Electronic Supplementary Information (ESI)

Biophysical investigation of liposome systems decorated with bioconjugated copolymers in the presence of amantadine

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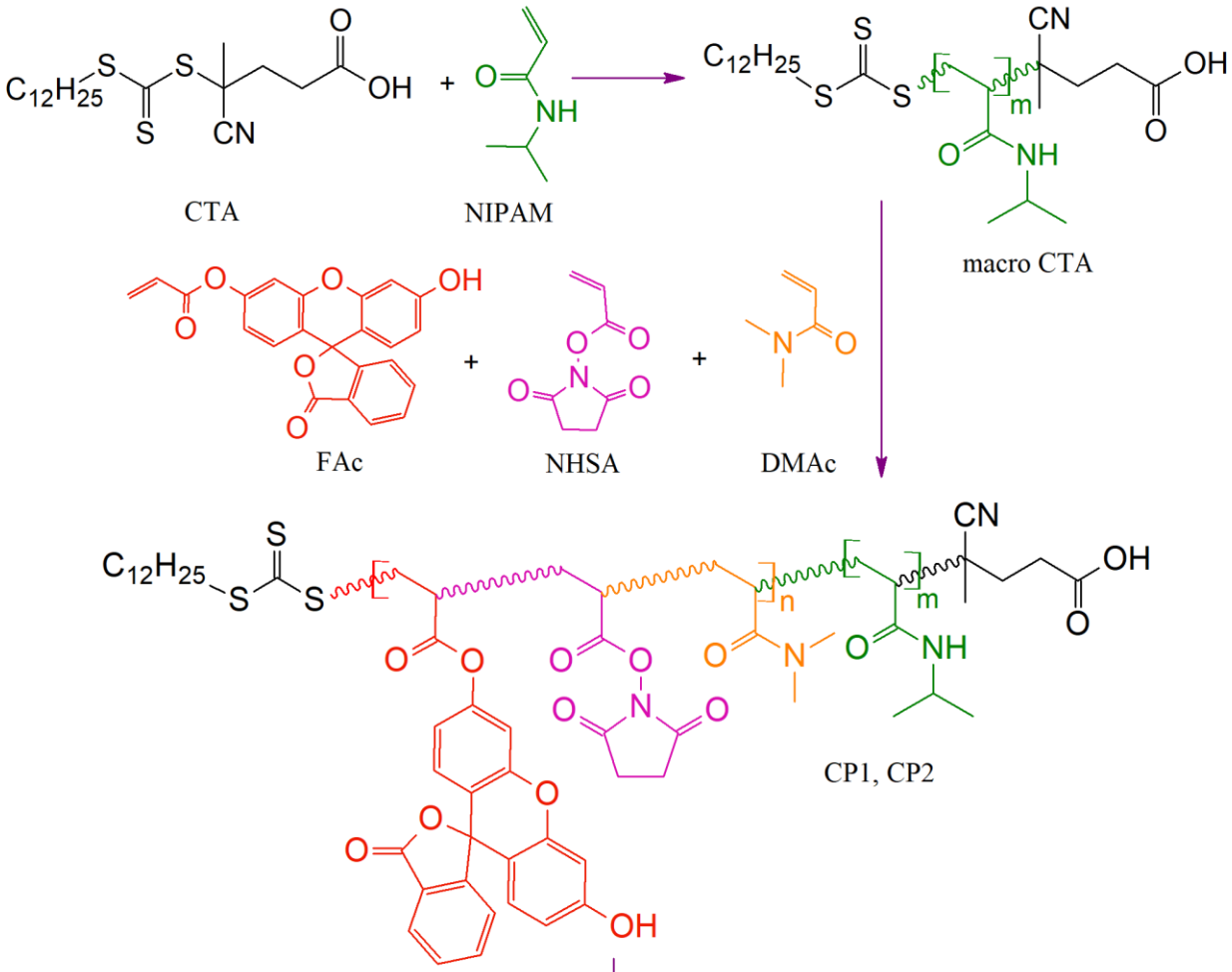
1. Summarized synthetic procedure for the preparation of peptide-copolymer conjugates via RAFT polymerization and a grafting through reaction

