Molecular tools for light-navigated therapy

Reeßing, Friederike

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Chapter 4:

A LIGHT-RESPONSIVE LIPOSOMAL AGENT FOR MRI CONTRAST ENHANCEMENT AND CARGO DELIVERY

Medical magnetic resonance imaging (MRI) produces high-resolution anatomical images of the human body, but has limited capacity to provide useful molecular information. The light-responsive, liposomal MRI contrast agent described herein could be used to provide an intrinsic theranostic aspect to MRI and enable tracking the distribution and cargo release of drug delivery systems upon light-triggered activation.

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Part of the synthetic work presented in this chapter was performed by Ms. Chantal Mulder in the context of her MSc research project.
A LIGHT-RESPONSIVE LIPOSOMAL AGENT FOR MRI CONTRAST ENHANCEMENT AND CARGO DELIVERY

INTRODUCTION

In the clinic, Magnetic Resonance Imaging (MRI) is widely used as a non-invasive medical imaging technique that provides anatomical information with excellent resolution without exposing the patient to damaging ionizing radiation. The contrast in MRI stems from the difference in local densities and relaxation times of protons in human tissues. In about 30 million clinical scans performed annually worldwide, the contrast is further enhanced by the administration of paramagnetic contrast agents (CAs), such as gadolinium(III) complexes, which significantly shorten the $T_1$ relaxation time of surrounding protons. This causes a higher intensity in the acquired MR image and enables the visualization of the distribution of the CA in the human body.

Tissue-specific CAs are currently available to image structures that are barely distinguishable on a regular scan, such as the vascularization of the brain. However, due to the low sensitivity of MRI, the requirement of relatively high local concentrations (0.01 mM) of CAs for effective signal enhancement presents a major limitation, especially regarding the development of CAs for the imaging of disease-specific biomarkers that are present at much lower concentrations. Therefore, while structures that are highly abundant in the human body, such as fibrin or collagen, can be readily visualized - targeted imaging of less abundant structures, such as receptors or other proteins that are associated with certain pathological conditions, remains challenging.

This problem has been previously addressed through the development of responsive CAs, that show increased contrast enhancement upon activation by enzymes leading to signal amplification. Other CAs that enable diagnostics beyond purely anatomical imaging, take advantage of changes in ion or neurotransmitter concentration as well as changes in pH, temperature or redox potential, inter alia. Even though the effectiveness of this strategy has been proven for these targets, certain limitations to this approach remain: for instance, the untimely and/or off-target activation, as the conditions for the activation of the responsive CAs are frequently also present outside the lesion(s) in normal, healthy tissues.

In this respect, local activation of a CA with light could be used as a general strategy for improved MRI contrast enhancement. Of note, the use of photons as CA activators does not interfere with endogenous physiological processes. Moreover, light can be delivered with a very high spatiotemporal resolution and is biocompatible within a broad wavelength range. Due to these advantages, the research fields focusing on the use of light for biomedical applications, e.g. for the selective activation of drugs (photopharmacology), in photodynamic therapy, or for the activation of genetically engineered ion channels (optogenetics) are expanding very quickly fueled by truly promising results.

The research presented in this chapter aims to establish a general strategy for signal amplification in contrast-enhanced MRI, which could be used for selective imaging of
low-concentration targets. This strategy envisions the use of targeted light-emitting systems that locally activate the MRI CA through the production of photons, resulting in signal amplification. A key advantage of this approach is that the use of light for activation would provide a CA that is readily adaptable to various targets, by changing the light-emitting component in contrast to the systems described above that are limited to one specific target.

DEVELOPMENT OF A VIOLET-LIGHT-ACTIVATABLE LIPOSOMAL AGENT FOR MRI CONTRAST ENHANCEMENT AND DRUG DELIVERY

As a key step towards this general goal, the synthesis and evaluation of a photoactivated MRI CA that changes its relaxivity in response to irradiation with violet light is described here. Furthermore, it is demonstrated how this liposomal CA can simultaneously be used as a responsive cargo delivery system.

For the successful design of a photoactivatable CA, it is crucial to consider the molecular characteristics influencing its relaxivity. As defined in the Solomon-Bloembergen-Morgan theory, these characteristics are: (i) the tumbling time, and thus the size of a CA, (ii) the number and (iii) the residence time of water molecules coordinated to the gadolinium complex. Especially, the molecular control over of the first two features is straightforward and can be used for the design of responsive CA, as shown in various molecular approaches. In order to prove the concept of light-responsive contrast agents, we designed a CA that, upon light-activation, converts from a relatively large nanoscopic complex to a single small molecule. This conversion is accompanied by a marked change in tumbling time, which results in a significant change in relaxivity. A related strategy has been successfully used in an enzyme-based approach by Aime and co-workers for the development of an MRI CA responsive to matrix metallo-proteinase.

We constructed the light-responsive MRI contrast agent by linking a gadolinium complex, via a photocleavable group, to a lipophilic alkyl chain, which functions as an anchoring group for liposomes (Fig. 4.2). We hypothesized that irradiation of such liposomes would induce photocleavage and subsequent release of the Gd complex with an additional free carboxylic acid group (Fig. 4.2). Besides the relaxivity change due to modulation of the tumbling time, also the hydration state of the Gd complex is expected to change, since the liberated carboxylate moiety may coordinate to the gadolinium ion replacing one water molecule from the complex. Altogether, these processes lead to a lengthening of the $T_1$ relaxation time and therefore to a signal reduction. Since it is generally preferred to obtain an increase in signal upon activation, we envision a ratiometric approach, analyzing the $T_1$ and $T_2$ relaxation time for future applications, following the example of Aime et al.
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Fig. 4.1: Design principle for light-activated MRI contrast agents for imaging (a, b) and theranostics (c, d); a) the Gd\(^{III}\) complex for \(T_1\)-signal enhancement is incorporated into the bilayer of liposomes. Upon irradiation with \(\lambda = 400\) nm light, the Gd\(^{III}\) complex is released, causing a decrease in \(T_1\) relaxation rate; b) the liposomes selectively penetrate into tumor tissue due to the enhanced permeability and retention (EPR) effect. A targeting moiety (here an antibody) binding to the tumor cells bears a light-emitting system that leads to the release of the Gd\(^{III}\) complex from the lipid bilayer of the liposomes; c) upon light irradiation, the liposomes release the Gd\(^{III}\) complex with concurrent release of the payload incorporated in their aqueous lumen; d) the liposomal CA can be used for site-selective drug delivery using local irradiation as a stimulus to release the liposome cargo. The response to light can be monitored by MRI due to a change in relaxivity.
Fig. 4.2: Molecular structure of the Gd\textsuperscript{III} complex of compound 1 (Gd-1) and its photo-product Gd-2. Gd-1 bears a Gd\textsuperscript{III} complex and an anchoring group for liposomes connected via a photocleavable linker. Upon light exposure, the compound cleaves and the Gd\textsuperscript{III} complex is released.

