Supplementary Material (ESI) for Chemical Communications

Supplementary information:

The binding of CNA35 contrast agents to collagen fibrils

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S1: Cryo-TEM sample preparation

R2/2 Quantifoil Jena grids, were purchased from Aurion. 300 mesh lacey carbon film grids were purchased from Agar Scientific. The TEM-grids were surface plasma treated using a Cressington 208 carbon coater operating at 5 mA for 40 s prior to the sample preparation. A solution of 1 mg/ml equine collagen type I was brought into contact with a sample grid. After a manual blotting step the grid was brought into contact with HBS, pH 7.4 for 10 minutes. In case of contrast agent incubation, the sample was blotted manually once more and brought into contact with a contrast agent solution. This entire procedure took place at room temperature and at 100% humidity in a glove box. The sample vitrification procedure was carried out using an automated vitrification robot (FEI Vitrobot Mark III).

S2: Cryo-TEM

Part of the cryogenic transmission electron microscopy (cryo-TEM) was performed on a Titan Krios (FEI) equipped with a field emission gun (FEG) operating at 300 kV and images were recorded using a 2k x 2k Gatan CCD camera equipped with a post column Gatan energy filter (GIF). The other part of cryo-TEM was performed on a FEI Tecnai 20, type Sphera TEM instrument equipped with an LaB_6 filament operating at 200 kV, in which the images were recorded with a bottom-mounted 1k x 1k Gatan CCD camera. A Gatan cryoholder operating at ~ -170 °C was used for the cryo-TEM measurements.

S3: Liposome binding statistics

Statistics was performed in order to quantify liposome binding efficiency to collagen bundles. A liposome count was carried out on low-magnification images showing either a loosely packed or well formed collagen bundle. A volunteer, who did not know whether functionalized or non-funtionalized liposomes were present in the cryoTEM images, classified liposomes to be not touching or touching the collagen bundle. The results are presented in table S1.

S4: Fluorescence binding assay

Wells of an 8-well strip plate (Corning, Schiphol-Rijk, the Netherlands) were incubated for 5 minutes at room temperature with 50 μL of 0.1 mg/mL equine collagen type I (kind gift from prof. G. Fallini, University of Bologna) in acetic acid (20 mM). Next, non-bound proteins were aspirated from the wells and the wells were rinsed 3 times with 300

μL Hepes Buffered Saline (HBS) pH 7.4 (20 mM HEPES and 135 mM NaCl) at room temperature. Next, wells were blocked with 250 μL of 5% (w/v) milk powder in HBS for 10 minutes at room temperature, aspirated and again rinsed 3 times with 300 μL HBS. Then, 50 μL of a solution of contrast agent in HBS was added to each well and incubated for 30 minutes at room temperature. Wells were aspirated and washed 10 times with 300 μL HBS. Fluorescence was subsequently measured using a Fluoroskan Ascent FL plate reader (excitation: 578 nm; emission: 620 nm).

S5: Materials

Gadolinium - diethylene triamine pentaacetic acid-bis(stearylamide) (Gd-DTPA-BSA) was obtained from Gateway Chemical Technology (St. Louis, MO), 1,2-distearoyl-sn-glycero-3- phosphoethanolamine-N-[methoxy(poly(ethylene glycol))- 2000] (PEG2000-DSPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(poly(ethylene glycol))-2000] (Mal-PEG2000-DSPE), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-PE) were obtained from Avanti Polar Lipids (Alabaster, AL).

CNA35-rhodamine preparation was carried out according to Krahn et al.[1] CNA35 containing a C-terminal thio-ester was expressed according to Reulen et al.[2] and the thio-ester was subsequently biotinylated according to Tolbert et al.[3] For the generation of mutant-CNA35, the Y175K mutation was introduced in the 6His-CNA35 gene from pQE30-CNA35 using the QuickChange Site-directed mutagenesis kit (Stratagene) using the primers: 5'-

CGGGAACAAGTAGTGTTTTCTATAAAAAAACGGGAGATATGCTACC-3' and 5'-GGTAGCCATATCTCCCGTTTTTTTATAGAAAACACTACTTGTTCCCG-3'

yielding pQE30-CNA35(Y175K). Expression and purification in *E.coli* was carried out similar to the expression of CNA35.