A series of copolymers of different molar mass and copolymer composition were synthesized via the reversible addition-fragmentation chain transfer (RAFT) polymerization technique. First, a macro chain transfer agent (macro-CTA) based on poly(*N*-isopropylacrylamide) (PNIPAM) was prepared using 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid as a RAFT agent; this polymer possesses thermo-responsive properties in aqueous media. Next, a chain extension reaction for the PNIPAM macro-CTA was carried out using a mixture of comonomers, which included a hydrophilic comonomer (i.e., dimethylacrylamide (DMA)), a photo-responsive comonomer (i.e., fluorescein acrylate (FAc)) and a reactive comonomer (i.e., acrylic acid-N-succinimide ester (NHSA)) for usage as grafting through sites for peptide conjugation in a subsequent reaction step [1]. Hence, the copolymers were conjugated with the L130 (Ala-Cys-Pro-His) and L220 (Arg-Asp-Gln-Glu-Gly) peptides via a transesterification reaction with the NHSA functional sites (Scheme 1); L130 and L220 mimic the amino acid sequences of the 130 and 220 loops at the receptor binding site of the hemagglutinin protein of influenza A virus [2].


CTA
NIPAM
macro CTA
FAc
NHSA
DMAc
CP1, CP2
L130
L220
PDMA-b-PNIPAM-L130 (l)
PDMA-b-PNIPAM-L130 (h)
PDMA-b-PNIPAM-L220 (l)
PDMA-b-PNIPAM-L220 (h)

Scheme 1. Schematic representation of the synthesis route of the investigated peptides-copolymer conjugates. Step 1: macro-CTA (PNIPAM). Step 2: copolymers CP1 and CP2 (PNIPAM-*b*-P(DMA-*co*-FAc-*co*-NHSA)). Step 3: conjugation of CP1 and CP2 with the L130 and L220 pepetides (PDMA-*b*-PNIPAM-L130 (*l*); PDMA-*b*-PNIPAM-L220 (*l*); PDMA-*b*-PNIPAM-L130 (*h*); PDMA-*b*-PNIPAM-L220 (*h*)).

2. Materials

Chain transfer agent (CTA) 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (Boron Molecular), radical initiator 4,4-azobis(4-cyanovaleric acid) (ACVA) (Sigma-Aldrich), trioxane (used as NMR internal reference) (Sigma-Aldrich), monomers fluorescein acrylate (FAc) (Sigma-Aldrich) and acrylic acid-N-succinimide ester (NHSA) (Sigma-Aldrich), 3-amino-1-propanol (AmP) (Sigma-Aldrich) and triethylamine (TEA) (Sigma-Aldrich) were used as received. Radical initiator azobis-isobutyronitrile (AIBN) (Sigma-Aldrich) was purified via crystallization in methanol. Dried dioxane, dried dimethyl formamide (DMF), methanol, diethyl ether, deuterated chloroform ($CDCl_3$) and deuterated methanol (MeOD) solvents (all from Sigma-Aldrich) were used as received. Monomers *N*-isopropylacrylamide) (NIPAM) and dimethylacrylamide (DMA) (Sigma-Aldrich) were stirred under the presence of inhibitor remover beads (Sigma-Aldrich) for 30 min to eliminate inhibitors / stabilizing agents (i.e., hydroquinone or 4-tert-butylcatechol).

