Synthesis and in vitro evaluation of a multifunctional and surface-switchable nanoemulsion platform

Anita Gianella[a], **Aneta J. Mieszawska**[a], **Freek J.M. Hoeben**[b], **Henk M. Janssen**[b], **Peter A. Jarzyna**[a], **David P. Cormode**[a], **Kevin D. Costa**[c], **Satish Rao**[c], **Omid C. Farokhzad**[d], **Robert Langer**[e], **Zahi A. Fayad**[a], and **Willem J.M. Mulder**[a,*]

[a]Translational and Molecular Imaging Institute, Department of Radiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA [b]SyMO-Chem B.V., Eindhoven, The Netherlands [c]Cardiovascular Cell and Tissue Engineering Laboratory, Cardiovascular Research Center, Mount Sinai School of Medicine, New York, NY, USA [d]Laboratory of Nanomedicine and Biomaterials, Department of Anesthesiology, Brigham & Women's Hospital, Harvard Medical School, Boston, MA, USA [e]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

Abstract

We present a multifunctional nanoparticle platform that has targeting moieties shielded by a matrix metalloproteinase-2 (MMP2) cleavable PEG coating. Upon incubation with MMP2 this surface-switchable coating is removed and the targeting ligands become available for binding. The concept was evaluated in vitro using the biotin and $\alpha v \beta 3$-integrin-specific RGD-peptide functionalized nanoparticles.

Intravenously administrable nanoparticles can be used in a variety of biomedical applications that range from targeted drug delivery, target-specific imaging, nucleic acid delivery to thermal therapy.[1,2] The nanoparticle coating is of key importance as it greatly influences pharmacokinetics and bioavailability as well as the nanoparticle's ability to target a diseased site.[3] Polymeric coatings, such as a polyethylene glycol (PEG) coating, provide shielding, reduce recognition and subsequent removal by the mononuclear phagocyte system (MPS), and therefore are applied to the majority of nanoparticles used for intravenous administration.[1] To enhance specificity and induce nanoparticle uptake by cells, target-specific molecules can be conjugated to the nanoparticle's coating.[1,2] However, the exposure of targeting moieties at the surface may counteract the shielding effect of the polymeric surface coating and can cause augmented recognition by the MPS. In addition, off-target binding to epitopes expressed by vascular components such as endothelial or circulating cells can also reduce accumulation of nanoparticles at their target site.

To deal with the aforementioned limitations of ligand functionalized nanoparticles we have developed a highly flexible nanoemulsion (Fig. 1a), based on a previously reported platform,[4] of which the coating can be removed by matrix metalloproteinase-2 (MMP2).[5] We have chosen for an MMP2 cleavable site as the enzyme is highly expressed at a variety of pathological sites, including solid tumors and atherosclerotic plaques.[6,7] Upon nanoparticle accumulation at the pathological tissue and exposure to MMP2, the coating is

'removed' and the targeting moieties become available for binding. The nanoemulsion core consists of soybean oil and the coating is comprised of a mixture of cholesterol, PEG350 phospholipids (mPEG350-DSPE), PEG phospholipids functionalized with targeting moieties (i.e. biotin or RGD functionalized PEG1000 lipids), as well as a MMP2 cleavable methoxy-polyethylene glycol (PEG)-lipid (mPEG-MMP2p-DSPE, Fig. 1b) to provide shielding. A description of the synthesis of the nanoemulsion and mPEG-MMP2p-DSPE is provided in the Supporting Information.

As a model for targeting and to robustly demonstrate the effect of shielded versus unshielded nanoemulsions we designed experiments where plain nanoemulsions, which had biotin-PEG1000-DSPE and the non-cleavable mPEG3000-DSPE incorporated, were used. Avidin, a 66 kDa protein with 4 binding sites for biotin, can induce nanoparticle aggregation, if the nanoparticles are unshielded and biotin is exposed, and can therefore serve as a model for targeting. By varying the lipid composition of the corona (cholesterol, mPEG350-DSPE, mPEG3000-DSPE, and biotin-PEG1000-DSPE) we were able to create nanoemulsions with different coating types (Table S.1). The avidin-induced aggregation was monitored with dynamic light scattering (DLS), while targeting of Rhodamine labeled nanoemulsions was evaluated on an avidin coated 96-well plate using a plate reader (Fig. S1, S2).

