Collagen binding MR-detectable liposomes

H. M. Sanders[1], M. Merkx[2], A. Mol[3], W. J. Mulder[1,4], G. J. Strijkers[1], and K. Nicolay[1]

[1]Biomedical NMR, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, Netherlands, [2]Laboratory of Macromolecular and Organic Chemistry, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, Netherlands, [3]Laboratory for Cell and Tissue Engineering, Deparment of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, Netherlands, [4]Department of Radiology, Mount Sinai School of Medicine, New York, New York, United States

Introduction

The extra cellular matrix (ECM) plays a very important role in the normal and abnormal development of tissues. Target specific imaging of the ECM will provide unique tools to monitor tissue maturation and remodelling. Since collagen is the major constituent of the extra cellular matrix providing strength to tissues, this protein was chosen as a first target. Magnetic resonance imaging (MRI) has emerged as the potential leading *in vivo* modality in several diagnostic protocols. Therefore the goal of this research was to develop a collagen specific MR contrast agent (CA). In a first approach a bimodal liposomal MR-CA (figure 1), carrying rhodamine functionalized lipids and a high payload of Gadolinium containing lipids was functionalized with CNA-35. This bacterial collagen adhesion protein of *Staphylococcus aureus* was chosen as the collagen binding motive, because it shows specific binding to collagen can be obtained in large quantities.[1]

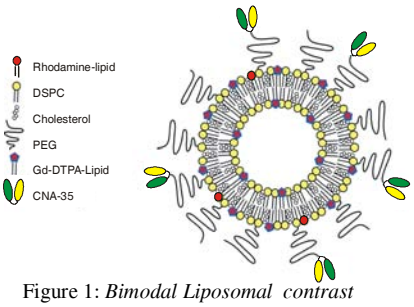

Figure 1: *Bimodal Liposomal contrast agent functionalized with CNA-35 (d = 200 nm)*

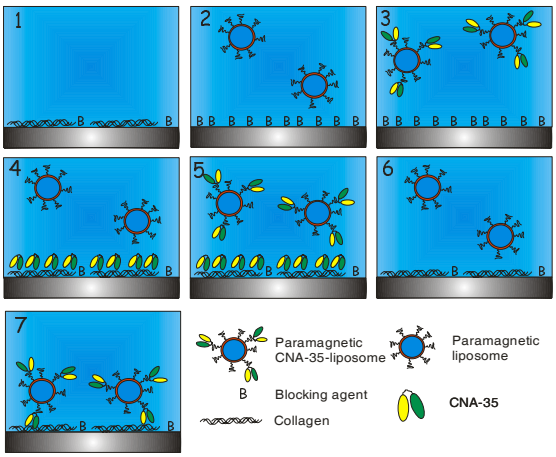

Figure 2: *Cartoon of binding experiments 1-7*

Materials and methods

The liposome formulation used was described earlier by Mulder et al.[2] The liposomes were prepared by lipid film hydration followed by extrusion through 200 nm membranes. CNA-35 was obtained as a fusion protein from an *E. coli* expression system[3] and was subsequently conjugated to the distal end of maleimide functionalised PEG-lipids after SATA-modification of its primary amines.[3] Untargeted liposomes were obtained by omitting the conjugation of CNA-35.