RESULTS

Synthesis

To achieve an efficient, short and high-yielding synthesis, we used a Passerini multicomponent reaction (MCR) for creating the photoactive scaffold\textsuperscript{28} that could in subsequent transformations be modified with a liposome-anchoring group and a chelator for gadolinium. For this key step in the synthesis, nitroveratryl aldehyde, 4-bromo-butanoic acid and isocyanide\textsuperscript{2} were reacted to afford the core structure\textsuperscript{3}, bearing an alkyne functionality. This alkyne was then reacted in a variant of the copper(I)-catalyzed azide-alkyne Huisgen cycloaddition\textsuperscript{29} with azide\textsuperscript{5,30,31} in dichloromethane, which ensured the solubility of both substrates. Subsequently, the Gd\textsuperscript{III} ligand\textsuperscript{32} was introduced into compound 6 through a nucleophilic substitution. Being aware of the recent concerns about the accumulation of gadolinium in tissues due to the release from complexes with linear ligands,\textsuperscript{33} we aimed at increasing the complex stability by choosing a cyclic ligand over the less stable linear variant.\textsuperscript{34} Deprotection of the tert-butyl groups gave the multifunctional compound 1, whose structure includes a photoreactive moiety, a ligand for Gd\textsuperscript{III} and two alkyl chains for docking into the liposomes.
Liposome preparation

The liposomes were prepared by hydrating a dry lipid film of an equimolar mixture of compound 1 and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with TBS buffer (pH 7.5) to give a concentration of 2.5 mM for each component. Repeated cycles of freezing, thawing and ultrasonication produced small, unilamellar vesicles (SUVs), to which a solution of gadolinium(III) chloride was added to form the lanthanide complex. By adding Gd\textsuperscript{III} to the pre-formed liposomes, we assume to form the complex with the molecules whose hydrophilic head group face to the outside only, resulting in the photo-triggered release of the Gd\textsuperscript{III} complex solely to outside and not the lumen. After removal of unselectively bound Gd\textsuperscript{III} ions by dialysis, cryoTEM (Fig. 4.4a), dynamic light scattering analysis (see experimental section) and EDX spectroscopy (Fig. 4.4c,b) were applied to confirm the formation of SUVs and accumulation of gadolinium in the liposomes. No free Gd\textsuperscript{III} was observed in the medium and the ratio of phosphorus to gadolinium signal was analyzed and determined to be 1:1.76 (Gd/P). The final concentration of gadolinium in the sample was 0.95 mM (150 ppm), as determined by inductively coupled plasma-optical emission spectrometry (ICP-OES), indicating that the complex was formed with 38% of all available ligands. Assuming that no free Gd\textsuperscript{III} is present (as indicated by a photometric assay, see experimental section) and that the complex was only formed with the ligands facing outside, it can be calculated that ca. 76% of all the available ligands coordinate to a Gd\textsuperscript{III} ion.
Fig. 4.4: CryoTEM and EDX analysis of the MR-active liposomes; a) cryoTEM image of dialyzed liposomes, containing 50% DOPC and 50% compound 1, with 1 eq. of GdCl₃ added; b) EDX spectrum of an area of liposome aggregation after addition of 2 equivalents of GdCl₃ and subsequent dialysis; c) EDX spectrum of the background of the same sample as in b). The absence of gadolinium signal in c) indicates the selective binding of Gd³⁺ to the liposomes.

**Fast-Field-Cycling NMR relaxometry**

Fast Field Cycling (FFC) NMR relaxometry is a method that allows the determination of the spin-lattice relaxation time (T₁) over a range of proton Larmor frequencies and is commonly used in the molecular evaluation of MRI CAs. Here, we used FFC NMR relaxometry to study the synthetic reproducibility, stability and photoresponsiveness of the liposome formulation.

Robustness of the method used for liposome preparation was evaluated by comparing the relaxation rate of three independently prepared samples and high uniformity was observed (Fig. 4.5b, Fig. 4.13a, samples 1-3). Subsequently, the stability of the liposomes over time was assessed. Storage of sample 3 at 4 °C for one week led to a decrease in relaxation rate of only 3% (measured at 10 MHz), and even after four weeks merely 7% decrease was observed (Fig. 4.5b). Importantly, the shape of the recorded nuclear magnetic resonance dispersion (NMRD) profiles persisted over time (Fig. 4.13b, experimental section), confirming the stability of the nanoscopic complex, as could be derived from the characteristic increase in relaxivity at proton Larmor frequencies above 7 MHz.
Next, we examined the effect of irradiation with light at $\lambda = 400$ nm on the relaxivity to evaluate whether the contrast agent showed the desired photoresponsiveness. The data confirmed that irradiation results in a marked decrease in relaxivity within 1 h of irradiation (Fig. 4.5a,c). Already after 10 min, a change $\Delta r_1$ of 21% (measured at 10 MHz) was observed, which is comparable to values reported for other light-switchable paramagnetic metal complexes.\textsuperscript{42–44} Moreover, the decrease in relaxivity coincided with
a change in the shape of the NMRD profile. The increase at higher field strength (>7 MHz), which is characteristic for nanoscopic contrast agents, diminishes with increasing irradiation time, suggesting that the Gd\textsuperscript{III} complex converged to a small molecule.\textsuperscript{26} These results indicated the successful uncaging of the Gd\textsuperscript{III} complex from the liposome. With prolonged irradiation (60 min in total), the decrease in relaxivity could be further enhanced to 49% of the initial value (from 10.7 m\textsuperscript{M}\textsuperscript{-1}s\textsuperscript{-1} to 5.2 m\textsuperscript{M}\textsuperscript{-1}s\textsuperscript{-1}). Likewise, the relaxation rate at 4.7 T, which is closer to the operating field of (pre-) clinical MRI scanners, was shown to clearly decrease from 4.8 s\textsuperscript{-1} to 1.9 s\textsuperscript{-1} (corresponds to 39% of the initial value) after 60 min irradiation (Fig. 4.15, see experimental section).

To confirm that the decrease in relaxivity stems from the photocleavage of compound Gd\textsuperscript{-1} docked into the liposome bilayer, we compared the kinetics of the relaxivity decrease (measured by FFC relaxometry) and the uncaging process, followed by UV-Vis spectroscopy. Towards this goal, we followed the changes in absorbance at \(\lambda = 365\) nm, the absorption maximum of the intact ortho-nitro-phenyl moiety, under the assumption that the decrease is quantitatively correlated with photocleavage.\textsuperscript{45–48} As anticipated, absorption at \(\lambda = 365\) nm diminished upon exposure to \(\lambda = 400\) nm light, which coincided with a decrease in relaxivity (Fig. 4.5c). The lifetimes for these two processes were determined to be 23.6 min for change in relaxivity and 25.1 min for the photocleavage. These findings confirm that the change in relaxivity indeed results from the photocleavage of compound \textsuperscript{1}.

A major concern of the application of gadolinium-based CAs is the instability of the Gd\textsuperscript{III} complex, as free Gd\textsuperscript{III} has unwanted long-term toxic effects on the human body.\textsuperscript{3} While cyclic complexes are generally considered to be stable,\textsuperscript{4,34,49} we nevertheless investigated the stability of our Gd\textsuperscript{III} complex upon irradiation, employing a photometric assay in which xylenol orange is used as a sensitive indicator for the presence of free Gd\textsuperscript{III} ions.\textsuperscript{50} The results showed that the complex stability is not affected by light as there is no substantial increase in free Gd\textsuperscript{III} concentration after 1 h of irradiation with light at \(\lambda = 400\) nm (Fig. 4.16b, see experimental section).