Liposomes were prepared according to Sanders et al.[5] Paramagnetic micelles were prepared by the lipid film hydration method. The lipid film was formed by rotary evaporation of lipid mixture dissolved in chloroform/methanol (4:1 v/v). The lipids Gd-DTPA-BSA, PEG2000-DSPE, mal-PEG2000-DSPE and rhodamine-PE were present in a molar ratio 4:1:5:0.05. After rotary evaporation to dryness, the lipid film was dried under N_2 for 30 minutes. The lipid film was hydrated with HEPES buffered saline (HBS) at pH 6.7, containing 20 mM HEPES (Sigma) and 135 mM NaCl (Sigma). The solution was slowly rotated at 65 ^{o}C for 1 hour, which resulted in a clear micelle solution.

CNA35 was coupled to the micelles by a sulfhydryl-maleimide coupling method as reported before.[4] CNA35 was reacted with succinimidyl S-acetylthioacetate (SATA) in a molar ratio of 1:8 for 1 hour in an $NaHCO_3$ buffer at pH 8.0. Unreacted SATA was removed by using a Satorius concentrator with a MWCO of 10 kDa. Subsequent deacetylation was conducted with a hydroxyl amine solution (0.5 M hydroxyl amine, 1 M HEPES, 32 mM EDTA, pH 7.0) for 1 hour. Modified CNA35 (lipid:CNA35 ratio was 50:1) was then allowed to react with pre-formed micelles at 4 ºC overnight in HBS pH 6.7. Uncoupled CNA35 (< 5% of total) was separated from CNA35-functionalized micelles using Sartorius concentrators with a MWCO of 100 kDa. During the latter process the pH was increased by washing extensively with HBS pH 7.4. The exact same procedure was used for mutant-CNA35 micelles.

S6: Roughness calculation

Surface profiles were manually drawn on high magnification (19000x) images of collagen fibrils either not incubated, incubated with 1 μM monomeric CNA35, 100 μM of monomeric mutant-CNA35 or CNA35-liposomes (7.5 mM lipid concentration, i.e. 10 μM of CNA35). Surface profiles were only drawn on collagen bundles with no natural bends in order to be able to judge the roughness without being influenced by any other effects. These surface profiles were analyzed using in-house build software according to the formula: $R_A = 1/n \sum_{i=1}^{n} |x_i - \bar{x}|$, where R_A is the surface roughness, n is the number of data points, x_i is the surface height at position i and $\bar{x}$ is the average surface height. The total test length was at least 500 nm in all cases, with a minimum of 200 data points.

S7: Cryo-TEM experiment using micelles

Next to incubation with liposomes, also incubation experiments with CNA35-functionalised micelles and mutant-CNA35-micelles were carried out. Incubation protocols were exactly the same for micelles as for liposomes and monomeric CNA35. The concentration of protein for CNA35 functionalized micelles and for mutant-CNA35 was 1-100 μM, which was similar as for the monomeric CNA35 and monomeric mutant-CNA35 experiments. Micelle incubation experiments yielded similar behavior for

micelles as for liposomes (supplementary information figure S7). Even in case of the high 100 μM CNA35 concentration, collagen fibrils stayed intact.

References:

1. Krahn, K. N.; Bouten, C. V.; van Tuijl, S.; van Zandvoort, M. A.; Merkx, M., Anal Biochem 2006, 350, (2), 177-85.

2. Reulen, S. W.; Dankers, P. Y.; Bomans, P. H.; Meijer, E. W.; Merkx, M., J Am Chem Soc 2009, 131, (21), 7304-12.

3. Tolbert, T.; Wong, C., J Am Chem Soc 2000, 122, (23), 5421-5428.

4. Mulder, W. J.; Strijkers, G. J.; Griffioen, A. W.; van Bloois, L.; Molema, G.; Storm, G.; Koning, G. A.; Nicolay, K., Bioconjug Chem 2004, 15, (4), 799-806.

