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SUPPORTING INFORMATION

Multifunctionalized Polyurethane-Polyurea Nanoparticles: Hydrophobically Driven Self-Stratification at the o/w Interface Modulates Encapsulation Stability

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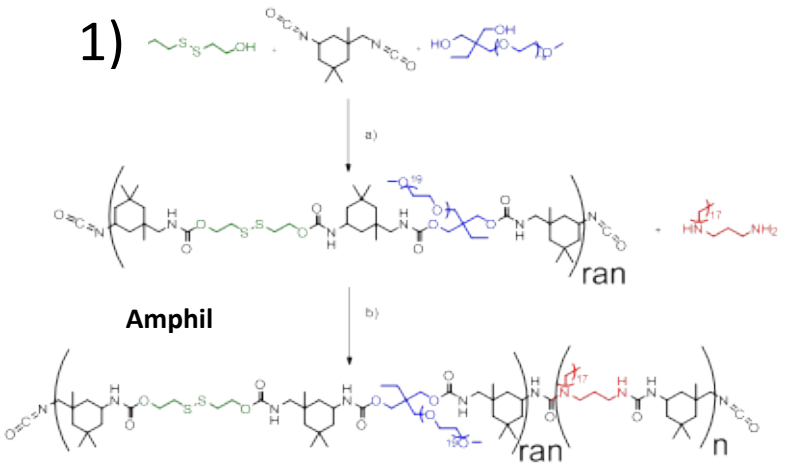
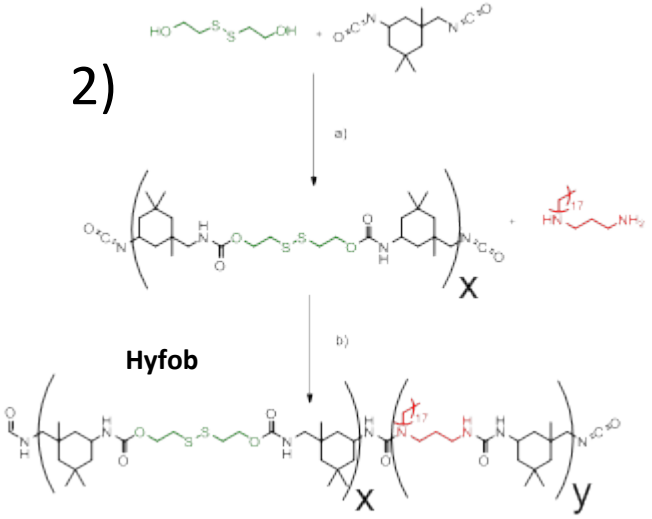
1. Monomers information

Ymer N120 is a polymeric non-ionic hydrophilic building block containing two primary hydroxyl groups and a long ethoxylated capped side chain. From a synthetic point of view, YMER N-120, was chosen because of its low melting point and viscosity, features that make it amenable for direct addition to the prepolymer acting as co-solubilizer in the formulation and eliminating the need of an extra co-solvent. We considered YMER N-120 an interesting intermediate to create a polymer backbone with longer side-chains sideways than equivalent PEG (polyethylene glycol, 1000 g/mol) which should fold up within itself upon contact with water.
Isophorone diisocyanate (IPDI) is an organic compound in the class known as aliphatic diisocyanates. Interestingly, aliphatic isocyanates are less pulmonary sensitizing and not carcinogenic compared to aromatic ones. Is particularly noteworthy that fully reacted isocyanate polymers to form its respective urethane or urea bonds do not contain toxicity referred to the isocyanate group[1]. In addition, degradation products of IPDI such IPDA (isophorone diamine) are rapidly eliminated via urinary excretion[2].
Genamin TAP 100D is a hydrophobic building block containing a C18 fatty chain and two reactive amines for polymerization. We focused on it attracted by its high hydrophobicity and low melting point which allowed us to perform a more green chemistry avoiding large amounts of solvents.
Bayhydur 3100 is a hydrophilic aliphatic polyisocyanate based on hexamethylene diisocyanate (HDI) and is solvent-free. It was interesting for our purposes due to its high hydrophilicity and high NCO functionality which allowed as to work in aqueous media and decorate it easily and fast with a cyclic RGD peptide via urea linkage. More interestingly was that adjusting the stoichiometry we got conjugates of just one peptide per B3100 molecule maintaining NCO reactivity for further shell functionalization. In addition it complies with FDA food contact regulations.

*Please refer to figure S1 in the supporting information to see their chemical structure in detail.



a) DBTL (cat), Crodamol GTCC, N_2, 50ºC, 4 h*. b) Acetone, N2, 40ºC, 30 min



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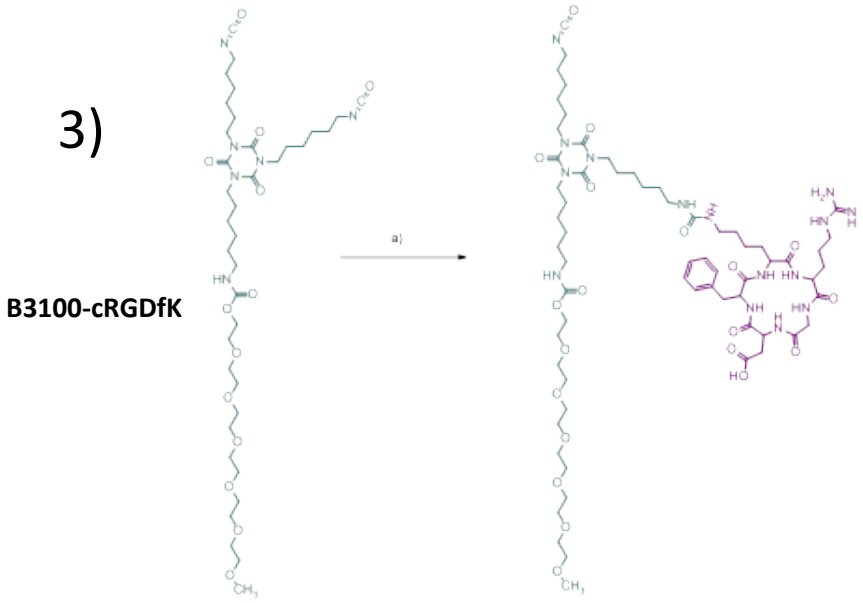
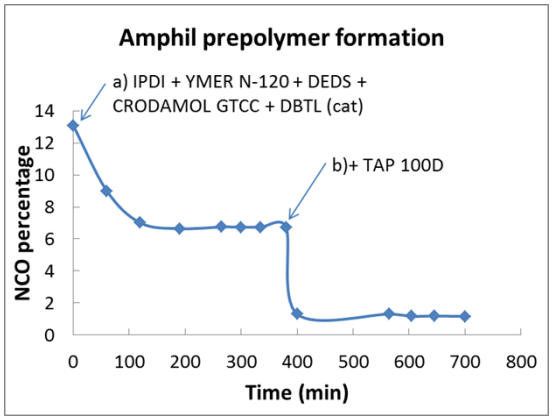


a) PBS, Et_3N (cat), 5ºC, 2 h.