3. Characterization techniques

Chemical structures of peptides-copolymer conjugates and precursor compounds were corroborated via proton nuclear magnetic resonance (^{1}H NMR) using a Bruker Avance 300 MHz spectrometer. Analyses were performed at room temperature and chemical shifts (δ) are reported in ppm. Molar mass and dispersity values of the macro-CTA, block copolymers and conjugates were estimated via ^{1}H NMR and size-exclusion chromatography (SEC) using an Agilent 1200 series set-up equipped with a G1310A pump, a RID-G1362A refractive index detector and a PSS GRAM guard/30/1000Å (10 μm particle size) column system; dimethyl acetamide containing 0.21 wt. % of LiCl was used as an eluent at a flow rate of 1 mL min^{-1} at 40 °C. The molar mass values were estimated against a calibration curve built with poly(methyl methacrylate) standards of narrow dispersity in the range from 400 to100,000 g mol^{-1}.

4. Synthesis of block copolymers and peptide-copolymer conjugates

4.1 Poly(N-isopropylacrylamide) (PNIPAM) (macro-CTA)

For the synthesis of the PNIPAM macro-CTA, 5g (44.2mmol) of NIPAM monomer, 200 mg (0.5 mmol) of CTA, 8.2 mg (0.05 mmol) of AIBN radical initiator, 135 mg (1.5 mmol) of trioxane (NMR internal reference) and 30 mL of dried dioxane were added into a 100 mL round-bottom glass flask. This reaction mixture was degassed via sparging argon gas for 15 min. Thereafter, the reaction system was magnetically stirred at 500

rpm in a pre-heated oil bath at 70°C for 3h. The reaction time was predetermined to reach a monomer conversion value of ca. 70% as monitored via ^{1}H NMR analysis; this value is recommended to keep suitable living characteristics of macro-CTA [3]. The purification process of the obtained polymer consisted in concentrating the reaction mixture with the aid of a rotary evaporator followed by precipitation in cold diethyl ether, the obtained precipitate was filtered and dried under vacuum at 40°C. The chemical structure of the PNIPAM macro-CTA was corroborated via ^{1}H NMR analysis (Fig. S1).

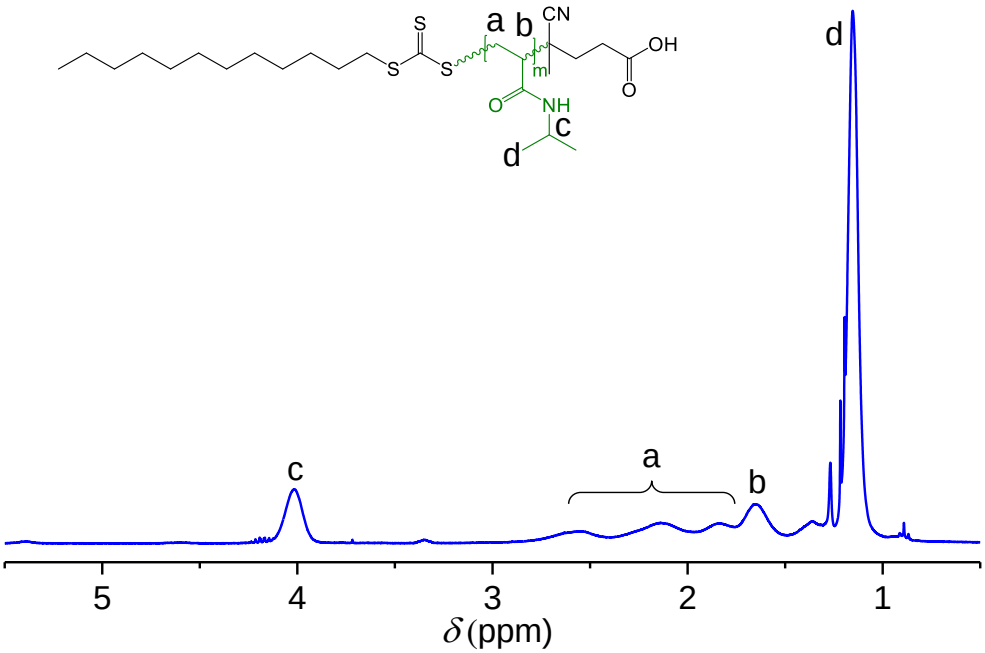


Fig. S1: ^{1}H NMR ($CDCl_3$) spectrum of the PNIPAM macro-CTA.