**Cytotoxicity studies**

In order to validate the applicability of the presented contrast agent in a biological setting, we evaluated the potential toxicity of the liposomal formulation towards human umbilical vein endothelial cells (HUVEC), human normal epithelial cells and M1 macrophages. Since the photocleavage of ortho-nitro phenyl-based photo-protecting groups is a complex process with a multitude of products,\textsuperscript{51} we aimed to ascertain that there are no toxic effects of the formulation throughout the course of cleavage. To this end, cell death was evaluated by flow cytometry using the Annexin V-FITC/PI method, which did not indicate any cytotoxic effect of the liposomes that contained Gd\textsuperscript{-1} in their bilayer, as compared to the control with cell medium (Fig. 4.6). Similarly, irradiation of
A light-responsive liposomal agent for MRI contrast enhancement and cargo delivery

the photoresponsive liposomes prior to addition to the cells did not enhance cell death either, indicating that no toxic products are formed upon photocleavage.

Fig. 4.6: Assessment of cytotoxic effects of liposomes containing Gd-1 evaluated by flow cytometry using Annexin V-FITC/PI method. The photoresponsive liposomes appear not to exert any obvious cytotoxic effects on HUVEC, normal epithelial cells or M1 macrophages, neither in the dark, nor after irradiation with λ = 400 nm for up to 60 min. The medium and medium supplemented with 70 µM Taxol were used as negative and positive control, respectively.

Cargo release from liposomes

Next, we explored the possibility of using the liposomal CA for MRI-guided drug delivery, as outlined in Fig. 4.1c. To this end, we examined the effect of the photocleavage on the liposome structure and integrity. In particular, we evaluated whether cleavage of compound Gd-1 destabilizes the lipid bilayer and thereby promotes the release of the liposome cargo, or if the liposomes stay intact without releasing their content.

To address this question, we probed the changes in permeability of the liposomes under irradiation, using calcein as a model for drugs that can be delivered as liposomal preparations. Calcein is a fluorescent dye that can be encapsulated at high, self-quenching concentrations in the aqueous lumen of liposomes. Release of calcein from the liposomes results in increased fluorescence. After storage for 1 h in the dark, the fluorescence intensity of the liposomes decorated with compound Gd-1 and loaded with calcein at self-quenching concentration (0.1 M) only marginally increased (Fig. 4.7), showing the stability of the nano-container in the dark. In contrast, upon irradiation with λ = 400 nm light, a clear increase in fluorescence was observed, indicating that photocleavage process destabilizes the integrity of the lipid bilayer of the liposomes.
Unfortunately, it was not possible to determine the exact release rate due to photobleaching of calcein under irradiation. Dynamic light scattering (DLS) analysis showed re-organization of the liposomes, leading to a net decrease in size (Fig. 4.18, experimental section). Altogether, the cargo release upon irradiation, concurrent with the change in magnetic relaxivity, may be exploited for using this light-responsive liposomal MRI CA also for theranostic purposes.

**Fig. 4.7:** Evaluation of the effect of photocleavage on liposome integrity. Fluorescence intensity ($\lambda_{ex} = 480\,\text{nm}$, $\lambda_{em} = 520\,\text{nm}$) of 50% DOPC/50% compound 1 liposomes loaded with calcein at self-quenching concentration (0.1 M), measured as a technical triplicate. Upon irradiation, liposome membrane integrity is reduced as is evident from an increase in fluorescence due to calcein release.

**DISCUSSION AND CONCLUSION**

The described research presents the proof of principle for an activated MRI contrast agent with intrinsic capability for drug delivery, offering prospects for diagnostics and image-guided therapy. In this context, we developed and evaluated a light-responsive liposomal gadolinium complex and we demonstrated that its exposure to light results in a marked decrease in relaxation rate, indicating the conversion of a nanoscopic Gd$^{3+}$ complex into a small molecule. Various experimental techniques, including cryoTEM imaging, EDX spectroscopy and FFC relaxometry, supported the concept we proposed for constructing light-responsive MRI CAs.
A LIGHT-RESPONSIVE LIPOSOMAL AGENT FOR MRI CONTRAST ENHANCEMENT AND CARGO DELIVERY

The increase in the permeability of the liposomes upon light exposure, as demonstrated by calcein release, opens new possibilities to employ this CA for theranostic applications. To date, there are only few examples of agents combining MR-imaging with pharmacotherapy,\textsuperscript{23,56} e.g. by incorporating MRI CAs into nanoparticles for the assessment of the integrity of the latter. Other promising approaches include the thermo-sensitive release of MRI contrast agents and therapeutics from liposomes and a combination of Gd\textsuperscript{III} complexes with porphyrins for photodynamic therapy.\textsuperscript{57,58} Our strategy, however, stands out due to the prospect of using internal light-emitting targeting moieties for activation, which makes the drug release system unbiased and independent of external stimuli.

In further perspective, we envision to use a two-step approach in which the patient is first injected with a disease-specific antibody (or derivate thereof) equipped with a bioluminescent enzyme-substrate system. After its injection, the conjugate is allowed to selectively accumulate in the lesion(s). Subsequently, the corresponding substrate of the light-producing enzyme is injected that is converted at the site of the lesion only, resulting in the localized generation of photons. In turn, these photons locally enhance MRI contrast of the light-activatable CA injected in a second step. The relaxivity of the presented example decreases upon light-activation, which could possibly be distinguished from clearance of the CA by a ratiometric approach, as mentioned earlier.\textsuperscript{27} Deliberate timing of the scans and stepwise administration of the respective components would result in distinguished resolution. Possible luminescent tools, that could be used for the activation of the CA, are luciferin/luciferase systems, which have been optimized for \textit{in vivo} bioluminescence imaging.\textsuperscript{59,60} In addition, the expression of horseradish peroxidase in mammalian cells has been explored aiming at local prodrug activation \textit{in vivo}.\textsuperscript{61} Both enzyme families are often used in \textit{in vitro} biomedical research, providing a good base for the development of suitable bioluminescent targeting moieties. Altogether, this method may be useful to reduce the side effects in systemic chemotherapy for the treatment of localized malignant disease.

For the clinical development of the CA reported here, it is crucial to shift the activation wavelength from $\lambda = 400$ nm to above $600$ nm in order to maximize tissue penetration of the light, while reducing wavelength associated toxicity.\textsuperscript{18} Recent developments in green and red light-responsive photocaging groups, such as BODIPY-based PPGs, which will be discussed in the following section, suggest that possibilities for efficient activation within a clinical setting are emerging.\textsuperscript{62–67} With regards to the use of our system for MRI guided drug delivery, a bathochromic shift in activation wavelength would open up the possibility to use clinically established light delivery systems,\textsuperscript{68–70} commonly used in photodynamic and photothermal therapies, for triggering the local drug release. Moreover, the introduction of polyethylene glycol (PEG) chains is also currently being studied in our laboratories in order to increase the stability and circulation time of the liposomes in the body.\textsuperscript{71}
TOWARDS RED-LIGHT-ACTIVATED MRI CONTRAST ENHANCEMENT AND DRUG DELIVERY

As outlined above, light-responsive MRI contrast agents have the potential to become a tool for obtaining molecular information about biological processes with high resolution. The research presented in the first part of this chapter focused on the development of MR-trackable liposomes that change relaxivity upon irradiation with $\lambda = 400$ nm (violet) light. Since light of this wavelength is strongly absorbed in the body, we aimed to bring this proof-of-principle study closer to application by establishing an analogous system responsive to red or near-infrared (NIR) light, which shows significantly deeper tissue penetration. A BODIPY moiety introduced in chapter 2 (Fig. 4.8, shown in orange) seemed to be a promising molecular scaffold to replace the ortho-nitro phenyl structure of the previous design. The activation wavelength for BODIPY-based photocleavable groups typically lies above $\lambda = 500$ nm and can be shifted bathochromically even further by extension of the chromophoric system with e.g two styryl groups (Fig. 4.8 in red).\textsuperscript{72}

Fig. 4.8: Molecular design of compound 8, a red-light-responsive, photocleavable MRI contrast agent that can be incorporated into the bilayer of liposomes.