5. H. M. Sanders, G. J. Strijkers, W. J. Mulder, H. P. Huinink, S. J. Erich, O. C. Adan, N. A. Sommerdijk, M. Merkx and K. Nicolay, Contrast Media Mol Imaging, 2009, 4, 81-88.

Table S1: Results from the liposome binding count. Liposomes were classified as in touch or not in touch with either loosely packed collagen bundles or well-assembled collagen fibrils. The percentage of specifically bound liposomes is given as specific (%). A comparison was made between functional (CNA) and non-functional liposomes (unfunctionalized)

	Not in touch	In touch	%In touch	Specific (%)
CNA liposomes				
Total	1715	664	28%	13%
Loosely	1021	538	35%	20%
Well-assembled	694	126	15%	0%
Unfunctionalized liposomes				
Total	942	171	15%	0%
Loosely	565	109	16%	1%
Well-assembled	377	62	14%	-1%

Figures Supporting information:

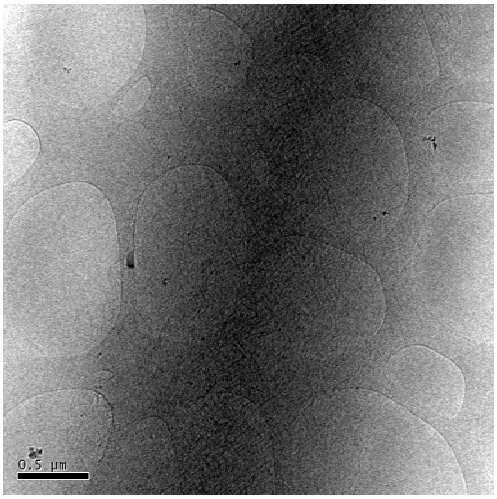

Figure S1: Equine collagen at pH 2, poorly defined collagen fibril. The scale bar is 0.5 µm.

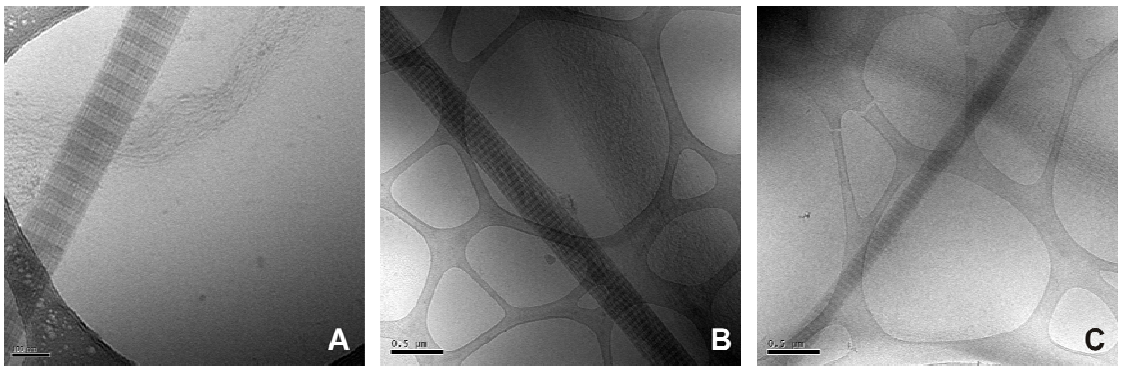

Figure S2: Equine collagen after an incubation period of 10 minutes at pH 7.4. All images show different collagen fibrils. Scale bars represent 0.5 μm.

