 2009 (Carl Zeiss) software.

Flow cytometry assay

To detect cell-nanocarrier-interaction and to analyze the expression of surface markers, cells were harvested and washed in staining buffer (phosphate buffer saline [PBS]/2% FCS). To block Fc receptor-mediated staining, cells were incubated with rat anti-mouse CD16/CD32 Ab (clone 2.4G2), purified from hybridoma supernatant, for 15 min at room temperature. After that, cells were incubated with eFluor450-conjugated Ab specific for MHC class II I-Ab,d,q/I-Ed,k(cloneM5/114.15.2),fluorescein isothiocyanate (FITC)-labeled Ab directed at CD80 (clone 16-10A1), phycoerythrin (PE) anti-CD86 (clone GL-1), PE-Cy7-labeled anti-CD11c (clone N418), for 30 min at 4 °C. Dead cells were stained by incubation with fixable viability dye (FVD) eFI506 for 30 min at room temperature in the dark. Samples were measured with a BD

FACSCanto IIflow cytometer equipped with BD FACSDiva software (BDBiosciences). Data were generated based on defined gating strategies and analyzed using FlowJo software (FlowJo,Ashland, USA).

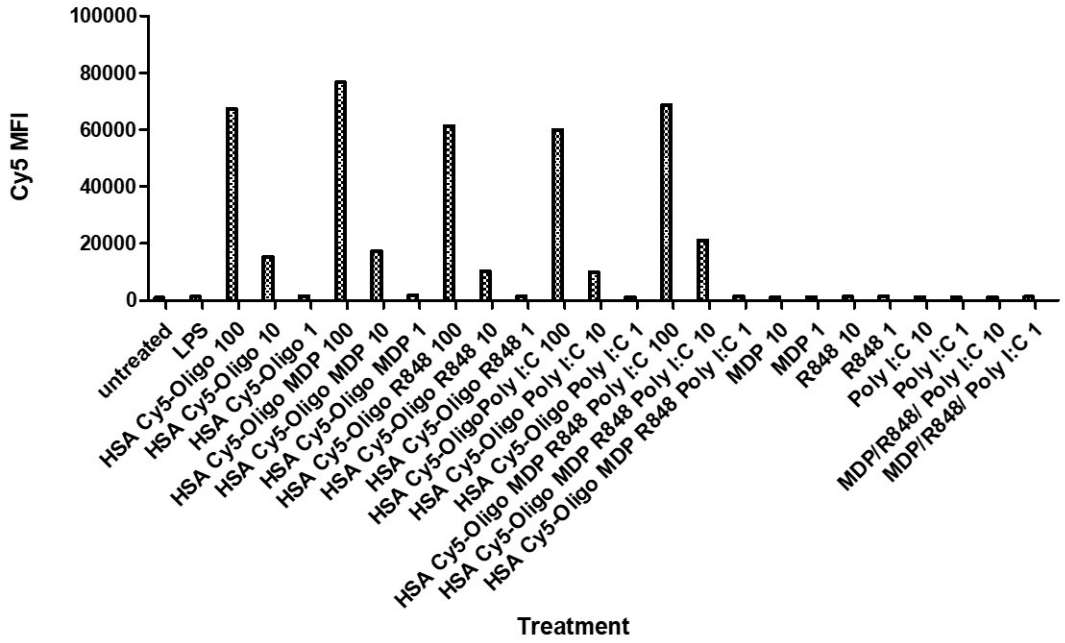


Figure S23. Cell binding/uptake into BMDC of loaded HSA-HDDP nanocarrier and free adjuvants measured by flow cytometry.

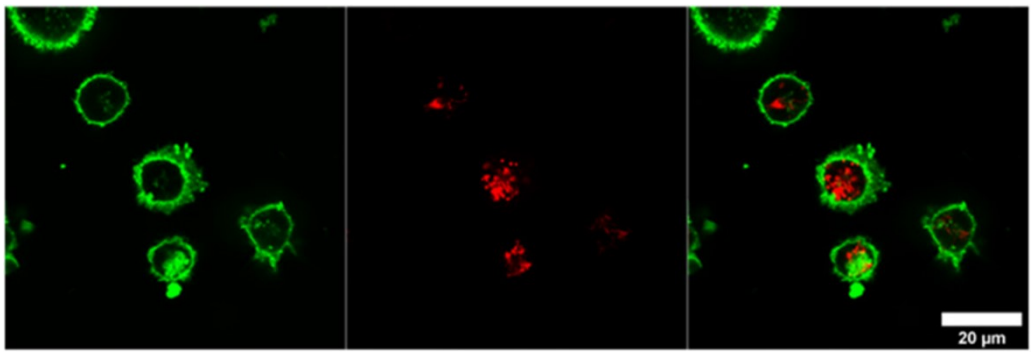


Figure S24. Confocal image of bone-marrow derived dendritic cells (green) and uptaken human serum albumin nanocarriers (red).

References

1. Goddard-Borger, E. D.; Stick, R. V., An efficient, inexpensive, and shelf-stable diazotransfer reagent: imidazole-1-sulfonyl azide hydrochloride. *Organic letters* **2007,** *9* (19), 3797-3800.