4.2 Chain extension reactions of PNIPAM macro-CTA (synthesis of block copolymers CP1 and CP2)

For chain extension reactions of PNIPAM macro-CTA (i.e., synthesis of block copolymers CP1 and CP2), PNIPAM macro-CTA, a mixture of monomers (DMA/NHSA/FAc) of predetermined molar ratio (see Table S1), ACVA radical initiator and a mixture of dioxane:DMF (50:50) were added into a 100 mL round-bottom glass flask. The reaction mixtures were degassed by sparging argon gas for 15 min. Thereafter, the reaction systems were magnetically stirred at 500 rpm in a pre-heated oil bath at 70°C for 5h. The purification process of the obtained block copolymers consisted in concentrating the corresponding reaction mixtures with the aid of a rotary evaporator and the resulting slurries were subjected to a dialysis process against methanol using 6-8 kDa mesh dialysis membranes to remove unreacted monomers and residual reaction solvents; the dialysis solvent was exchanged every 24 h (3 exchanges). Finally, the products were subjected to rotary evaporation to eliminate methanol and dried under vacuum at 40 °C. The chemical structures of block copolymers CP1 and CP2 were corroborated via ^{1}H NMR analyses. For instance, Fig. S2 displays the ^{1}H NMR spectrum of CP1 where the characteristic signals of each functional group can be observed.

Table S1. Formulation and nomenclature assigned to copolymers.

copolymer		macro-CTA	block DMA			initiator	$^{a}M_n$ (kDa)
		NIPAM	DMA	NHSA	FAc	ACVA	
CP1	ratio	1	88	10	2	0.1	^{b}17.13
	mmol	0.156	13.7	15.6	0.3	0.156	
CP2	ratio	1	176	20	4	0.1	^{c}28.65
	mmol	0.156	27.4	31.2	0.6	0.156	

[a] Molar mass values were determined via ^{1}H NMR analysis. [b]Considering a global monomer conversion of 89 % as a reference. [c]Considering a global monomer conversion of 93 % as a reference.

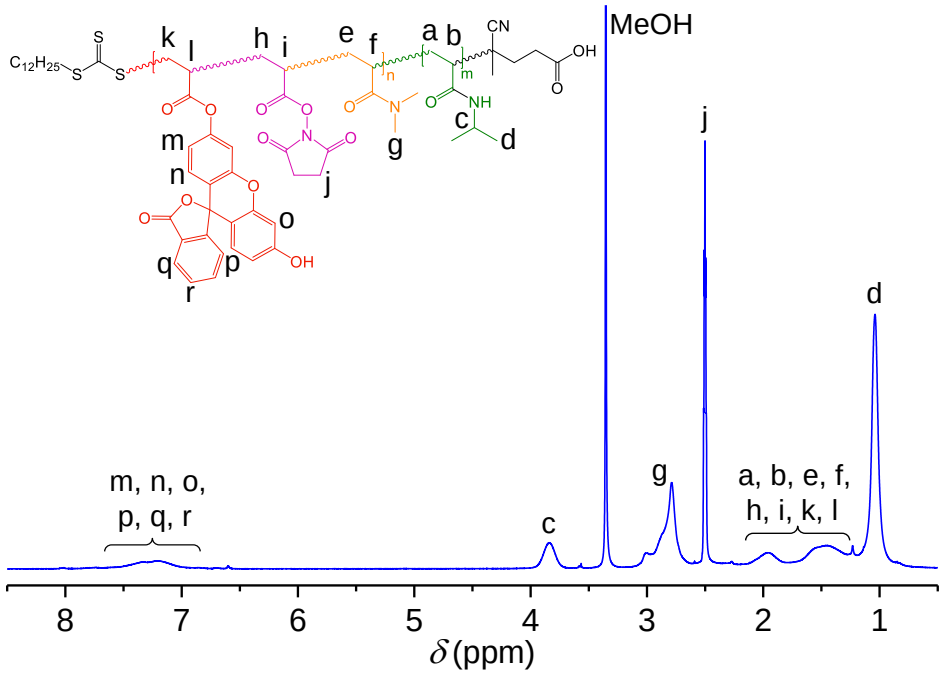


Fig. S2: ^{1}H NMR (MeOD) spectrum of block copolymer CP1.