Surface switchable nanoemulsions were obtained by incorporation of mPEG-MMP2p-DSPE into the formulation. Based on the aforementioned model aggregation and binding experiments, we prepared nanoemulsions using the minimum of 10% PEG shielding (thereby permitting maximum MMP2 access to the cleavable peptide), 2.5% biotin to ensure good binding to avidin, and optionally replacing the uncleavable mPEG3000-DSPE by the MMP2 cleavable lipid mPEG-MMP2p-DSPE (Table S.2). Through HPLC analysis we evaluated the nanoparticle composition after synthesis (Fig. S.10), which was found to be very similar to the starting phospholipid mixture (Table S.3), thereby proving the integrity of the nanoemulsion synthesis.

The nanoemulsions were left untreated or treated with MMP2 before conducting aggregation or binding experiments. We observed that mPEG-MMP2p-DSPE containing nanoemulsions that were not pre-incubated with MMP2 (Fig. 2a) or that were incubated with inactive MMP2 (Fig. S.11) did not aggregate upon incubation with avidin, similar to the control nanoemulsions that contained either 10% or 20% mPEG3000-DSPE (Fig. S.2a). Conversely, MMP2-treated mPEG-MMP2p-DSPE containing nanoemulsions did aggregate and the relative particle size was found to increase by a factor 2 to 3, similar to the control nanoemulsions with the freely exposed biotin-PEG-DSPE (and 0% mPEG3000-DSPE) (Fig. 2a). MMP2 dose effects on the cleavage are shown in Fig. S.12.

To mimic binding to epitopes expressed at cells, we performed binding experiments with an avidin monolayer using the same samples and conditions as for the above-described aggregation experiment. To that end we used avidin coated 96-well plates and incubated the wells with the different samples for 30 min. Rhodamine labeled phospholipids were included in the lipid corona of the nanoemulsions so that their binding could be measured by recording the fluorescence with a plate reader. Similar to the aggregation experiment, and in line with the aforementioned hypothesis, we observed binding for the unshielded control nanoemulsions and, crucially, for the mPEG-MMP2p-DSPE nanoemulsions that were pretreated with MMP2 (Fig. 2b).

In a further evaluation of the nanoemulsion binding characteristics, we developed a self-assembled monolayer (SAM) method that enabled the functionalization of gold-silicon (Au-Si) wafers with avidin and imaging with atomic force microscopy (AFM). The 2D-AFM

image in Fig. 3c clearly shows elevated binding of mPEG-MMP2p-DSPE containing nanoemulsion treated with MMP2 to the surface as compared to the control sample (Fig. 3b).

To confirm that the aggregation/binding was induced by cleaving of the mPEG2000 moiety we performed mass spectrometry on mPEG-MMP2p-DSPE that was left untreated or incubated with MMP2 for 12 hrs. Mass spectrometry revealed the mPEG2000 cleavage product in the sample incubated with MMP2 (Fig. S.13).

The kinetics of PEG cleavage was also investigated. To that end the nanoemulsions were incubated with MMP2 for different time spans, ranging from 1 to 12 hours, while avidin-induced aggregation was used as the readout method. As shown in Fig. S.14b, 12 hours of MMP2 treatment was necessary to induce the ligand exposure. A similar timeframe has been shown by others to induce the in vitro cleavage of cancer pro-drugs conjugated with MMP2 sensitive peptides.[16] However, it is important to consider that in vitro conditions do not fully reflect the in vivo situation. We purposely and carefully designed our nanoparticles to have a PEG-density and ligand density that requires relatively long MMP2 exposure in order for the PEG to be cleaved off. Indeed, in experiments where we have exposed the nanoemulsions to serum protein (FBS), we have seen a high stability of the nanoparticles (Fig. S.15).