Figure S1. Synthetic process of the idealized structures of reactive prepolymers that form the PUUa NP. 1) Amphil polymerization process. *Hyfil was formed by random polymerization of DEDS and YMER N-120 with IPDI. 2) Hyfob polymerization process. 3) B3100-cRGDfK conjugation reaction.

	IPDI	DEDS	YMER N-120	GENAMIN TAP 100D	L-LYSINE	DETA	B3100	cRGDfK
w/w % Amphil[a]	24.29	1.08	39.50	10.41	-	-	-	-
w/w % Hyfob[a]	3.48	1.08	-	1.08	-	-	-	-
w/w % B3100-cRGDfK[a]	-	-	-	-	-	-	6.49	1.95
w/w % PUUa NP[b]	27.77	2.15	39.5	11.49	7.2	3.39	6.49	1.95
Equivalents fraction[c]	9.29	1.04	2.93	2.65	1.83	1.83	1	0.12

Table S1. The quantitative reaction of the monomers was ensured by automatic titration and HPLC (Fig. S2 and Fig. S9 respectively) and qualitatively by FT-IR (Fig. S3 and S4). a) Relative mass proportion of each monomer in the prepolymer regarding the multiwalled PUUa NP. b) Total mass proportion of each monomer in the multiwalled PUUa NP. c) Equivalents fraction of reactive species (either NCO, OH or NH2)



$NCO\%\ Hyfil = 6.7\ \%\quad NCO\%\ Amphil\ = 1.2\ \%$

Figure S2. Back titration of free isocyanate. Disappearance of NCO by a first random reaction with –OH groups to form urethane prepolymer (a), Hyfil) and a second stage with –NH2 groups to form urethane-urea prepolymer (b), Amphil). As expected, urethane bonds were formed progressively (almost 3h). Urea bonds were formed immediately after addition of Genamin TAP 100D hydrophobic diamine. When every monomer is totally reacted NCO percentage remains constant over time, which proves its scalability. As desired, remaining isocyanate functionality for further crosslinking is maintained after total consumption of Genamin TAP 100D free amino groups.

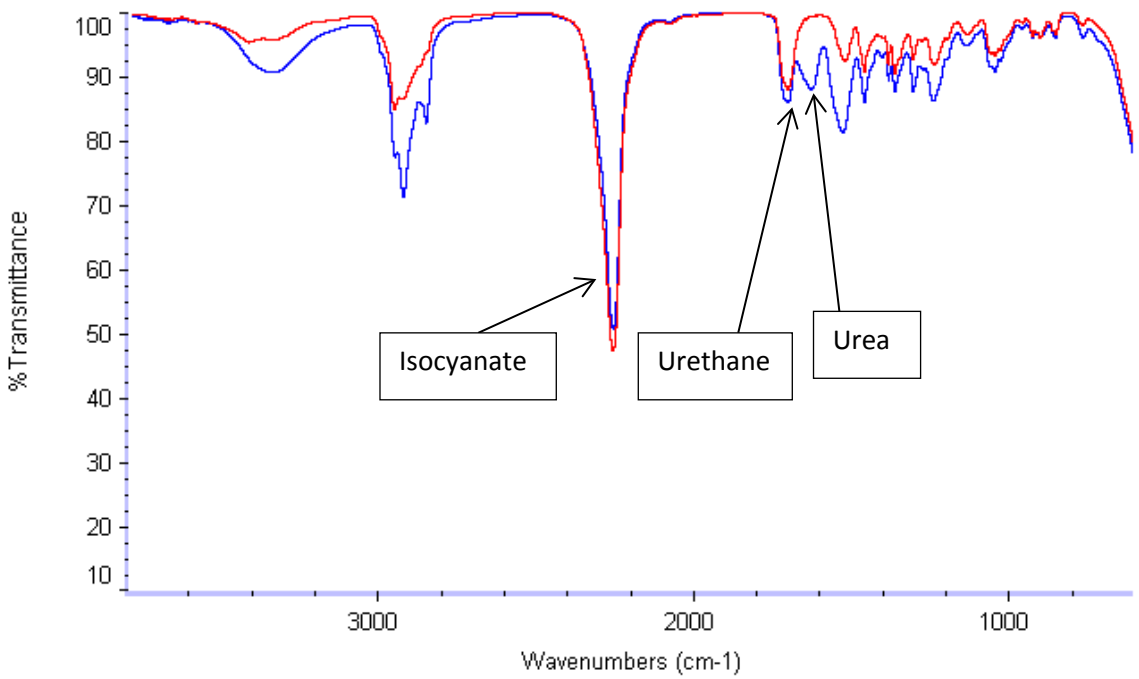


Figure S3. IR spectra corresponding to the synthetic process of Hyfob reactive prepolymer. It can be observed how isocyanate band of polyurethane prepolymer (red) decreases when Genamin TAP 100D is added and polyurea bonds (Hyfob) are formed (Blue)