A total of seven binding experiments (figure 2) was performed in triplicate to verify the binding specificity. Table 1 shows which incubations were performed in each of the binding experiments. The wells (clear, flatbottom Costar 8-well strip-plates) used for experiment 1 and 4-7 were incubated overnight at 4° C with 50 µL of 55 µg/mL rat-tail collagen type I (C7661, Sigma-Aldrich) in HEPES-buffered saline (HBS), containing 20 mM HEPES, 135 mM NaCl (pH 7.4). Protein solutions were then aspirated from the wells using an automated Wellwash AC plate washer and the wells were rinsed 3 times with 300 µL HBS at 20° C. The wells of all experiments were blocked with 250 µL of 5% (w/v) milk powder in HBS for 2 h at 20° C, aspirated and rinsed 10 times with 300 µL HBS. A 50 µL solution of CNA-35 in a 0.1 M $NaHCO_3$ buffer (pH 8.3) was added to the wells of experiment 4 and 5 and incubated 3 h at 20 °C. Afterwards the solution was aspirated and the wells were rinsed 5 times with 300 µL HBS. A 50 µL solution of liposomes (untargeted or CNA-functionalized) in HBS was added to wells and incubated 3 h at 20 °C. Wells were aspirated and washed 5 times with 300 µL HBS, after which the fluorescence was read with an automated Fluoroskan Ascent FL plate reader (excitation: 578 nm; emission: 620 nm). The same wells as used in the fluorescence platereader were imaged using a 6.3 T-scanner. An inversion recovery sequence using 12 inversion times ranging from 12 till 5000 ms and 2 averages was used to obtain a T1-map with a field of view of 3 x 3 cm^2 with a slice thickness of 1 mm, perpendicular to the bottom of the wells. A matrix size of 512 x 512 yielded an in plane spatial resolution of 60 µm.

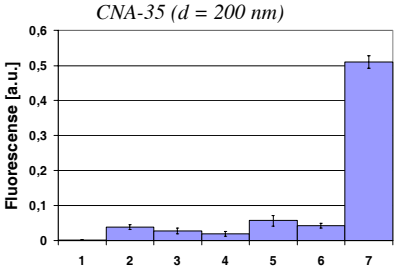

Figure 3: *Fluorescence data from experiment 1 to 7 proving CNA-35 mediated binding*

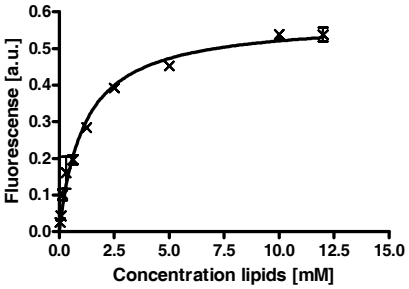

Figure 4: *Titration experiment, varying the concentration of contrast agent, yielding a dissociation constant in the nano-molar range*

Results and Discussion

The results obtained using a fluorescence platereader, shown in figure 3, indicate CNA-35-mediated liposomal binding to collagen. No fluorescence of the collagen layer (experiment 1) was detected. Experiment 2 and 3 show that no binding of the liposomes to the blocking agent took place. Experiment 4 indicates that a covalent bond between liposomes and CNA-35 was required for liposome binding. Pre-blocking with CNA-35 prevented liposome binding as shown by the fifth experiment. Experiment 6 shows that no binding between untargeted liposomes and collagen took place. Finally experiment 7 demonstrated CNA-mediated binding between collagen and liposomes.

Assuming 400,000 lipids per liposome, a dissociation constant (Kd) of 0.325 nM was calculated from the titration experiment shown in figure 4. This sub-nano-molar value is very promising for future experiments.

An inversion recovery sequence was used to obtain a T1-map of the well plate (figure 5). A clear T1-shortening was observed at the bottom of the wells of experiment 7 and not for any other system, which is in excellent agreement with the fluorescence data.

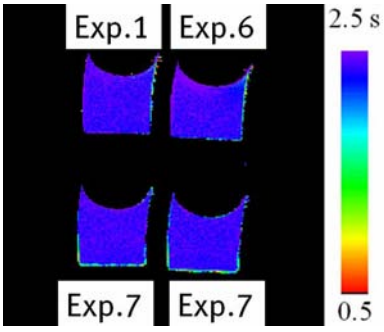

Figure 5: *T1-map of experiments 1, 6 and 7 obtained via an inversion recovery sequence*

Conclusions

The CNA-35 functionalized liposomal contrast agent binds to collagen and can be visualised using MRI. This specific contrast agent might be suitable to follow collagen formation during the maturation of tissues. We plan to monitor disease processes that involve collagen. Also the formation of collagen in tissue engineered constructs might be monitored due to the high binding affinity.

References: [1] Krahn et al. Anal. Biochem. 2006: 350: 177-185, [2] Mulder et al. Bioconjug. Chem. 2004: 15: 799-806 [3] Zong et al. EMBO J. 2005: 24: 4224-4236