The binding of CNA-35 conjugated nanoparticles to assembled versus disassembled collagen fibrils

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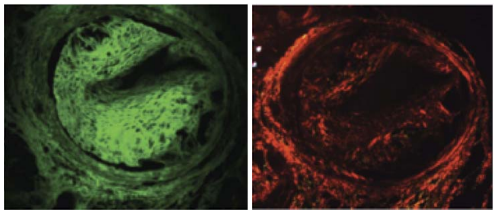
Introduction: Imaging of collagen can be an important diagnostic and therapeutic tool. CNA-35, a bacterial adhesion protein [1] has been conjugated to paramagnetic micelles [2] and liposomes [3] for collagen-targeted MRI. Recently, we reported the successful use of CNA-35 micelles for atherosclerotic plaque phenotyping [2]. However, the spatial distribution of collagen on regular stainings differed from that probed by CNA-35-oregon green (Fig 1). CNA-35 wraps around collagen triple helices [4]. Consequently, CNA-35 conjugated nanoparticles likely bind preferentially to isolated triple helices rather than mature collagen fibrils and might thus offer unique insights in collagen turnover. This study aimed to test the hypothesis that CNA-35 bearing nanoparticles preferentially bind to unstructured collagen, using cryogenic transmission electron microscopy (cryo-TEM). Particles bearing a non-binding CNA-35 mutant were used as a control.

Materials and Methods: CNA-35 liposomes were prepared as in [3]. Micelles were prepared from Gd-DTPA-BSA, PEG2000-DSPE, mal-PEG2000-DSPE and rhodamine-PE (molar ratios 4:1:5:0.05) in HBS at pH6.7, followed by CNA-35 coupling [2,5]. The same procedure was used for micelles and liposomes conjugated with mutant CNA-35, in which tyrosine 175 was replaced by a lysine. Cryo-TEM was performed at -170°C, using a Titan Krios (FEI). Liposome counting to quantify collagen binding was done on low-magnification images. A blinded observer classified these as touching or not touching the collagen bundle. Particle binding to collagen was also quantified with fluorescence, using the rhodamine-PE lipid. Wells of strip plates were coated with 0.1mg/ml equine collagen type I in acetic acid (20mM), rinsed with HBS, blocked and again washed with HBS, and incubated for 30min @ RT with 50µl contrast agent solution in HBS. After extensive washing with HBS, fluorescence was measured with a plate reader (excitation: 578nm; emission: 620nm).

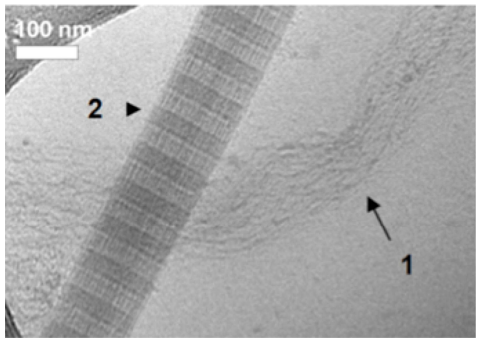


^ Fig 1: Histology of collagen in atherosclerosis. CNA-35-oregon green staining (left) of plaque shows a diffuse distribution compared to the more localized collagen visualization with picrosirius red (right).

Results and Discussion: Fig 2 shows a cryo-TEM image of a loosely packed collagen bundle and a fully assembled fibril, demonstrating the ability of cryo-TEM to visualize the structural organization of collagen. Fig 2 depicts CNA-35 liposomes (A,C) and control liposomes (B,D) incubated with loosely-packed collagen bundles (A,B) and structured collagen fibrils (C,D). These data suggest a high degree of association of CNA-35 functionalized liposomes with loosely-packed collagen (Fig 2A) and a much lower association to highly ordered collagen (Fig 2C). Non-functionalized liposomes exhibit minor association to both ordered and disordered collagen (Fig 2B,D). Quantitative analyses showed that 35% of CNA35-liposomes was in contact with loosely packed collagen and only 15% with a well-assembled fibril. Since a similar percentage of un-functionalized liposomes was in contact with loosely-packed and well-assembled collagen (14% and 16%, respectively), it was concluded that CNA35-liposomes preferentially bind to loosely packed collagen bundles and have little affinity for well-assembled fibrils. Liposomes coupled with mutant CNA-35 showed similar values as control liposomes.