Protease induced nanoparticle aggregation has been suggested to be useful in medical and biochemical assays as well as to preserve good pharmacokinetics of target-specific nanoparticles.[8,9] In for example tumor targeting, a number of epitopes of interest that are expressed at tumor cells are also expressed by cells of the MPS and, more importantly, by the diseased endothelium. An example of such an epitope is the αvβ3-integrin, which is an excellent tumor vasculature marker for nanoparticle targeting,[10] but targeting of this integrin expressed at tumor cells is difficult as, upon intravenous administration, the nanoparticles are exposed and bind to the vascular endothelium first and therefore can only marginally extravasate into the tumor interstitium.[11] Because RGD peptides are known to have a high affinity for αvβ3-integrin, we have functionalized our platform with c[RGDfK]-DSPE (full synthetic details are included in the Supporting Information). As for biotin-PEG1000-DSPE, c[RGDfK]-DSPE has been designed such that the c[RGDfK] targeting units protrude from the nanoemulsion platform after MMP2 cleavage of the mPEG-MMP2p-DSPE lipid.

Human umbilical vein endothelial cells (HUVEC) and breast cancer cells (MDA-MB-231), that both are known to express high levels of αvβ3-integrin, as well as murine macrophage (J774A1) were treated with RGD functionalized control nanoemulsions and surface switchable nanoemulsions. Flow cytometry revealed a clear difference in nanoparticle-cell association when the RGD moieties were available for targeting, i.e. at 0% mPEG3000 and at 10% mPEG-MMP2p-DSPE after MMP2 treatment (Fig. 4).

Lastly, to examine the versatility of our nanoemulsion platform, we included either oleic acid coated iron oxide (IO) or oleyl mercaptan coated gold (Au) nanocrystals as well as the lipophilic drug simvastatin in mPEG-MMP2p-DSPE functionalized nanoemulsion samples to render so-called theranostic nanoparticles, exhibiting both therapeutic and diagnostic properties. In Supp. Fig. 20a and 20b we present DLS size and polydispersity measurements as well as transmission electron microscopy (TEM) images of these theranostic nanoemulsions. For the Au nanoemulsions the attenuation characteristics, measured in Hounsfield Units (HU) per mM (SFig. 20a), revealed good CT contrast generating properties (Fig. 5, top), similar to that of a clinically applied iodinated CT contrast agent (Isovue, Bracco Diagnostic). As a measure for the IO nanoemulsion's potential to serve as an MRI contrast generating material we acquired the longitudinal and transverse relaxivities $r1$ and $r2$ at 60 MHz (Supp. Fig. 20a). Clear MR hypo-intensities are generated by IO loaded

nanoemulsions as is shown in Fig. 5 (bottom). The drug inclusion for the final Au formulation was measured using 1H-NMR spectroscopy (Fig. S.21). Simvastatin encapsulation efficiency (encapsulated drug/input) was established to be 57.2% of the initial input value, and the loading efficiency was 1.43% (weight drug/weight oil).