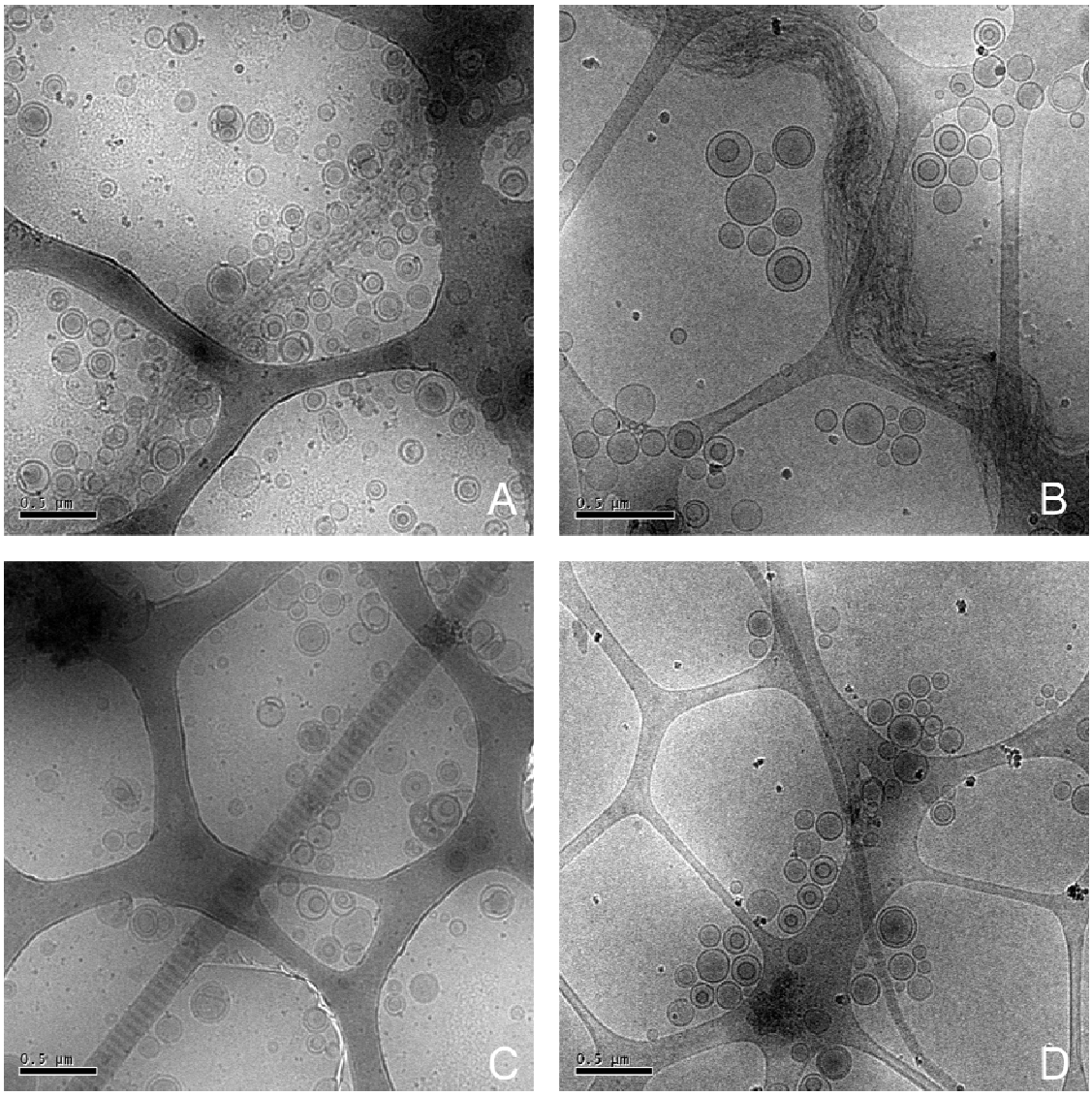

Figure S3: Low magnification images of A) poorly assembled collagen fibril incubated with CNA35-liposomes, B) poorly assembled collagen fibril incubated with unfunctionalized liposomes, C) well-assembled collagen fibril incubated with CNA35-liposomes, D) well-assembled collagen fibril incubated with unfunctionalized liposomes. Scale bars represent 0.5 μm.