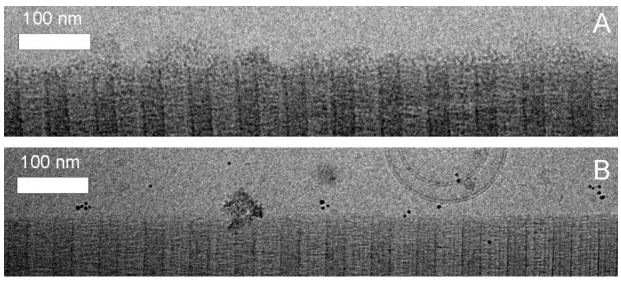
These data suggest that 15% of the liposomes were in contact with collagen due to non-specific interactions. To investigate whether liposomes exhibit a basal affinity for collagen, fluorescence-binding assays were done. These showed a high fluorescence signal in case of CNA35-liposomes (0.36 au), compared to un-functionalized liposomes (0.04 au). The latter value was also measured for controls without collagen. We therefore concluded that background binding of liposomes to collagen is negligible. The high apparent background binding on cryo-TEM can also be explained from the fact that during cryo-TEM sample preparation liposomes and collagen fibril are confined in a thin aqueous film and are forced to be in close proximity, consequently readily being classified as touching without actually being bound.

Cryo-TEM studies with monomeric CNA-35 showed that the protein by itself is capable of disordering collagen tertiary structure (Fig 4A). Mutant CNA-35 caused no change in collagen structure, while also incubations with CNA-35 liposomes (Fig 4B) or CNA-35 micelles had no such effect of collagen architecture.

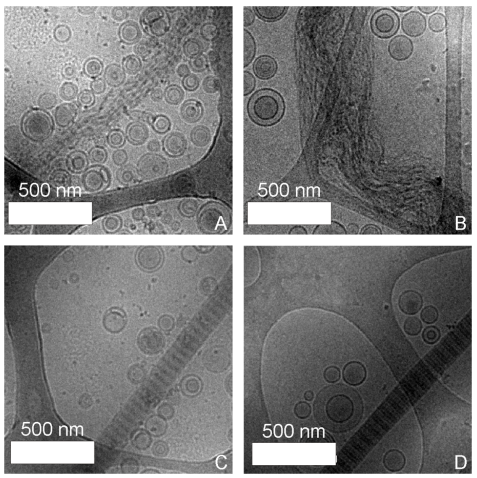


^ Fig 2: A) Loosely packed collagen bundle (1) and fully assembled fibril (2).

Conclusion: In contrast to monomeric CNA-35, CNA-35-conjugated liposomes and micelles preferentially bind to disordered collagen, yielding possibilities for designing collagen-specific MRI contrast agents that primarily report on collagen remodeling.



< Fig 4: A) 1µM CNA-35 incubated on structured collagen, leading to surface disordering. B) Structured collagen incubated with CNA-35 liposomes. Note: the collagen fibril is strongly ragged in A, while unaffected in B.



^ Fig 3: A) CNA-35 liposomes associated with loosely packed collagen bundle. B) Control liposomes and a loosely-packed collagen bundle. C) CNA-35 liposomes together with a highly structured collagen fibril. D) Control liposomes together with a structured collagen fibril.

References: [1]. Rich *et al.* J Biol Chem 274: 24906-13, 1999; [2]. De Smet *et al.* Proc ISMRM 16: 515, 2008; [3]. Sanders *et al.* CMMI 4: 81-8, 2009; [4]. Zong *et al.* EMBO J 24: 4224-36, 2005; [5]. Mulder *et al* Bioconj Chem 15: 799-806, 2004.