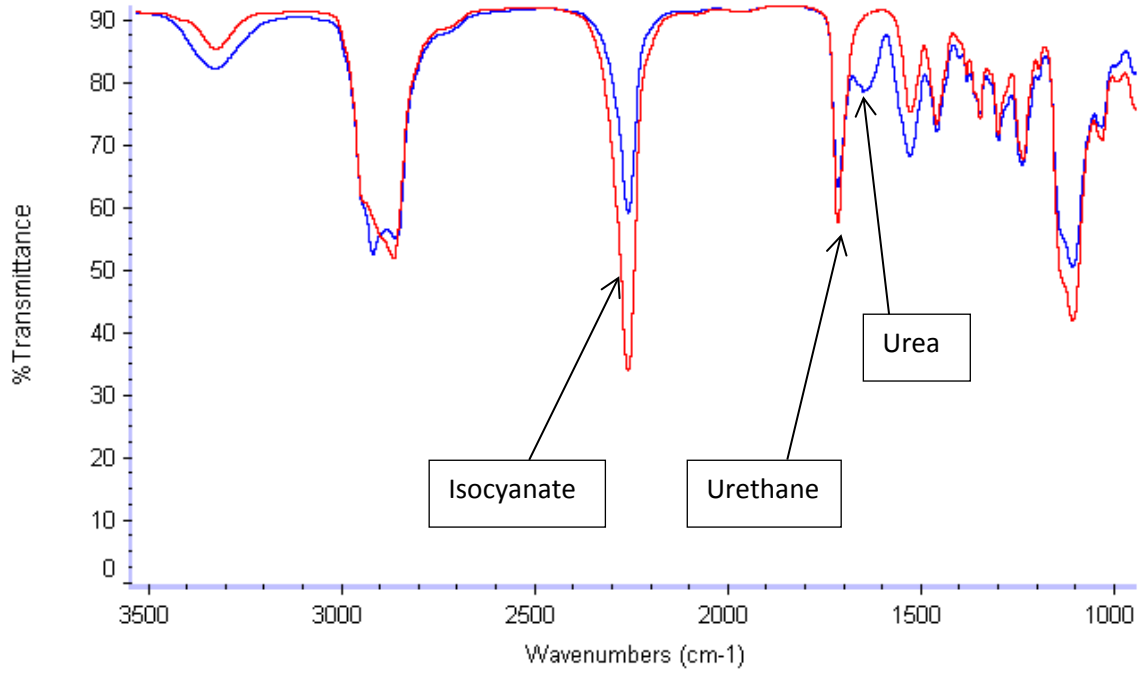


Figure S4. IR spectra corresponding to the synthetic process of Amphil reactive prepolymer. It can be observed how isocyanate band of polyurethane prepolymer (red) decreases when Genamin TAP 100D is added and polyurea bonds (Amphil) are formed (Blue).

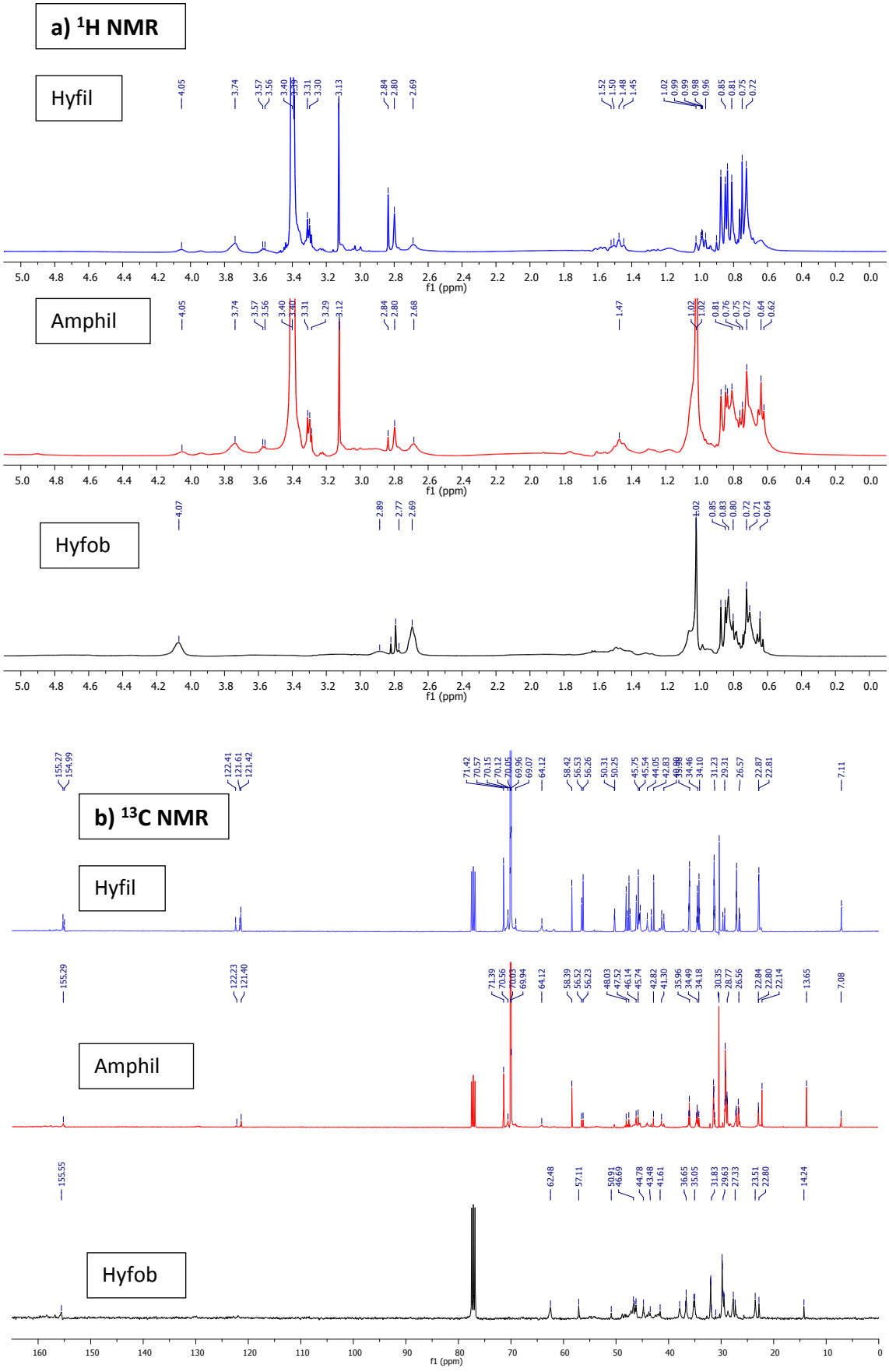
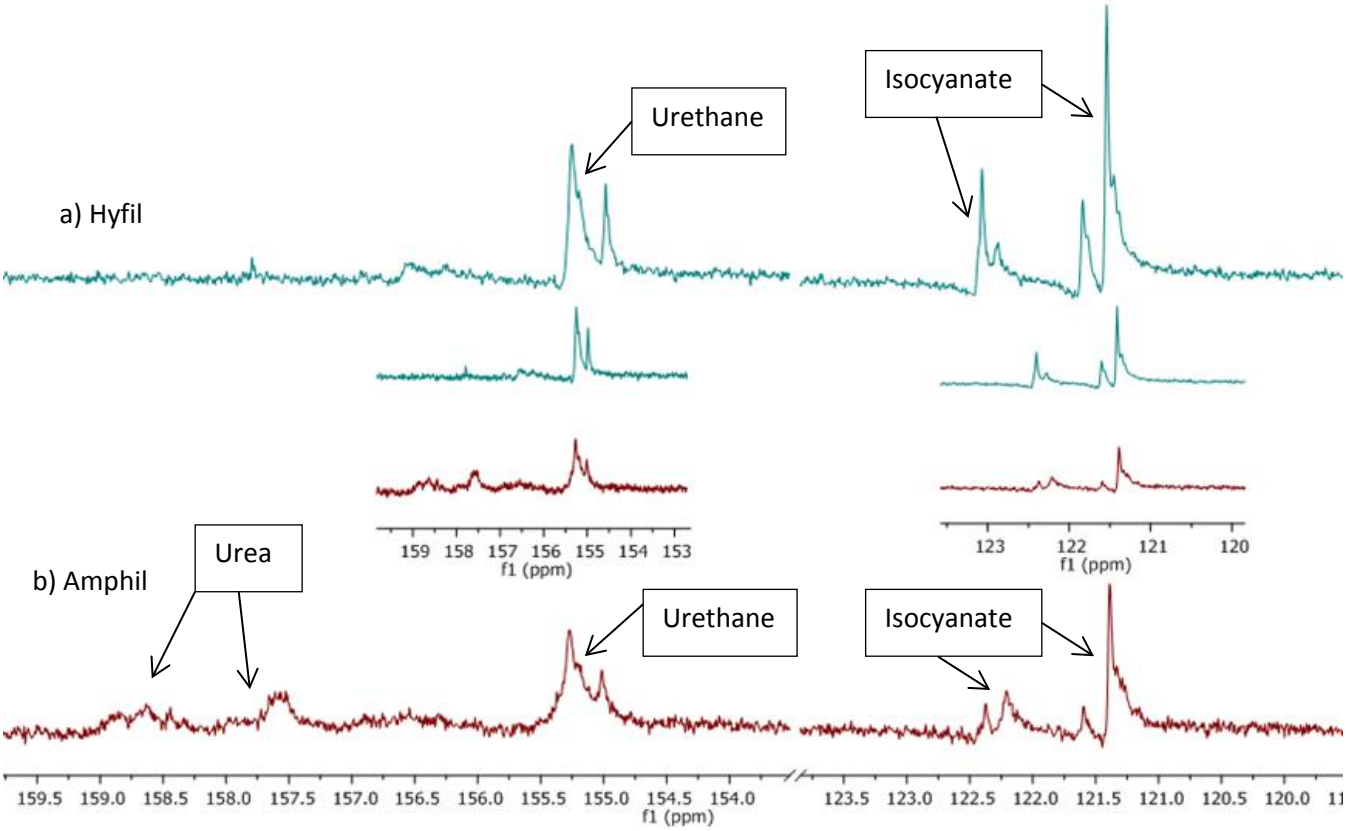