4.3 Conjugation of block copolymers CP1 and CP2 with peptides via a grafting through reaction (PDMA-b-PNIPAM-L130 (l); PDMA-b-PNIPAM-L220 (l); PDMA-b-PNIPAM-L130 (h); PDMA-b-PNIPAM-L220 (h)). For the conjugation reactions, the corresponding amounts of CP1 or CP2, peptides L130 or L220, and TEA (Table S2), and 7 mL of DMF were added into a 50 mL round-bottom glass flask. These reaction mixtures were degassed by sparging argon gas for 15 min. Next, the reaction systems were magnetically stirred at 500 rpm in a pre-heated oil bath at 50°C for 6h. Thereafter, a predetermined amount of AmP (Table S2) diluted in 2 mL of DMF was added into the reaction systems maintaining the previous reaction parameters for 18 h.

The purification process of the obtained copolymer conjugates consisted in subjecting the obtained reaction mixtures to a dialysis process against a solvent mixture of methanol:distilled water (80:20) using 10-12 kDa mesh dialysis membranes to remove unreacted compounds and residual reaction solvent; the dialysis solvent was exchanged every 24 h (3 exchanges). Finally, the products were subjected to rotary evaporation to eliminate solvents and dried under vacuum at 40 °C.

Table S2. Nomenclature of the prepared copolymer conjugates and utilized amounts of reagents for the corresponding conjugation reactions.

conjugated nanostructure		copolymer		peptide		Amp	TEA
		CP1	CP2	L130	L220		
PDMA-*b*-PNIPAM-L130 (*l*)	ratio	[a]1	-	0.5	-	1	2
	mmol	[b]0.167	-	0.084	-	0.167	0.334
PDMA-*b*-PNIPAM-L220 (*l*)	ratio	1	-	-	0.5	0.5	2
	mmol	0.167	-	-	0.084	0.167	0.334
PDMA-*b*-PNIPAM-L130 (*h*)	ratio	-	1	0.25	-	1.25	2
	mmol	-	[c]0.334	0.084	-	0.418	0.668
PDMA-*b*-PNIPAM-L220 (*h*)	ratio	-	1	-	0.25	1.25	2
	mmol	-	0.334	-	0.084	0.418	0.668

[a] *Refereed to NHSA groups;* [b] [c]*mmol corresponding to the NHS units in CP1 or CP2*

Fig. S3 summarizes the evolution of the M_n values throughout the different synthetic steps of the prepared copolymer conjugates (from the precursor macro-CTA to the copolymer conjugates). Furthermore, Fig. S4 displays comparisons of the recorded SEC chromatograms of the synthesized copolymer conjugates with their corresponding precursor materials where a shift of the SEC traces to shorter elution times can be observed as an indication of a molar mass increase throughout the different synthetic steps of the copolymer conjugates. Note that the nomenclature assigned to the copolymer conjugates has been simplified to the acronym PDMA for describing of the entire hydrophilic block (i.e., P(DMA-*co*-FAc-*co*-NHSA) as the DMA units represent the highest comonomer composition (88%) of this segment.