Since prior research (chapter 2) indicated that Passerini MCRs employing BODIPYs with extended $\pi$-systems is challenging and low yielding, we chose to take another synthetic approach for this project. Specifically, we decided to attach two alkyl chains as anchoring group for liposomes as para substituent of the styryl groups (Fig. 4.8, shown in dark blue) and the Gd$^{3+}$ ligand in meso-position of the BODIPY (Fig. 4.8, shown in light blue). Photochemical activation of the BODIPY core is expected to release the Gd$^{3+}$ complex, resulting in a similar change in relaxivity as observed for the system described above.
RESULTS AND DISCUSSION

Fig. 4.9 illustrates the envisioned synthetic route towards the red-light-activatable amphiphilic molecule \( \text{8} \) starting from acetylated BODIPY \( \text{9} \) (see chapter 2). This compound was reacted with aldehyde \( \text{10} \) in a Knoevenagel condensation to yield the corresponding BODIPY-derivative with an extended conjugated \( \pi \)-electron system and two lipophilic chains for the incorporation of the compound into the bilayer of liposomes. Similarly to the difficulties we encountered when we aimed to establish reproducible synthetic procedures for MCRs with BODIPYs (chapter 2), the isolated yield varied from 6 - 57\% under identical reaction conditions. Notably, initial problems regarding the instability of the product during purification via silica column chromatography were solved by addition of a base (triethylamine) to the eluents, which led to a general increase in isolated yield but still could not solve the problems of irreproducibility. Subsequent hydrolysis of the ester yielded compound \( \text{11} \).

Previous work in our group had shown that photocleavage of similar BODIPYs with efficient release of an amine can be achieved, when the corresponding amine is connected via a carbamate linkage in meso position.\(^{62,63}\) Since our aim was to use light to trigger the release of an Gd\(^{III} \) complex for MRI signal alteration, we synthesized a suitable ligand bearing an amine functionality (compound \( \text{13} \), Fig. 4.9a).\(^{74}\) In order to install the carbamate functionality for connecting compounds \( \text{11} \) and \( \text{13} \), two strategies can be envisioned: either the alcohol or the amine can be activated with e.g. para-nitro-phenyl chloroformate, followed by reaction with the respective amine or alcohol, as illustrated in Fig. 4.9b-d. Inspired by prior research,\(^{62}\) we first proceeded with the activation of the alcohol. After optimization of the reaction conditions, activated carbonate \( \text{15} \) could be obtained in moderate yield. However, subsequent coupling to compound \( \text{13} \) was not successful and therefore we explored the alternative approach. Hence, Gd\(^{III} \) ligand \( \text{13} \) was reacted with \textit{para}-nitro-phenyl chloroformate to afford carbamate \( \text{16} \). Unfortunately, even after thorough screening of numerous reaction conditions using various bases and equivalents of substrates, at room temperature or 40 \(^\circ\)C in different solvents, together with addition of a coupling reagent (DMAP) and the use of microwave irradiation (see Table 4.1), we did not succeed to obtain compound \( \text{14} \) in more than trace amounts.
Fig. 4.9: Synthetic approaches to afford compound 8. a) Synthesis of BODIPY 11 with extended chromophoric system and of Gd\(^{III}\) ligand 13 bearing an amine group, b-d) Attempted strategies for the coupling of compound 11 and 13, or their derivatives, with a carbamate functionality as a linker.
Table 4.1: Screened reaction conditions for the coupling of compound 11 and 16.

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<td>1</td>
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<td>1</td>
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In order to tackle the problem of low conversion, we decided to investigate another way to introduce the carbamate functionality, namely by reacting amine 13 with triphosgene, followed by addition of the alcohol. For assessment of the best possible synthetic procedure, we used the unsubstituted BODIPY alcohol 9 as a model substrate. Treatment of amine 16 with triphosgene for 1-3 h with subsequent reaction for 1 h with the alcohol that has been pre-treated with NaH, in a one-pot procedure, gave the best results, even though the conversion to product was generally still quite low (Fig. 4.11, see experimental section). With those optimized conditions, we explored the scope of the reaction by testing different alcohols that are precursors of PPGs, such as 6-nitroverartryl alcohol or 7-(diethyl-amino)-4-hydroxymethylcoumarin, both of which gave even better results in terms of conversion. Finally, we reacted alcohol 11 with compound 13 in the same procedure and were delighted to observe conversion to the desired conjugate 14, as confirmed by high resolution mass spectrometry (see Fig. 4.12, experimental section) performed on a fraction isolated from silica gel chromatography. Using this sample, we employed UV-Vis spectroscopy to test if irradiation with red light induces photolysis of the molecule: While in the dark no changes in the UV-Vis absorption spectrum were observed (Fig. 4.10a), irradiation with λ = 650 nm led to a clear decrease in absorbance (Fig. 4.10b). After deprotection of the tert-butyl protecting groups and GdIII complexation, we aim to incorporate the molecule into liposomes and assess its potential as a red-light sensitive probe for contrast enhancement and cargo delivery.

**CONCLUSION**

We designed and synthesized a red-light-responsive system, based on a BODIPY scaffold, as a promising tool for prospective in vivo use in biological systems. We optimized key steps in the synthetic route, such as the carbamate formation, and were able to prove the red-light responsiveness of the synthesized precursor of the final molecule. Unfortunately, the synthesis of this type of derivatives still remains challenging.
and low-yielding. The respective molecules typically only need low-energy light for their excitation to the excited state, due to a smaller HOMO-LUMO gap. With regard to extended π-conjugated systems as the one at hand, the energy difference between HOMO and LUMO is smaller due to both: a rise in energy level of the HOMO and decrease of the LUMO. This leads to higher susceptibility of the molecule for nucleophilic as well as electrophilic attack and hence reduced stability, possibly explaining the observed problematic reactivity.

Despite the challenges encountered in the research described herein, we are still convinced that the future of light-activatable therapeutic or imaging systems lies in the development of red- or NIR-light responsive probes. As an alternative to shifting the activation wavelength of the light-responsive moiety, one could envision the use of upconverting nanoparticles (UCNP) instead of a liposomal formulation, in combination with a comparable photolabile group as used in the first section of this chapter. This way, incoming light of lower energy will be upconverted by the nanoparticles and may ultimately serve to cleave the photolabile molecule and release the contrast agent.75,76 This system, however, would only allow imaging of the light activation without additional therapeutic drug delivery.