Figure S5. 400 MHz ^{1}H NMR spectra a) and 400 MHz ^{13}C NMR spectra b) of reactive prepolymers in $CDCl_3$. The blue and red spectra correspond to Hyfil and Amphil prepolymers respectively. All the peaks in the reactive prepolymers spectra have

been assigned by a previous singular ^{13}C and ^{1}H NMR spectra of each monomer (spectra not shown) and previosuly reported bibliography[3]. As seen from Amphil 1H NMR spectra, the peaks at 0.72, 0.85, 1.47, 1.61 and 2.80 ppm are assigned to IPDI methylene groups. The peak at 0.64, which can not be found in the Hyfil spectra, corresponds to the fatty diamine methyl group. The methylene groups of the fatty diamine appear at 1.02, 1.47 and 1.76. The chemical shifts at 0.72 and 3.30 are ascribed to YMER methyl groups and the peaks at 3.12 and 3.40 correspond to YMER methylene groups. The peak at 2.68 is ascribed to DEDS (CH_3-S-) and those at 3.74 and 4.07 to Amphil and Hyfob (CH_3-O-) respectively.

In the ^{13}C NMR spectra the peaks at 22.80, 42.81, 43.27, 45.40, 45.75, 47.53, 48.03, 56.24 and 56.52 are ascribed to methylene groups of IPDI stereoisomers mixture. The methyl groups of IPDI mixture correspond to the signals at 26.53, 27.08 and 29.21. The IPDI methine group was ascribed to the small signals at 36.05, 34.51 and 31.30. It can be observed some IPDI peaks appearing as doblets due to stereoisomers and rotamers. The peaks at 7.10, 58.39 and 22.84, 70.03, 70.56, 71.39 are assigned to YMER N-120 methyl and methylene groups respectively. The peak appearing only in the Amphil spectra at 13.65



correspond to methyl groups of Genamin TAP 100D and its methylene groups are ascribed to the singlet at 22.14 and the fatty chain multiplet at 29 ppm. The DEDS methylene groups are ascribed to the broad signals at 40.86 (CH_3-S-) and 64.06 (CH_3-0-).

Figure S6. The cut-out is an amplification of 400 MHz ^{13}C NMR spectra to identify isocyanate, polyurethane and polyurea carbons during prepolymers formation. a) The signals between 121 and 123 ppm are ascribed to isocyanate carbons of the prepolymer. Polyurethane carbon signal is ascribed to the bands at 155 ppm. b) In the Amphil, when Genamin TAP 100D diamine is added multiple bands between 157 and 159 appear due to urea bonds formation.