In summary we have presented a self-assembled and multifunctional nanoemulsion platform that is surface-switchable upon exposure to MMP2. The inclusion of diagnostically active materials and drugs is straightforward. We believe our nanoemulsion platform may be useful in a variety of conditions that are associated with MMP2 (over)expression, including cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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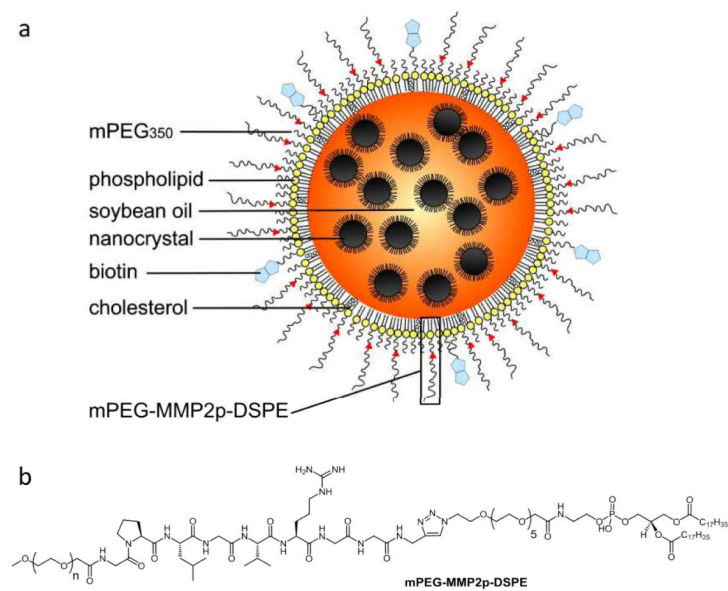
Fig. 1.
(a) Schematic of the biotin functionalized and surface-switchable nanoemulsion platform.
(b) Structure of MMP2 cleavable mPEGMMP2p-DSPE (with mPEG being mPEG2000).
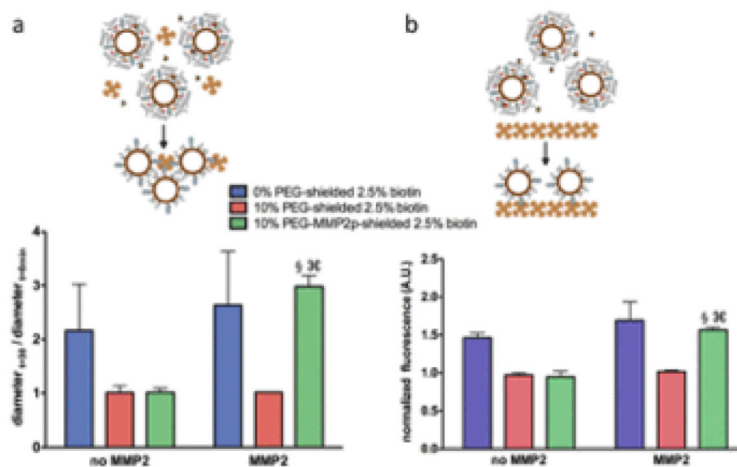
Fig. 2.
(a) Avidin-induced aggregation of biotin functionalized nanoemulsions. Nanoparticle relative size was measured by DLS. (b) Nanoemulsion binding to an avidin monolayer. Normalized fluorescence of unshielded control nanoemulsions, as well as non-treated and MMP2-treated surface-switchable nanoemulsions. Mean ± SD (N=3). § vs 10% PEG-shielded 2.5% biotin nanoparticles (P 0.05), ⌘ vs 10% PEG-MMP2p-shielded 2.5% biotin nanoparticles before MMP2 treatment (P 0.05).