AUTHOR CONTRIBUTIONS

F. Reeßing: Synthesis of compounds 1-7 and S1-S2, (photo-)chemical and relaxometric analysis, liposome preparation, cargo release assay.

Dr. M.C.A. Stuart: Acquisition of cryoTEM pictures and EDX analysis.

D.F. Samplonius: Cytotoxicity assays on HUVEC and epithelial cells.

C. Mulder: Synthesis and analysis of compounds 9-19, S3-S4.

ACKNOWLEDGEMENTS

The financial support from the Dutch Organization for Scientific Research (NWO VIDI grant no. 723.014.001 for W.S.), the Dutch Cancer Society (KWF grant RUG2014-6986 for W.H.) and the Dutch Ministry of Education, Culture and Science (Gravitation program 024.001.035 for B.L.F.) is gratefully acknowledged. We thank Ms. Verena Böhmer for helpful discussions, eng. Theodora Tiemersma-Wegman for MS analysis and eng. Hans van der Velde for ICP-OES analysis. Pieter van der Meulen for help with determination of relaxation rates at 4.7 T and Mark A.J.M. Hendriks for the cytotoxicity assay on macrophages.
EXPERIMENTAL SECTION

GENERAL INFORMATION

Starting materials, reagents and solvents were purchased from Sigma–Aldrich, Acros, Fluka, Fisher Scientific, TCI, Combi blocks, Sterling Chemicals and were used as received. Solvents for the reactions were of quality puriss., p.a.. Anhydrous solvents were purified by passage through solvent purification columns (MBraun SPS-800). For aqueous solutions, deionized water was used. Thin Layer Chromatography analyses were performed on commercial Kieselgel 60, F254 silica gel plates with fluorescence-indicator UV254 (Merck, TLC silica gel 60 F254). For detection of components, UV light at λ = 254 nm or λ = 365 nm was used. Alternatively, oxidative staining using aqueous basic potassium permanganate solution (KMnO₄) or aqueous acidic cerium phosphomolybdic acid solution (Seebach's stain) was used. Drying of solutions was performed with MgSO₄ and volatiles were removed with a rotary evaporator. Flash column chromatography was performed with Silicagel, pore size 60 Å, 40-63 µm particle size.

Nuclear Magnetic Resonance spectra were measured with an Agilent Technologies 400-MR (400/54 Premium Shielded) spectrometer (400 MHz) or Bruker Avance 600 NMR Spectrometer (600MHz). All spectra were measured at room temperature (22–24 °C). The multiplicities of the signals are denoted by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad signal). All ¹³C-NMR spectra are ¹H-broadband decoupled. High-resolution mass spectrometric measurements were performed using a Thermo scientific LTQ OrbitrapXL spectrometer with ESI ionization. The ions are given in m/z-units. Melting points were recorded using a Stuart analogue capillary melting point SMP11 apparatus. For spectroscopic measurements, solutions in Uvasol® grade solvents were measured in a 10 mm quartz cuvette. UV/Vis absorption spectra were recorded on an Agilent 8453 UV/Vis spectrophotometer with diode array detection. Temperature-control was exerted through a Peltier based temperature controlled cuvette holder (QuantumNorthwest). Fluorescence measurements were performed on a BioTek Synergy H1 microplate reader. ICP-OES analysis was performed on a Perkin Elmer Optima 7000 DV spectrometer. NMRD profiles were recorded on a Stelar 0.25 T FFC relaxometer SMARtracer.

Irradiation experiments were performed with a λ = 400 nm (3x Roithner VL-400-Emitter, optical power 1000 mW, λₘₐₓ = 400 nm, FWHM 11.9 nm) and λ = 650 nm (3x XML PD01, optical power 1200 mW, λₘₐₓ = 652 nm, FWHM 26.4 nm) LED system (Sahlmann Photochemical Solutions).
SYNTHETIC PROCEDURES AND COMPOUND CHARACTERIZATION

2: 2-Isocyano-N-(prop-2-yn-1-yl)acetamide

Compound 2 was prepared by a modification of a literature procedure.\textsuperscript{77,78} A mixture of methyl isocynoacetate (7.82 mmol, 711 µL) and propargylamine (15.6 mmol, 1.00 mL) was stirred at room temperature overnight. The formed solid was filtered off and washed with Et\textsubscript{2}O to provide compound 2 as light brown powder (759 mg, 78%). \(R_f = 0.63\) (pentane/AcOEt, 1:1 v/v); Mp. 109°C; \(^1H\) NMR (400 MHz, DMSO-d\textsubscript{6}): \(\delta 3.15\) (t, \(J = 2.4\) Hz, 1H, CH\textsubscript{3}), 3.88 (dd, \(J = 5.6\) Hz, \(J = 2.4\) Hz, 2H, CH\textsubscript{2}NH), 4.35 (s, 2H, CH\textsubscript{2}NC), 8.61 (br s, 1H, NH). \(^1H\) NMR spectrum is in agreement with published data.\textsuperscript{77}

3: 1-(4,5-Dimethoxy-2-nitrophenyl)-2-oxo-2-(2-oxo-2-(prop-2-yn-1-ylamino)ethyl) amino)-ethyl 4-bromobutanoate

A solution of compound 2 (1.62 mmol, 200 mg), 6-nitroveratraldehyde (1.34 mmol, 285 mg) and 4-bromobutyric acid (1.62 mmol, 270 mg) in chloroform (2.7 mL) was stirred at room temperature for 48 h. The volatiles were evaporated and the product was purified by flash column chromatography (pentane/AcOEt, 8:2 to 2:8 v/v) to give a white powder (373 mg, 56%). \(R_f = 0.18\) (pentane/AcOEt, 1:1 v/v); Mp. 142-143°C; \(^1H\) NMR (400 MHz, DMSO-d\textsubscript{6}): \(\delta 2.03-2.12\) (m, 2H, CH\textsubscript{2}CH\textsubscript{2}Br), 2.51-2.66 (m, 2H, CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{2}Br), 3.09 (t, \(J = 2.4\) Hz, 1H, CCH), 3.48-3.63 (m, 2H, CH\textsubscript{2}Br), 3.74 (d, \(J = 6.0\) Hz, 2H, CH\textsubscript{2}NH), 3.82-3.85 (m, 2H, CH\textsubscript{2}CC), 3.87 (s, 3H, CH\textsubscript{3}O), 3.90 (s, 3H, CH\textsubscript{3}O), 6.60 (s, 1H, CHO), 7.14 (s, 1H, ArH), 7.64 (s, 1H, ArH), 8.38 (t, \(J = 5.2\) Hz, 1H, NH), 8.60 (t, \(J = 6.0\) Hz, 1H, NH). \(^13C\) NMR (100 MHz, DMSO-d\textsubscript{6}): \(\delta 28.1, 28.3, 32.3, 34.3, 42.1, 56.6, 56.8, 70.8, 73.6, 81.3, 108.7, 111.9, 124.7, 141.8, 149.0, 153.2, 167.7, 168.6, 171.5. HRMS (ESI+) calc. for [M+H]+ (C\textsubscript{19}H\textsubscript{23}N\textsubscript{3}O\textsubscript{8}Br): 500.0663, found: 500.0652.
4: 1-(Dodecan-2-yloxy)-3-(dodecyloxy)propan-2-ol