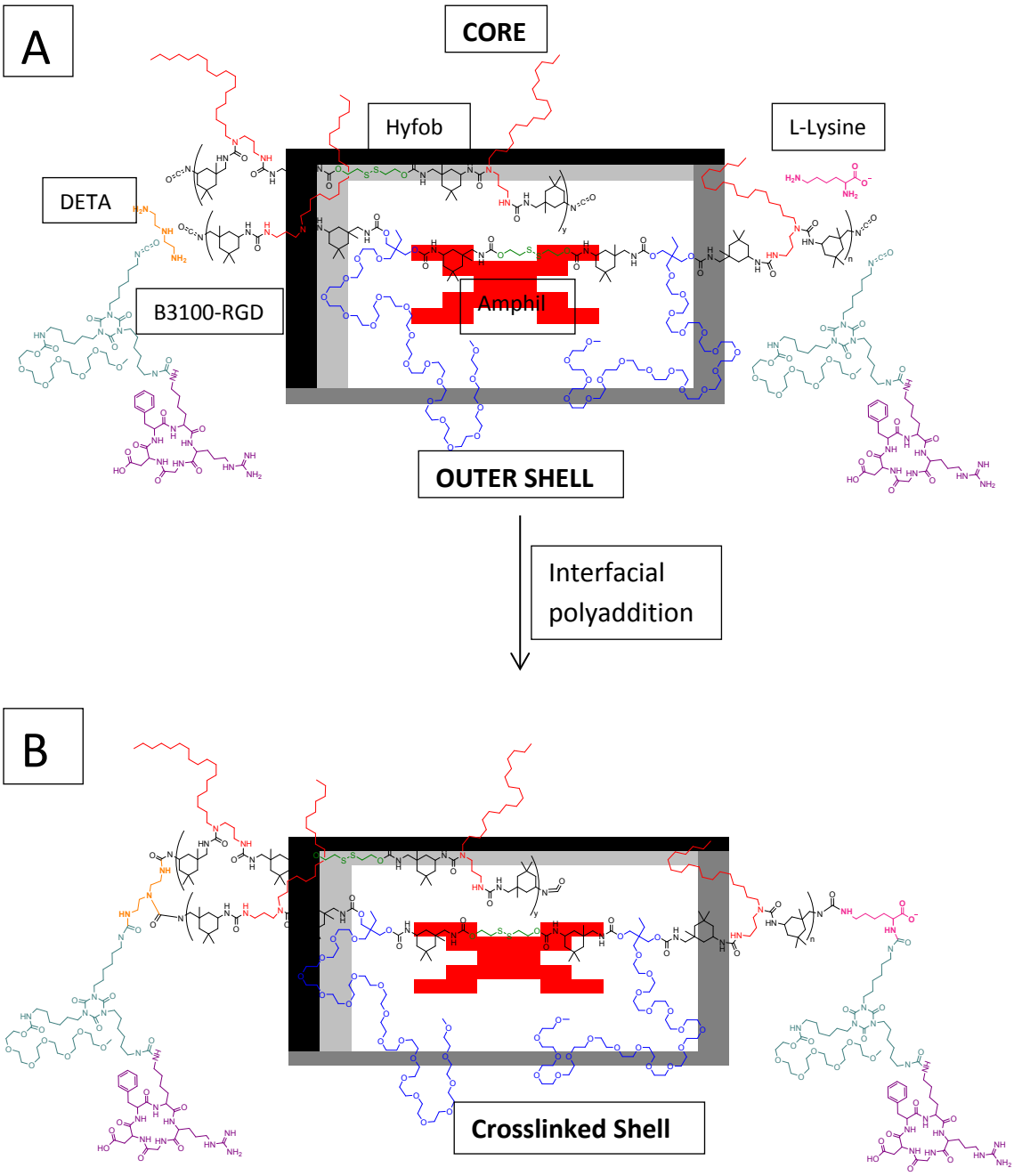


Figure S7. Crosslinking process of the nanoparticle. Amphil, Hyfob and B3100-cRGDfK self-stratify after emulsification with water A). After addition of crosslinkers, interfacial polyaddition with the reactive prepolymers occurs to form the crosslinked shell. Presumably, due to L-Lysine higher hydrophilia, it will join the most hydrophilic prepolymers (Amphil and B3100-cRGDfK). DETA, which is more reactive and less hydrophilic, will penetrate the inner shell and join Hyfob, Amphil and B3100-cRGDfK creating the PUUa NP B).

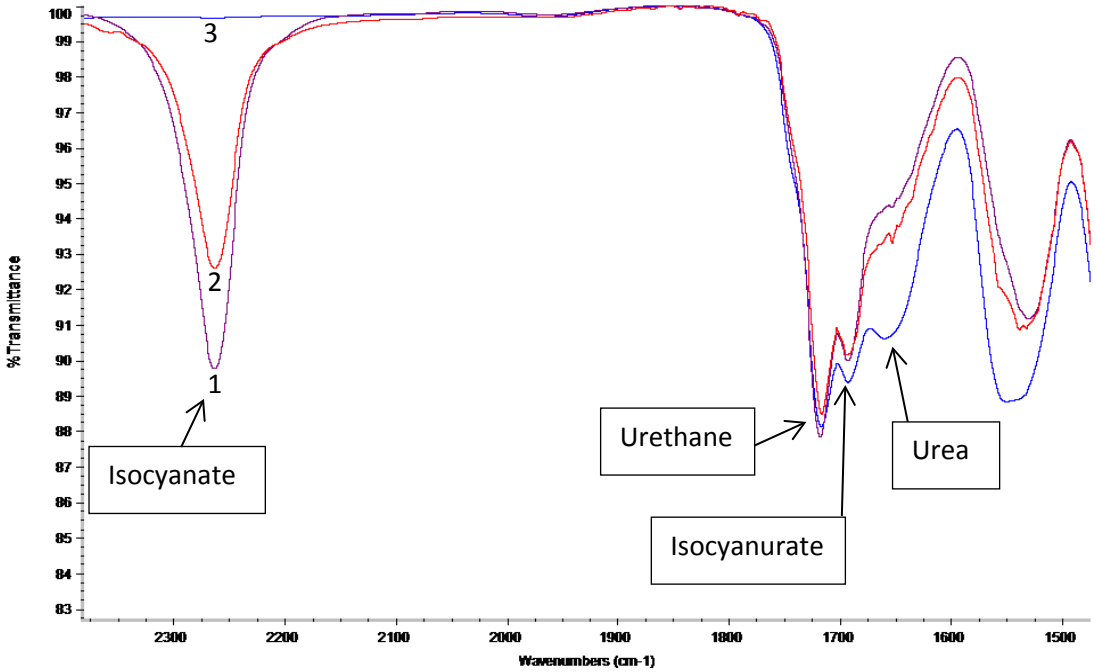


Figure S8. 1, 2, 3 spectra corresponding to the emulsified nanoparticles before Lys addition, 30 min after Lys addition and 10 min after DETA addition respectively. Isocyanate band decreases over time as amino crosslinkers are added and polyurea shell (PUUa NP) is formed. The IR bands at 2270 cm^{-1} correspond to the stretching of the –N=C=O functional group. The sharp band at 1715 cm^{-1} is ascribed to urethane C=O stretching. At 1690 cm^{-1} appears the isocyanurate C=O stretching band corresponding to the B3100 linker structure. The increasing band at 1653 cm^{-1} corresponds to the urea C=O stretching.