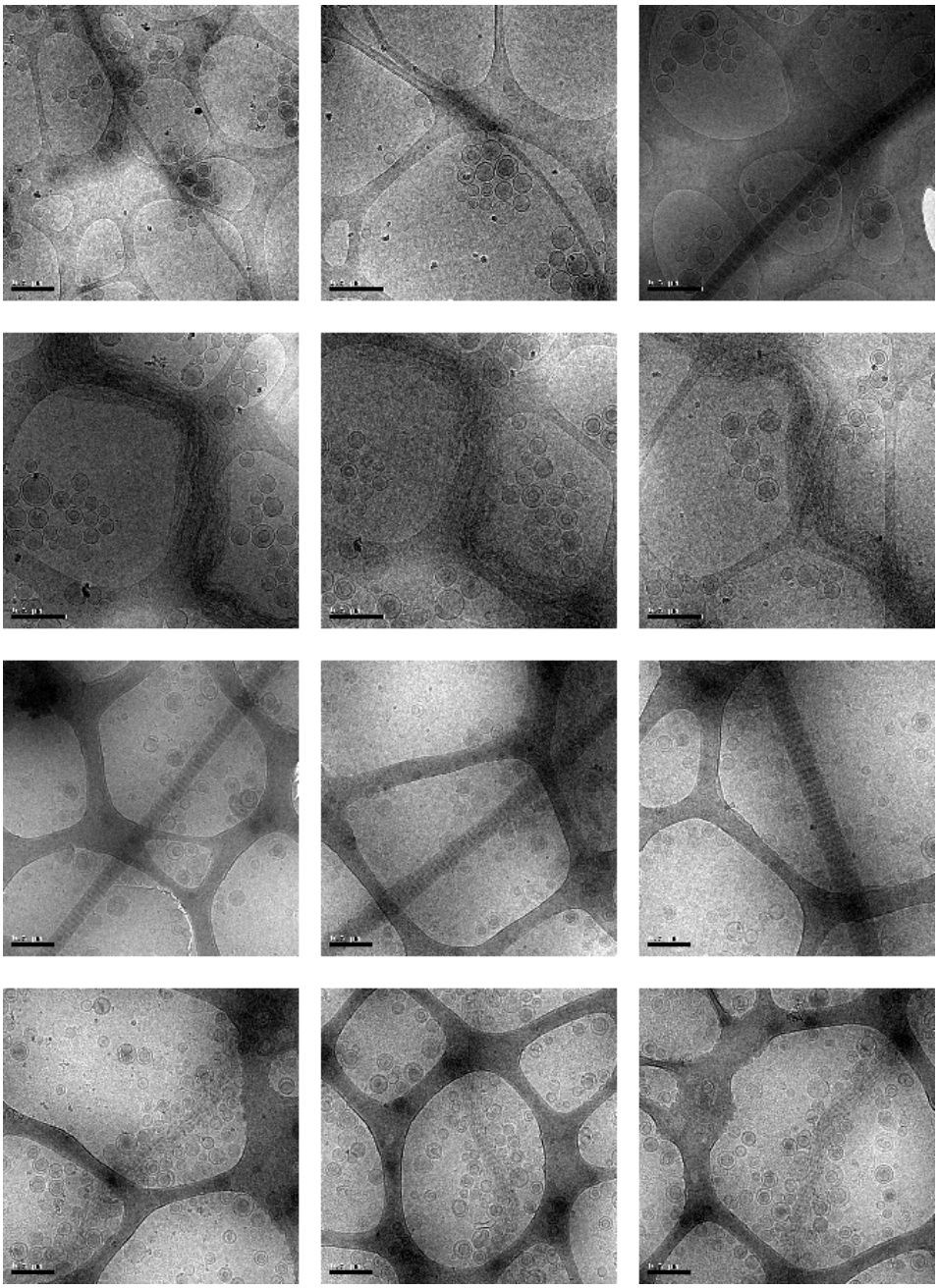

Figure S4: Typical images used for statistics. Well organized fibrils (rows 1 and 3) and loosely packed collagen fibrils (rows 2 and 4) incubated with unfunctionalized liposomes

(rows 1 and 2) and CNA35-functionalized liposomes (rows 3 and 4). Scale bars represent μm.