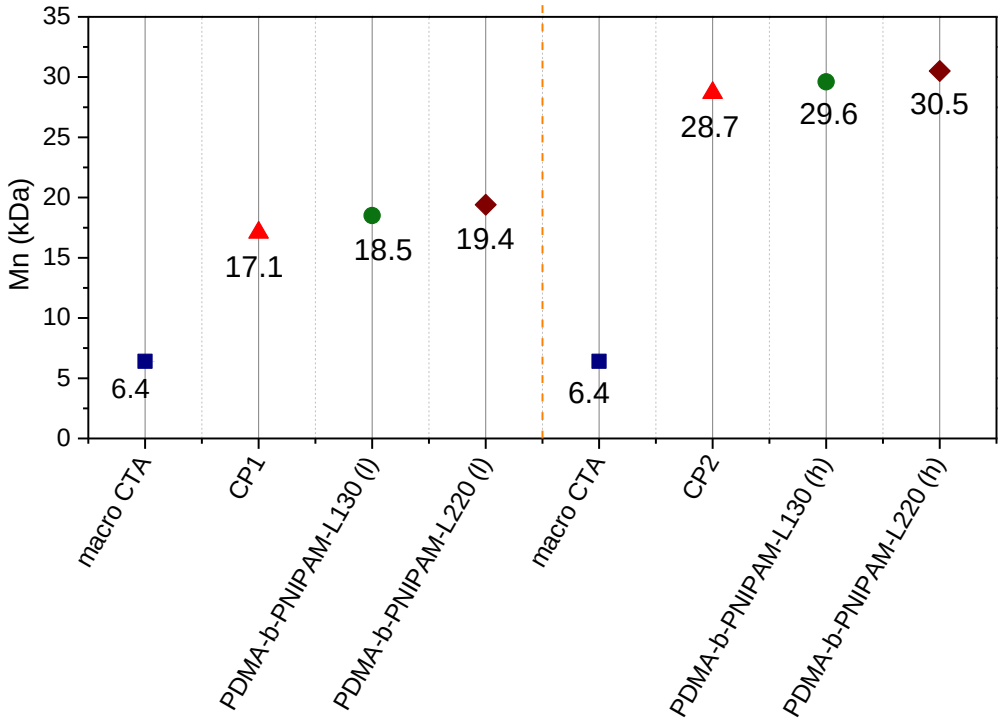


Fig. S3: Average number molar mass (M_n) values of the synthesized materials.