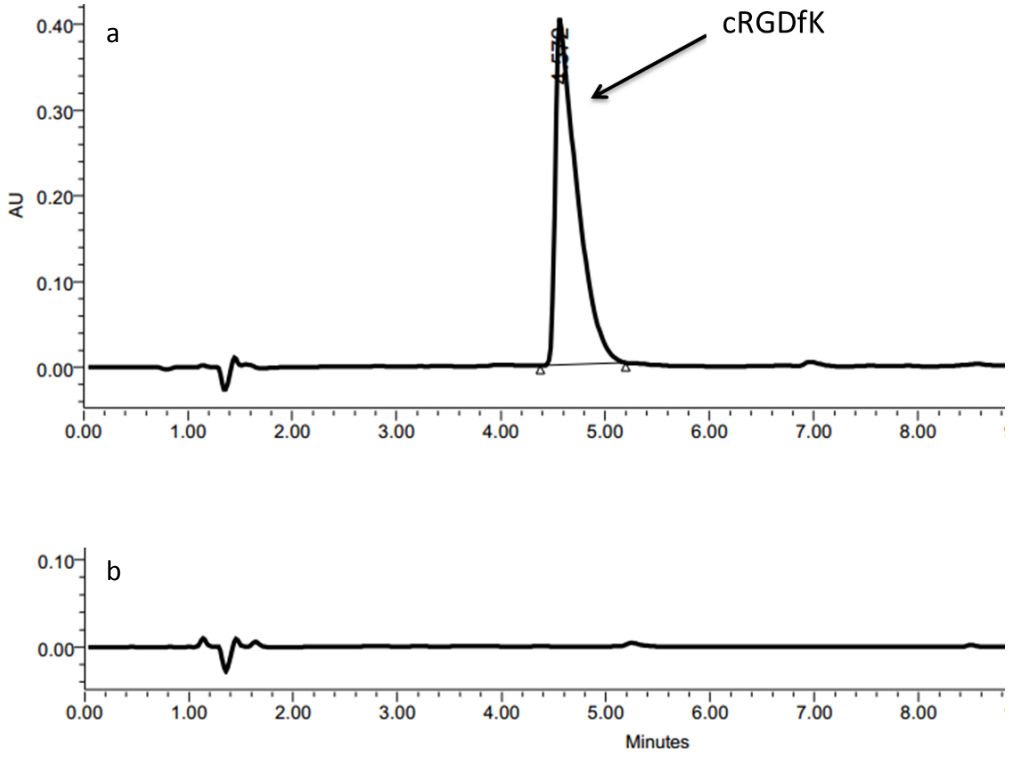


Figure S9. Conjugation reaction monitoring of cRGDfK peptide with B3100 linker by HPLC at 0 h a) and after 2 h reaction b).

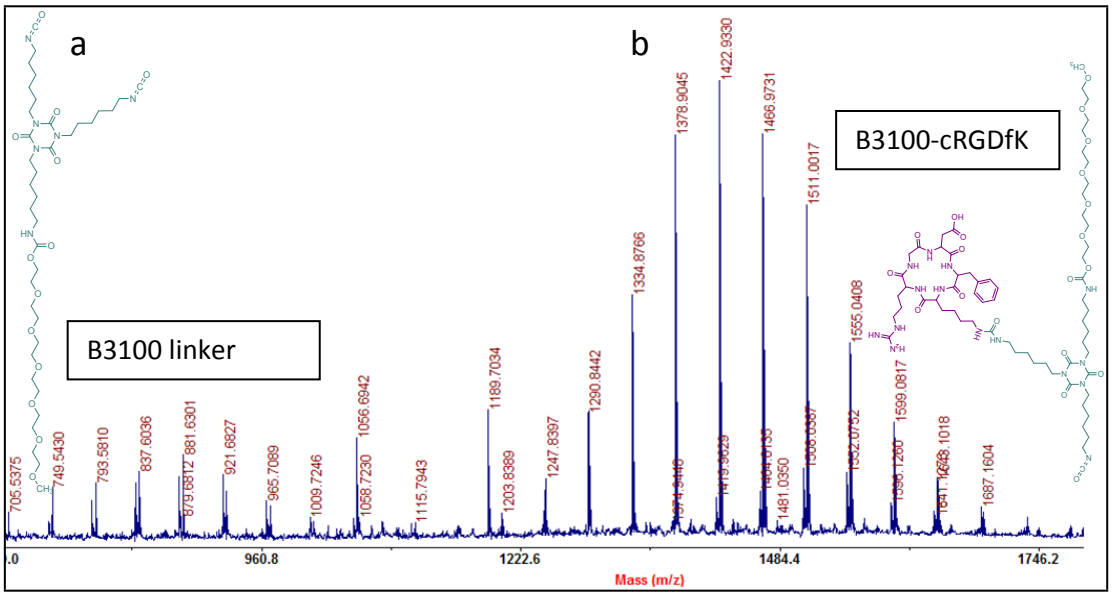
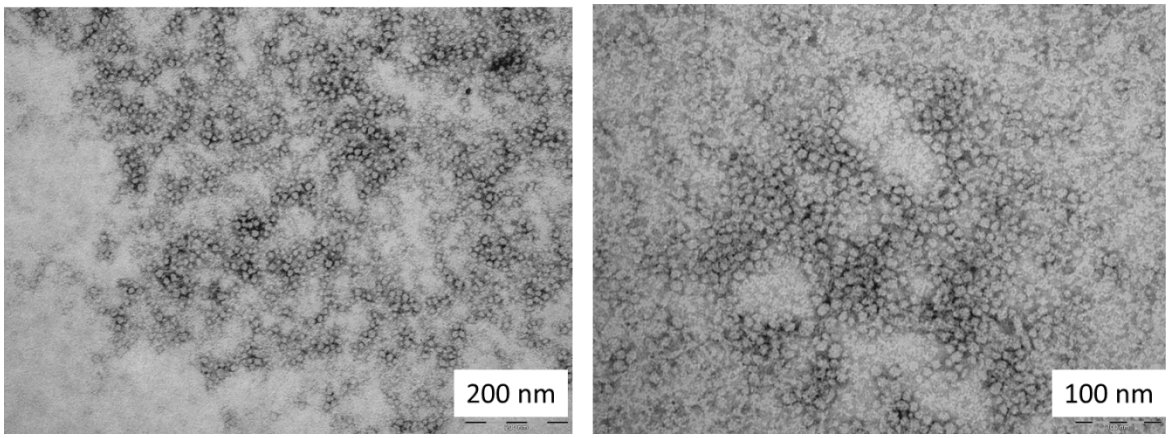


Figure S10. As seen from MALDI-TOF MS spectrum, the linker Bayhydur 3100 (B3100) has a gauss distribution of 700-900 g/mol (average 800 g/mol)) **a).** After 2h reaction a new gauss distribution of masses appears centred on 1400 (800 + 603.68 g/mol) which is consistent with the mass of the B3100-cRGDfK conjugate **b).**

FREE cRGDfK (mM)	FREE cRGDfK (mg/mL)	Conjugated cRGDfK (mg/mL)	Yield (%)
0.046	0.028	1.93	98.5

Table S2. Quantification of non-reacted cRGDfK peptide to PUUa NP-RGD. A calibration curve by HPLC analysis was performed with standard solutions of cRGDfK using L-Phenylalanine as internal standard.