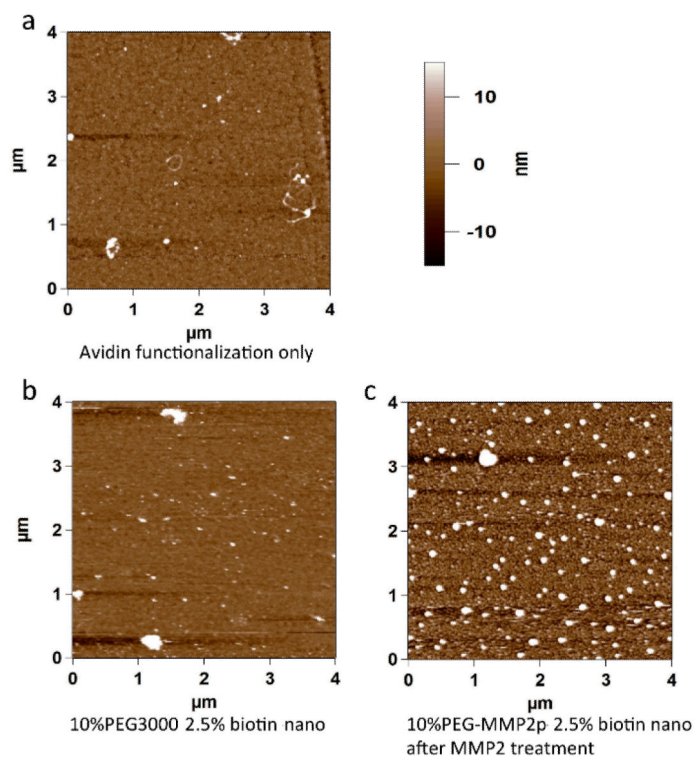
Fig. 3.
Typical 2D-AFM images of Au-Si wafers after incubation with (a) avidin only, (b) avidin and then a 10% mPEG3000-DSPE with 2.5% biotin-PEG1000-DSPE nanoemulsion and (c) avidin and then a 10% mPEG-MMP2p-DSPE with 2.5% biotin-PEG1000-DSPE nanoemulsion previously treated with MMP2.cells incubated with (i) 0% m PEG3000 and 2.5% RGD nanoemulsions, (ii) 10% mPEG3000 and 2.5% RGD nanoemulsions and (iii) 10% PEG-MMP2p nanoemulsion phantoms.
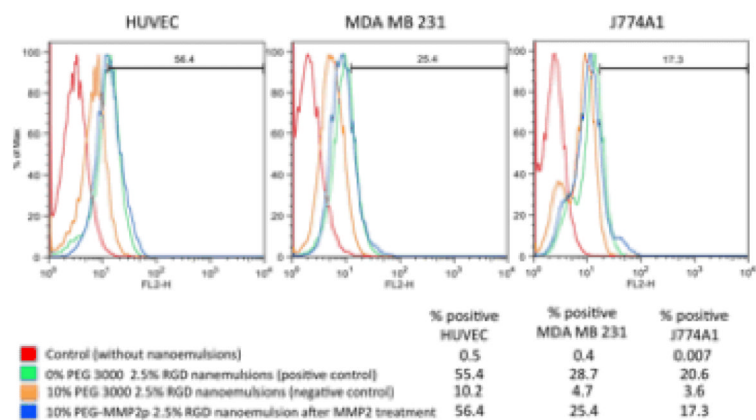
Fig. 4.
Flow cytometry histograms of HUVEC, MDA MB 231 and J774A1 cells incubated with (i) 0% mPEG3000 and 2.5% RGD nanoemulsions, (ii) 10% mPEG3000 and 2.5% RGD nanoemulsions and (iii) 10% PEG-MMP2p and 2.5% RGD nanoemulsions after MMP2 treatment.
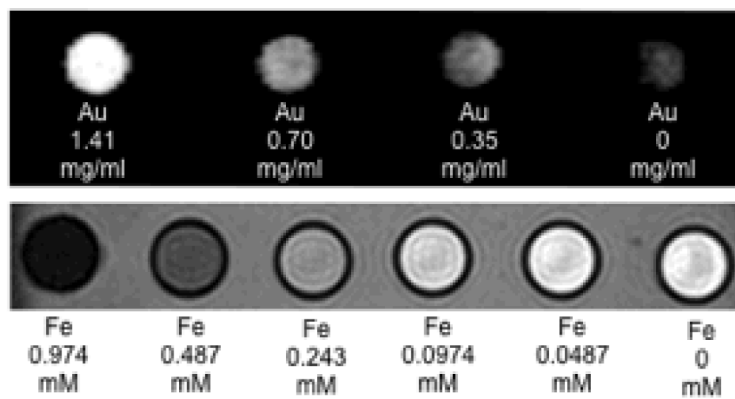
Fig. 5.
CT (top) and MR (bottom) images of Au and IO theranostic nanoemulsion phantoms.