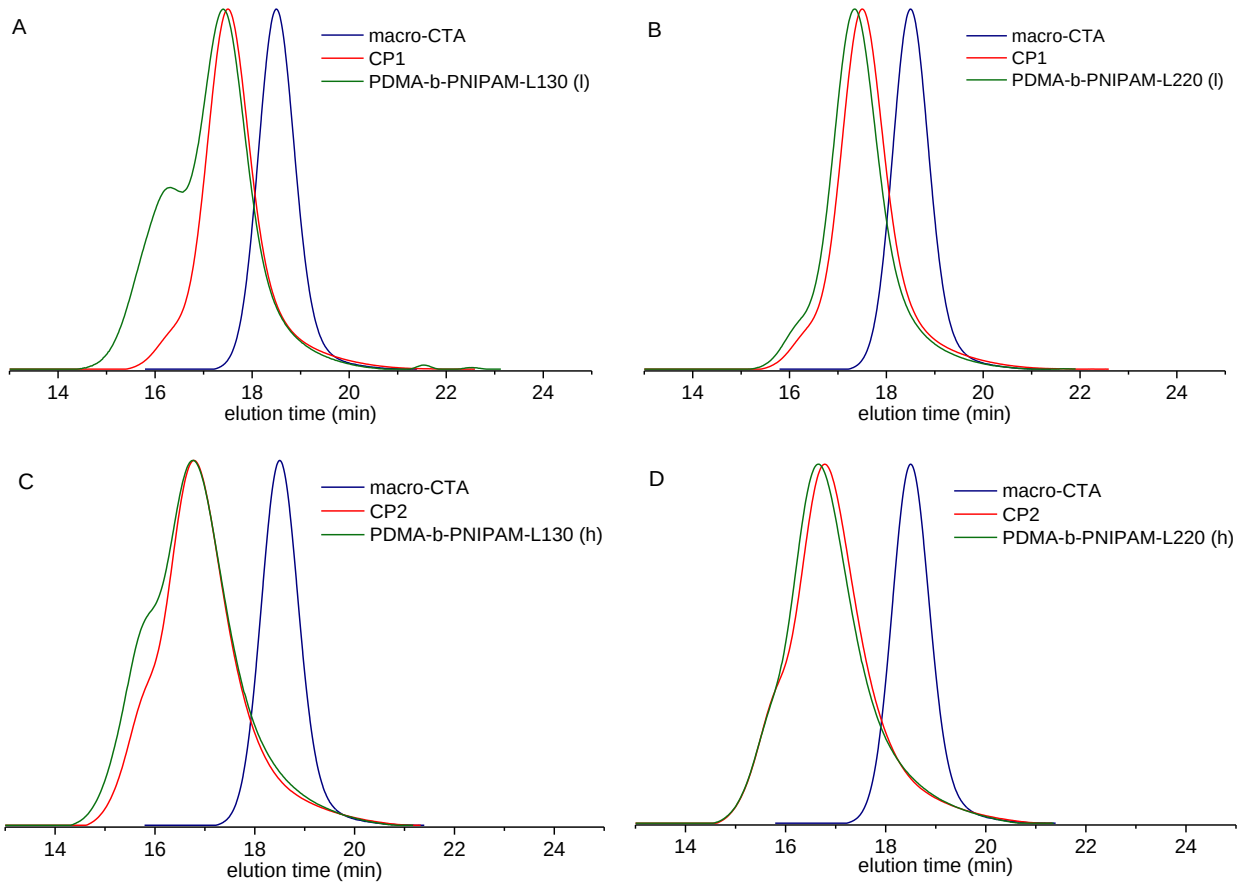


Fig. S4: SEC chromatograms utilized for the determination of the molar mass of the synthesized materials.

5. Lipids to prepare large unilamellar vesicles (LUVs). The MSC system.

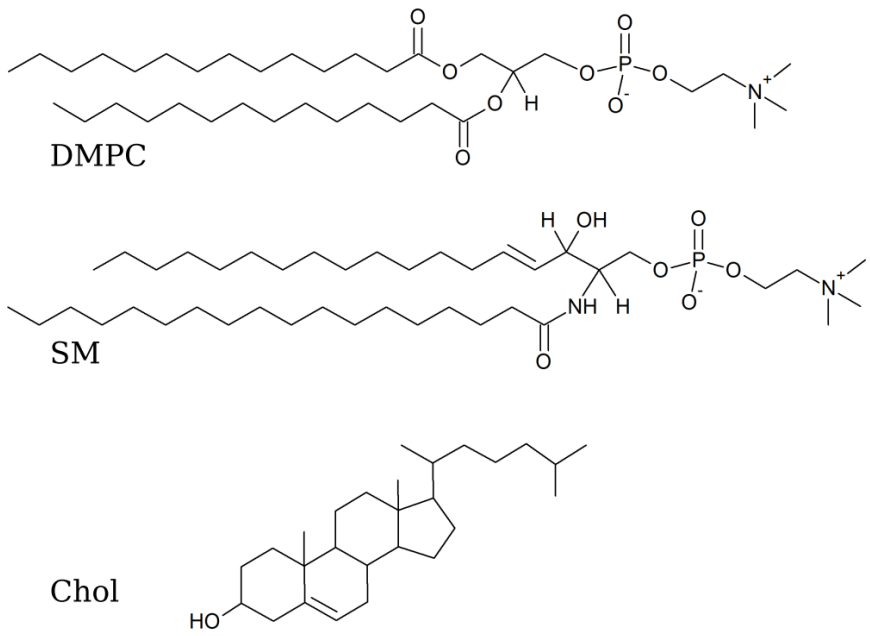


Fig. S5: Schematic representations of the chemical structures of DMPC, SM, and Chol. Such lipids were used to assemble the MSC system.