Sample	Diameter (nm)	SD (nm)	PDI
Monowalled PUUa NP	11.3	1.3	0.01

Figure S11. TEM micrographs of monowalled NPs. Monowalled NPs did not contain Hyfob prepolymer, just Amphil. Amphil prepolymer was then crosslinked with Lys and DETA. The absence of Hyfob lead to PUUa NPs with sizes around 10 nm. Monowalled NPs showed a much more diffuse and less defined shell compared to Multiwalled ones.

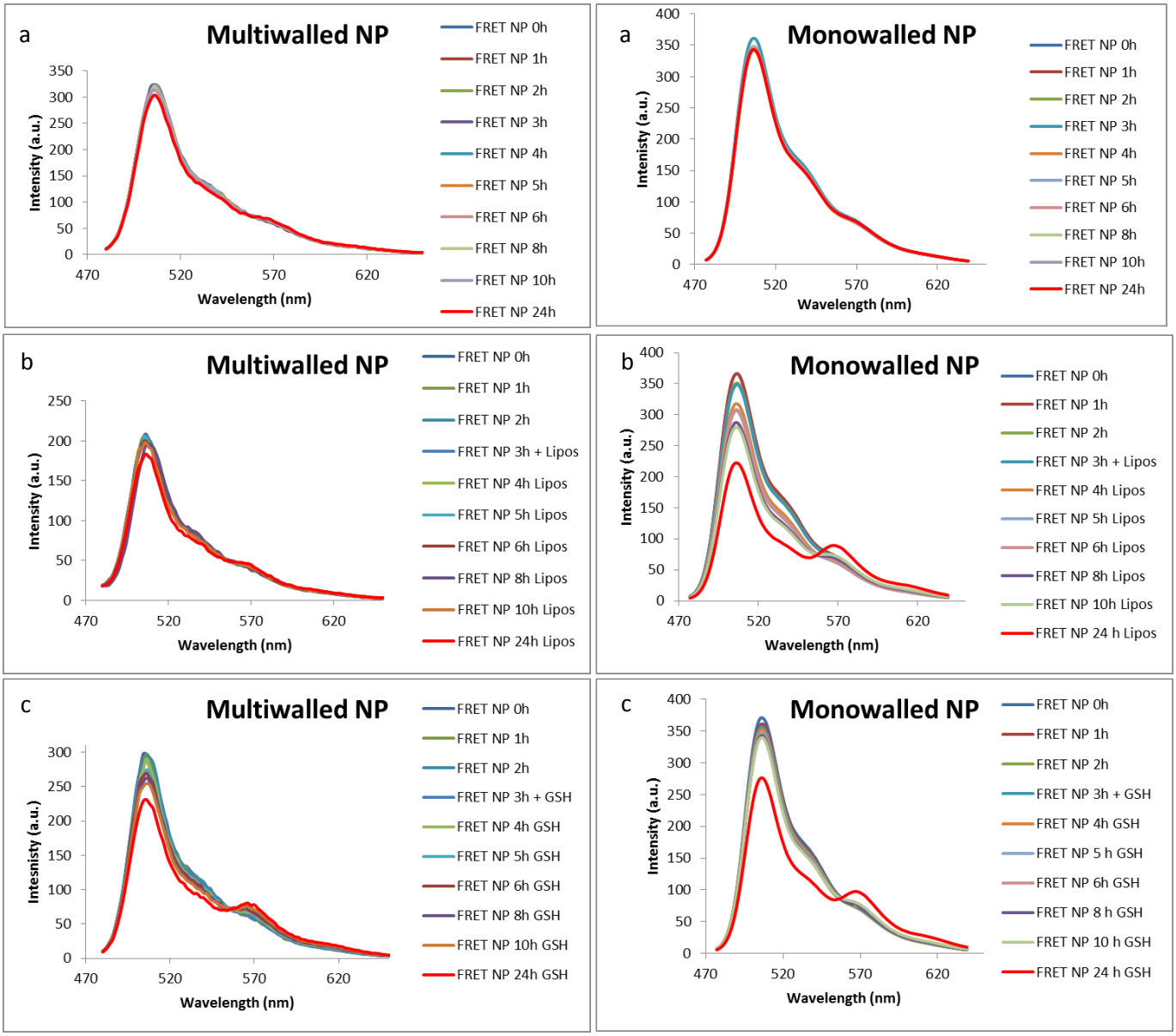


Figure S12. Comparison of fluorescence emission spectra over 24 h of multiwalled and monowalled NPs. Control FRET NPs a), FRET NPs mixed with Phosphatidyl choline-Cholesterol in PBS b), and FRET NPs with GSH c).