NaH (60% in mineral oil, 8.58 mmol, 206 mg) was added to dodecan-1-ol (18.0 mmol, 3.35 g) at 100 °C. After stirring for 30 min, epichlorohydrin (4.2 mmol, 0.39 g) was added dropwise and the mixture was stirred at 100 °C overnight. H₂O was added to quench the reaction, followed by DCM (50 mL). The aqueous phase was extracted with DCM, the collected organic phases were washed with brine and dried over MgSO₄. Evaporation of volatiles and purification with flash column chromatography (pentane/AcOEt, 95:5 v/v) gave the product as a pale yellow solid (572 mg, 32%). Rᵣ = 0.69 (pentane/AcOEt, 95:5 v/v); Mp. 30-32 °C; ¹H NMR (400 MHz, CDCl₃): δ 0.88 (t, 6H, C₃H₃), 1.26-1.29 (m, 36H, CH₃(C₉H₂)₉), 1.57 (m, 4H, C₉H₂CH₂O), 2.50 (d, 1H, OH), 3.41-3.51 (m, 8H, (C₉H₂OCH₂)₂CHOH), 3.92-3.96 (m, 1H, C₉H₂O); ¹³C NMR (100 MHz, CDCl₃): δ 14.1, 22.7, 26.1, 29.3, 29.5, 29.6, 29.7, 31.9, 69.5, 71.7, 71.8. ¹H NMR spectrum is in agreement with published data.

S1: 1-(Dodecan-2-yloxy)-3-(dodecyloxy)propan-2-yl methanesulfonate

A solution of methanesulfonyl chloride (1.46 mmol, 167 mg) in DCM (1 mL) was slowly added to a solution of compound 4 (1.17 mmol, 500 mg) and triethylamine (1.46 mmol, 148 mg) in DCM (3 mL) at room temperature. The mixture was stirred for 2 h. AcOEt was added and the organic phase was washed with H₂O and brine and dried (MgSO₄). Evaporation of volatiles gave the product as a pale yellow solid (484 mg, 82%). Mp. 57°C (lit. 58-59 °C); ¹H NMR (400 MHz, CDCl₃): δ 0.88 (t, 6H, C₃H₃), 1.24-1.32 (m, 36H, CH₃(C₉H₂)₉), 1.54-1.57 (m, 4H, C₉H₂CH₂O), 3.08 (s, 3H, SO₂C₃H₃), 3.41-3.51 (m, 4H, CH₂OCH₂CH₂), 3.60-3.67 (m, 4H, CH₂CH₂O), 4.81 (q, 1H, CH₂OH). ¹H NMR spectrum is in agreement with published data.

5: 1-(2-Azido-3-(dodecan-2-yloxy)propoxy)dodecane

Compound S1 (0.79 mmol, 400 mg) and NaN₃ (6.30 mmol, 410 mg) were dissolved in DMF (3 mL) and the reaction mixture was stirred at 100 °C overnight. AcOEt was added and the organic phase was washed with H₂O and brine and dried (MgSO₄). Evaporation of the volatiles gave the product as a yellow oil (294 mg, 82%). Rᵣ =
0.53 (AcOEt); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 0.88 (t, 6H, CH$_3$), 1.26-1.30 (m, 36H, CH$_3$(CH$_2$)$_9$), 1.54-1.60 (m, 4H, CH$_2$CH$_2$O), 3.43-3.57 (m, 8H, CH$_2$OC$_2$H$_5$H), 3.65-3.71 (q, 1H, CH$_2$Azide). $^1$H NMR spectrum is in agreement with published data.$^{31}$

6. 2-((2-(((1-(1,3-Bis(dodecyloxy)propan-2-yl)-1H-1,2,3-triazol-4-yl)methyl)amino)-2-oxoethyl)amino)-1-(4,5-dimethoxy-2-nitrophenyl)-2-oxoethyl 4-bromobutanoate.

The compound was prepared by a modification of a literature procedure for a different target.$^{29}$ To a solution of compounds 3 (0.12 mmol, 61 mg), and 5 (0.15 mmol, 66 mg) were added DIPEA (5.5 µmol, 0.72 mg) and acetic acid (5.5 µmol, 0.33 mg) in DCM (220 µL) followed by copper(I) iodide (3 µmol, 0.6 mg). The reaction mixture was stirred at room temperature overnight. The product was purified by flash column chromatography (pentane/AcOEt, 1:9-0:1 v/v) to obtain the product as a yellow oil (67.7 mg, 58%). R$_f$ = 0.80 (AcOEt); $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 0.88 (t, $^3$J = 6.0 Hz, 6H, (CH$_2$)$_3$CH$_3$), 1.26-1.28 (m, 40H, CH$_3$(CH$_2$)$_9$), 1.53 (m, 4H, (CH$_2$)$_3$CH$_2$O), 2.19 (q, $^3$J = 7.0 Hz, 2H, CH$_2$CH$_2$Br), 2.60-2.73 (m, 2H, CH$_2$CH$_2$CH$_2$Br), 3.39-3.54 (m, 5H, CH$_2$Br, (CH$_2$)$_3$CH$_2$O), 3.81 (d, $^3$J = 8.0 Hz, 4H, CH$_2$CH-TriazoleCH$_2$), 3.96 (s, 3H, OCH$_3$), 3.89 (dd, $^3$J = 6.0 Hz, 4H, HNCOC$_2$H$_5$NH), 4.01 (s, 3H, OCH$_3$), 3.89 (dd, $^3$J = 6.0 Hz, 2H, HNCOC$_2$H$_5$NH), 4.01 (s, 3H, OCH$_3$), 4.06 (dd, $^3$J = 16.0 Hz, 2H, CH$_2$CH-TriazoleCH$_2$), 7.13 (t, $^3$J = 6.0 Hz, 1H, NH), 7.20 (s, 1H, ArH), 7.38 (t, $^3$J = 6.0 Hz, 1H, NH), 7.60 (s, 1H, ArH), 7.70 (s, 1H, ArH); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 14.2, 22.8, 26.2, 27.7, 29.5, 29.6, 29.7, 29.8, 29.8, 30.4, 32.0, 32.3, 32.6, 35.1, 43.1, 56.6, 56.8, 60.9, 69.3, 71.1, 71.8, 108.1, 111.0, 114.1, 122.5, 124.6, 124.9, 140.8, 149.2, 153.8, 167.7, 168.2, 171.7. HRMS (ESI+) calc. for [M+H]$^+$ (C$_{46}$H$_{77}$BrN$_6$O$_{10}$): 953.4957, found: 953.4966.
7: 4,7,10-Tris(2-(tert-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-ium bromide

The compound was prepared according to a literature procedure. A suspension of cyclen (5.81 mmol, 1.00 g) and sodium acetate (19.1 mmol, 1.57 g) in N,N-dimethylacetamide (DMA, 12 mL) was stirred at -20 °C. A solution of tert-butyl bromoacetate (19.2 mmol, 2.83 mL) in DMA (4 mL) was added dropwise over 15 min at -20 °C. The reaction mixture was stirred at room temperature for 24 h, after which it was poured into H₂O (60 mL), resulting in a clear solution. Solid KHCO₃ (30.0 mmol, 3.00 g) was added portion-wise and a precipitate was formed. The precipitate was collected by filtration and dissolved in DCM (5 mL) and the solution was washed with H₂O (20 mL), dried over MgSO₄, filtered and concentrated to about 4-5 mL. Et₂O (50 mL) was added and compound 7 precipitated as a white solid (1.64 g, 48%). Rf = 0.63 (DCM/MeOH, 9:1 v/v); Mp. 179-181 °C; ¹H NMR (500 MHz, CDCl₃) δ 1.45 (s, 9H), 1.46 (s, 18H), 2.87 (m, 4H), 2.93 (m, 8H), 3.10 (m, 4H), 3.29 (s, 2H), 3.38 (s, 4H), 10.03 (br s, 1H). ¹H NMR spectrum is in agreement with published data.