6. Cryogenic transmission electron microscopy (cryoTEM)

Copolymer bioconjugates / liposomes were dispersed in 1 mL of a buffer solution of HEPES/NaCl 10 mM/145 mM) at a pH value of 7.4; the utilized concentrations are summarized in Table S3. These aqueous dispersions were utilized for CryoTEM investigations.

Table S3. Summary of the utilized concentrations of copolymer bioconjugates / liposomes in aqueous dispersions subjected to CryoTEM analysis.

Description	**Concentration**
MSC	0.3 mM (MSC)
MSC / AMT	0.3 mM / 2.0 mM
MSC / PDMA-*b*-PNIPAM-L220 (*l*)	0.3 mM / 0.03 mM
MSC / PDMA-*b*-PNIPAM-L130 (*l*)	0.3 mM / 0.03 mM
MSC / AMT / PDMA-*b*-PNIPAM-L220 (*l*)	0.3 mM / 2.0 mM / 0.03 mM
MSC / AMT / PDMA-*b*-PNIPAM-L130 (*l*)	0.3 mM / 2.0 mM / 0.03 mM
MSC / AMT / PDMA-*b*-PNIPAM-L220 (*h*)	0.3 mM / 2.0 mM / 0.03 mM
MSC / AMT / PDMA-*b*-PNIPAM-L130 (*h*)	0.3 mM / 2.0 mM / 0.03 mM
MSC / PDMA-*b*-PNIPAM-L220 (*h*)	0.3 mM / 0.03 mM
MSC / PDMA-*b*-PNIPAM-L130 (*h*)	0.3 mM / 0.03 mM

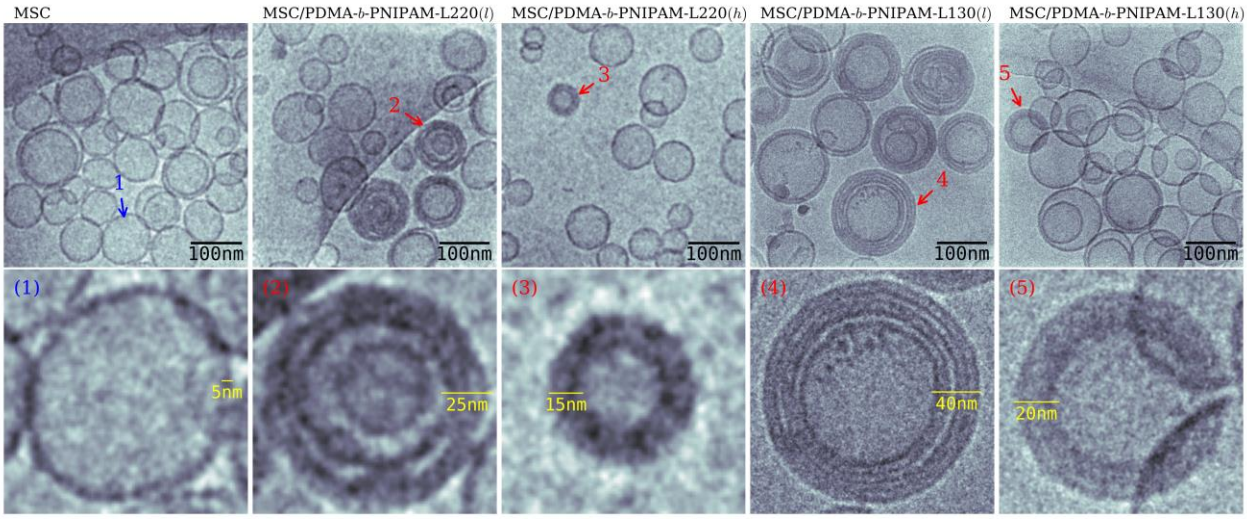


Fig. S6: Representative cryoTEM micrographs for MSC liposomes decorated with conjugated copolymers.

References

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