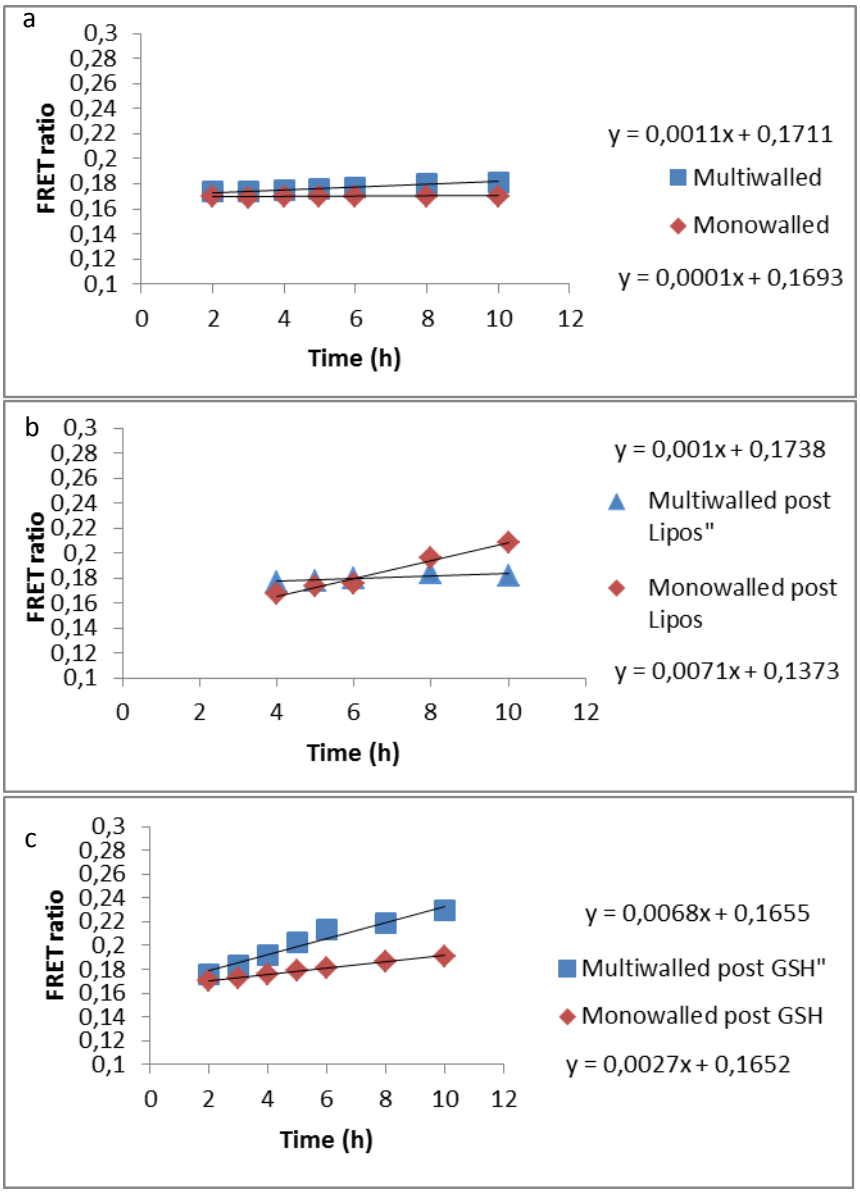


Figure S13. Comparison of measured FRET ratios between multiwalled and monowalled NPs during the first 10 h. FRET ratio of NPs in water a) FRET ratio of NPs mixed with Phosphatidyl choline-Cholesterol in PBS b) FRET ratio post GSH addition in PBS c).

	Integrin expression (% positive cells)	
Cell line	**$\alpha_V\beta_3$**	**$\alpha_V\beta_5$**
U87-MG	98.41	0.0
HT-29	3.63	60.30

Table S3: Expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ in U87-MG and HT-29 human cancer cell lines (from glioma and colon cancer, respectively).

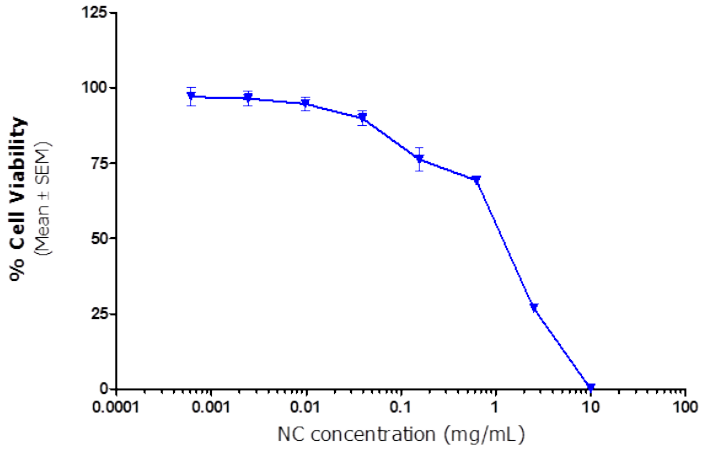


Figure S14. Cytoxicity of DiI loaded NP after 72 h incubation as measured by MTT cell viability assay. Concentrations below 0.1 mg/mL of NP are clearly non-toxic to HeLa cells.

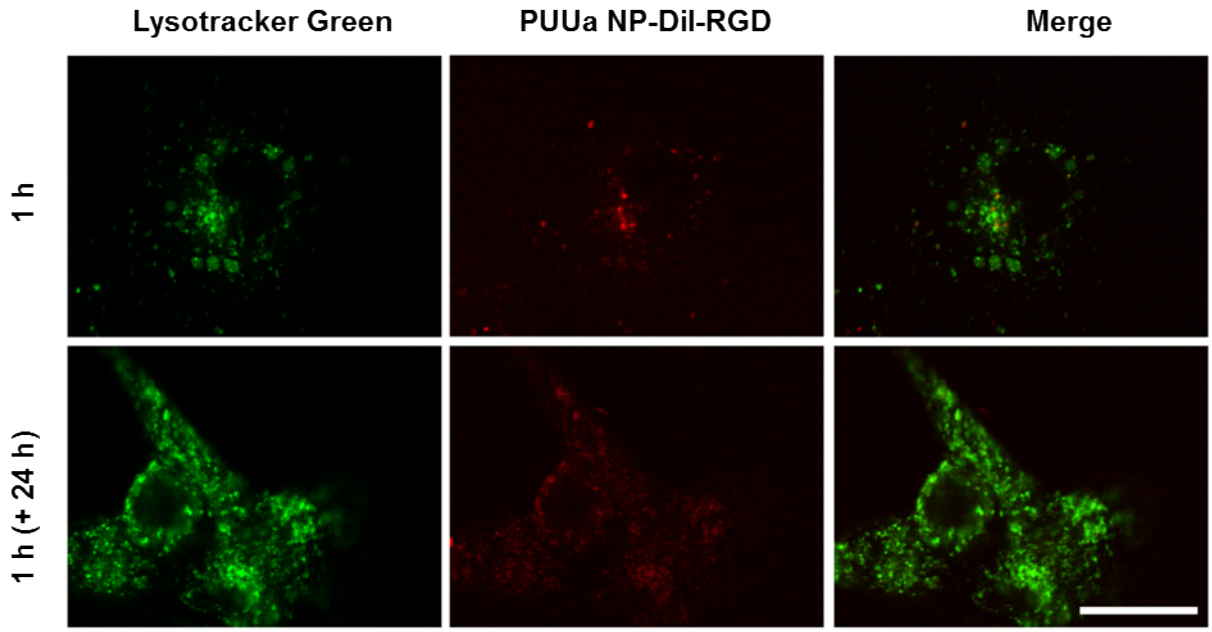


Figure S15. NP trafficking. Confocal images of U87-MG cells incubated 1 h with 1 µg/mL of DiI and stained with Lysotracker Green right after the incubation period (upper panel) or 24 h later (lower panel). Significant differences were observed in confocal images after quantification of the fluorescence signal (z-stacks) confirming that cells pulsed during 1 h and imaged 24 h later showed up to 20 % increase of DiI signal outside lysosomes compared to cells imaged after 1 h incubation ($p < 0.001$). Magnification bar corresponds to 20 µm. For more details see section 2.5.3. of Materials and Methods.

1 US Environmental Protection Agency_Diisocyanates Toxicology. http://www.epa.gov/oppt/auto/profile/toxicology1a.pdf (accessed May 7, 2015).

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