S2: Tri-tert-butyl 2,2',2''-(10-(4-(2-((2-(((1-(1,3-bis(dodecyl)propan-2-yl)-1H-1,2,3-triazol-4-yl)amino)-2-oxoethyl)amino)-1-(4,5-dimethoxy-2-nitrophenyl)-2-oxoethoxy)-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate

A solution of compound 6 (0.114 mmol, 109 mg), compounds 7 (0.1 mmol, 50 mg) and triethylamine (0.24 mmol, 34 µL) in acetonitrile (3 mL) was stirred at 60 °C overnight. The volatiles were evaporated and the product was purified by flash column chromatography (DCM/MeOH 98:2 – 90:10 v/v) to give a yellow oil (35 mg, 22%). Rf = 0.24 (DCM/MeOH, 95:5 v/v).
A solution of compound S2 (0.020 mmol, 25 mg) in trifluoroacetic acid (1.4 mL) and tri-iso-propylsilane (1.4 mL) was stirred at room temperature for 5 h. The volatiles were evaporated under reduced pressure and the residue was triturated with Et₂O to give yellow solid (TFA salt, 16 mg, 53%). Rₜ = 0.73 (DCM/MeOH, 9:1 v/v).
A light-responsive liposomal agent for MRI contrast enhancement and cargo delivery

$^1$H NMR (400 MHz, MeOD): $\delta$ 0.9 (t, 6H, 1, 1'), 1.28 (m, 42 H, 2-11, 2'-11', 23), 1.5 (m, 4 H, 24-25), 3.05-3.7 (m, 22H, 26-28), 3.21 (m, 4H, 12, 12'), 3.82 (m, 4H, 13, 13'), 3.88-3.99 (m, 2H, 17), 3.93 (s, 3H, 22/21), 3.96 (s, 3H, 22/21), 4.48 (s, 2H, 16), 4.9 (m, 1H, 14), 6.81 (s, 1H, 18), 7.25 (s, 1H, 19/20), 7.69 (s, 1H, 19/20), 7.91 (s, 1H, 15). $^{13}$C NMR (100 MHz, MeOD): $\delta$ 14.5, 23.8, 27.2, 30.5 (m), 30.8 (m), 31.6, 33.1, 35.7, 43.6, 57.0, 57.3, 62.5, 70.6, 72.5, 109.5, 113.1, 116.8 (TFA), 119.7 (TFA), 124.0, 125.5, 142.9, 145.7, 150.8, 154.9, 162.8 (TFA), 163.2 (TFA), 170.5, 171.1, 173.0. HRMS (ESI+) calc. for [M+H]$^+$ (C$_{60}$H$_{102}$N$_{10}$O$_{16}$): 1219.7556, found: 1219.7548.

10: 4-(Dodecyloxy)benzaldehyde

Compound 10 was prepared according to a literature procedure.$^{73}$ 4-Hydroxybenzaldehyde (8.9 mmol, 1.09 g) was dissolved in dimethylformamide (45 mL). 1-Bromododecane (12.3 mmol, 3 mL) and cesium carbonate (1.6 mmol, 5.37 g) were added. The reaction mixture was stirred at 65 °C overnight. Upon completion, the reaction mixture was cooled down to room temperature and the precipitated salts were removed by filtration. The filtrate was diluted with AcOEt (150 mL) and washed with H$_2$O (3x) followed by brine and dried with MgSO$_4$. The solvent was removed under reduced pressure. The product was purified by column chromatography (pentane/AcOEt 4:1 – 0:1 v/v). The product was obtained as a white solid (1.76 g, 74%). $R_f$ = 0.66 (pentane/AcOEt, 95:5 v/v); Mp. 26-29°C; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 0.78 (t, $J = 6.7$ Hz, 3H), 1.16 (s, 12H), 1.28 – 1.18 (m, 6H), 1.35 (t, $J = 7.8$ Hz, 2H), 1.68 (quint, $J = 6.8$ Hz, 2H), 3.88 (t, $J = 6.6$ Hz, 2H), 6.85 (d, $J = 8.4$ Hz, 2H), 9.74 (s, 1H), 7.67 (d, $J = 8.4$ Hz, 2H). $^1$H NMR spectrum is in agreement with published data.$^{73}$ $^{13}$C NMR (101 MHz, CDCl$_3$): $\delta$ 13.9, 22.5, 25.8, 28.9, 29.2, 29.4, 29.5, 29.5, 29.6, 31.8, 68.2, 114.5, 129.7, 131.6, 164.0, 190.1.
S3: Tri-tert-butyl 2,2',2''-(10-(2-(1,3-dioxoisoindolin-2-y)ethyl)-1,4,7,10-tetraazacyclocododecane-1,4,7-triy)triacetate

Compound S3 was prepared according to a literature procedure. Compound 12 (0.840 mmol, 497 mg) was dissolved in acetonitrile (15 mL) under nitrogen atmosphere. N-(2-Bromoethyl)phthalimide (1.00 mmol, 256 mg) and cesium carbonate (1.68 mmol, 547 mg) were added. The reaction mixture was heated to 80 °C for 24 h. Upon completion, the crude reaction mixture was filtered. The yellow filtrate was concentrated under reduced pressure. Chloroform was added and the precipitated impurities were filtered off. The solvent was evaporated, yielding crude product S3 as a yellow oil. Rf = 0.67 (DCM); 1H NMR (400 MHz, CDCl3): δ 1.44 (s, 27H), 2.69-2.79 (m, 18H), 3.21 (s, 6H), 3.74-3.77 (t, 2H), 7.68-7.71 (m, 2H), 7.82-7.84 (m, 2H).

1H NMR spectrum is in agreement with published data.

13: Tri-tert-butyl 2,2',2''-(10-(2-aminoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triy)triacetate

Compound 13 was prepared according to a literature procedure. Crude compound S3 was dissolved in MeOH (15 mL) in a pressure tube. Hydrazine hydrate (reagent grade, 50-60%, 120 μL) was added. The reaction mixture was stirred at 70 °C overnight. The solvent was evaporated, yielding crude product 13 as an orange oil (361.1 mg, 78% over two steps). Rf = 0.42 (DCM/MeOH 9:1 v/v); 1H NMR (400 MHz, CDCl3): δ 1.41 (d, 27H), 2.90 – 2.41 (m, 20H), 3.28 (two overlapping signals, 6H). 1H NMR spectrum is in agreement with published data.