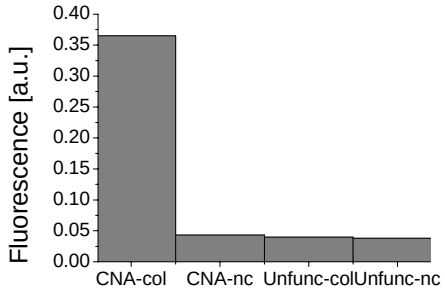

Figure S5: Fluorescent intensities of wells with (col) or without collagen (nc) incubated with CNA35-liposomes (CNA) or unfunctionalized liposomes (Unfunc).

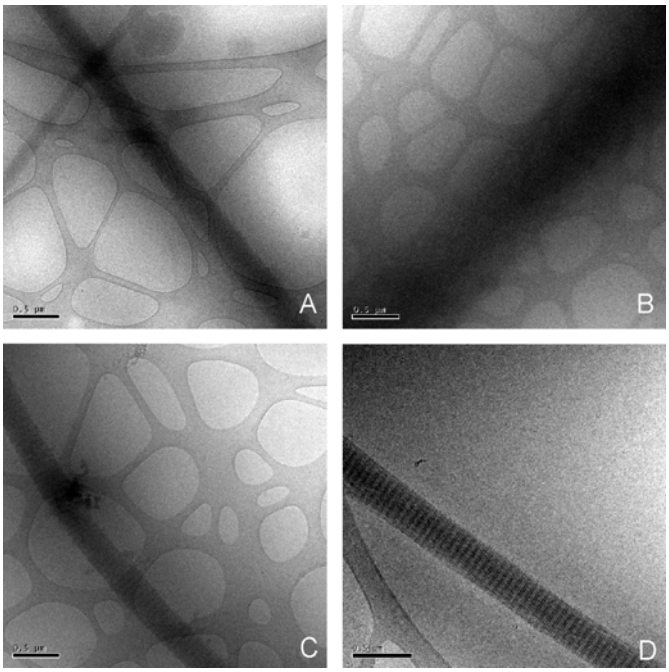

Figure S6: Collagen fibrils incubated with A) 100, B) 10 and C) 1 μM of CNA35. D) Collagen fibril incubated with 100 μM of mutant CNA35. Scale bars represent 0.5 μm.

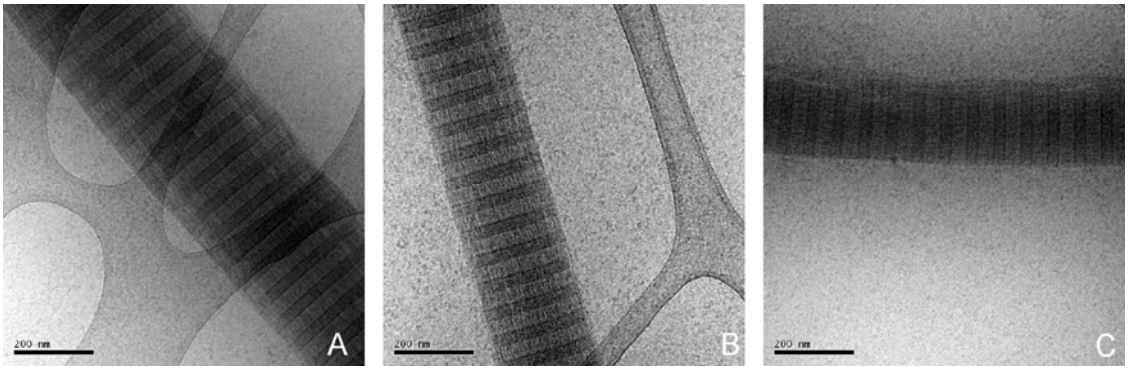

Figure S7: Collagen incubated with 1 (A) and 100 (B) μM of CNA35-micelles and 100 μM of mutant-CNA35-micelles (C). Scale bars represent 200 nm.