13C NMR (101 MHz, CDCl3): δ 28.3, 28.3, 28.3, 51.8, 52.1, 52.2, 52.9, 56.3, 56.6, 56.8, 77.4, 80.7, 171.5.
**S4:** (3,7-Bis((E)-4-(dodecyloxy)styryl)-5,5-difluoro-1,9-dimethyl-5H-4λ4,5λ4-dipyrrolo [1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)methyl acetate

Compound 9 (0.15 mmol, 49 mg) was mixed with compound 10 (1.93 mmol, 559 mg) and piperidine (1 drop). The reaction mixture was stirred in the dark at 60 °C under vacuum for 5 h. The product was purified by column chromatography (pentane/DCM 4:1 – 0:1 v/v with 1% triethylamine). Product S4 was obtained as a blue/green solid (31 mg, 23%). The isolated yield in other runs ranged from 6% to 57% while following the same procedure. Rf = 0.65 (DCM); Mp. 126–131 °C; 1H NMR (400 MHz, CDCl3): δ 0.88 (t, J = 6.7 Hz, 6H), 1.80 – 1.27 (m, 40H) 2.15 (s, 3H), 2.41 (s, 6H), 4.00 (t, J = 6.6 Hz, 4H), 5.34 (s, 2H), 6.72 (s, 2H), 6.92 (d, J = 8.6 Hz, 4H), 7.24 (d, J = 17.4 Hz, 2H), 7.61 – 7.52 (two overlapping signals, 6H); 13C NMR (101 MHz, CDCl3): δ 14.3, 16.0, 20.9, 22.9, 26.2, 29.4, 29.5, 29.6, 29.7, 29.8, 29.8, 32.1, 58.3, 68.3, 115.0, 118.7, 129.3, 129.4, 134.0, 136.8, 160.4, 170.8; 19F NMR (376 MHz, CDCl3): δ -138.56; HRMS (ESI–) calc. for [M–H]– (C54H72BF2N2O4): 863.5704, found 863.5718.

**11:** (3,7-Bis((E)-4-(dodecyloxy)styryl)-5,5-difluoro-1,9-dimethyl-5H-4λ4,5λ4-dipyrrolo [1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)methanol

0.1 N NaOH (0.35 mmol, 3.5 mL) was added to MeOH (20 mL) and the solution was stirred for 10 min. Afterwards, the solution was added to a stirred solution of compound S4 (0.35 mmol, 305 mg) in DCM (20 mL). The reaction was stirred in the dark for 18 h. The reaction mixture was extracted with DCM (3x). The combined organic layers were washed with 1 N HCl and brine and dried with MgSO4. Product 11 was obtained as a blue/green solid (270 mg, 93%). Rf = 0.49 (DCM); Mp. 109–114 °C; 1H NMR (400 MHz, CDCl3): δ 0.92 – 0.79 (m, 6H), 1.36 – 1.23 (m, 32H), 1.53 – 1.40 (m, 4H), 1.80 (quint, J = 6.7 Hz, 4H), 2.57 (s, 6H), 4.00 (t, J = 6.6 Hz, 4H), 4.94 (d, J = 5.4 Hz, 2H), 6.95 – 6.88 (m, 4H), 6.72 (s, 2H), 7.23 (d, J = 16.2 Hz, 2H), 7.61 – 7.53 (two overlapping signals, 6H); 13C NMR (101 MHz, CDCl3): δ 14.3, 16.0, 22.9, 26.2, 29.4, 29.5, 29.6, 29.7, 29.8, 29.8, 32.1, 68.3, 115.0, 129.3, 129.4, 134.1, 136.5, 160.3; 19F NMR (376 MHz, CDCl3): δ -138.63; HRMS (ESI–) calc. for [M–H]– (C52H73BF2N2O3): 821.5599, found 821.5615.
**15**: (3,7-Bis((E)-4-(dodecylxyloxy)styryl)-5,5-difluoro-1,9-dimethyl-5H-4λ4,5λ4-dipyrrolo [1,2-c:2′,1′-f][1,3,2]diazaborinin-10-yl)methyl (4-nitrophenyl) carbonate

*p*-Nitrophenyl chloroformate (0.06 mmol, 12 mg) was dissolved in DCM (1 mL), together with DIPEA (0.12 mmol, 21.2 μL). Compound 11 (0.012 mmol, 10.0 mg) was dissolved in DCM (1 mL) together with DIPEA (0.12 mmol, 21.2 μL). The *p*-nitrophenyl chloroformate solution was added to the solution of compound 11 at 0 °C over a period of 0.5 h under nitrogen atmosphere. The reaction was allowed to reach room temperature and was kept at room temperature for 22 h, after which the solvent was evaporated under reduced pressure. Compound 15 was purified by column chromatography (DCM) to afford a green solid (5 mg, 41%). *R* <sub>f</sub> = 0.92 (DCM); Mp. 109–114 °C.; ¹H NMR (400 MHz, CDCl₃): δ 0.88 (t, *J* = 6.7, 2.0 Hz, 6H), 1.54 (m, 40H), 2.53 (s, 6H), 4.01 (t, *J* = 6.5 Hz, 4H), 5.62 (s, 2H), 6.76 (s, 2H), 6.93 (d, *J* = 8.6 Hz, 4H), 7.26 (d, 2H), 7.42 (d, *J* = 9.1 Hz, 2H), 7.57 (two overlapping signals, 6H), 8.30 (d, *J* = 9.1 Hz, 2H); ¹³C NMR (101 MHz, CDCl₃): δ 14.1, 16.0, 22.7, 26.0, 29.2, 29.4, 29.6, 29.6, 29.6, 29.7, 31.9, 62.1, 68.2, 114.9, 119.0, 121.7, 125.4, 129.1, 129.3, 134.5, 137.1, 139.7, 153.7, 160.4; ¹⁹F NMR (376 MHz, CDCl₃): δ -138.38; HRMS (ESI–) calc. for [M–H]⁻ (C₅₉H₇₆BF₂N₃O₇): 986.5661, found 986.5687.

**16**: Tri-tert-butyl 2,2′,2″-(10-(2-(((4-nitrophenoxy)carbonyl)amino)ethyl)-1,4,7,10-tetraaza-cyclododecane-1,4,7-triy)triacetate

A solution of *p*-nitrophenyl chloroformate (0.18 mmol, 35.7 mg) in dry DCM (0.3 mL) was added to compound 13 (0.05 mmol, 29.1 mg). The reaction mixture was stirred at room temperature for 2 h, after which the solvent was evaporated. Et₂O (1 mL) was added, the solid was filtered off and washed twice Et₂O. The residue was dissolved in MeOH, filtered and the filtrate dried under vacuum to afford product 16 as an orange oil (11.9 mg, 32%). ¹H NMR (400 MHz, MeOD): δ 1.47-1.57
(overlapping signals, 27 H), 2.9-4.2 (broad m, 27H), 7.41 (d, 2H), 8.29 (d, 2H); \textsuperscript{13}C NMR (151 MHz, MeOD): \( \delta \) 27.1 (several overlapping signals), 115.1, 122.0, 122.0, 122.1, 124.7, 125.7, 145.0, 154.3, 155.9; HRMS (ESI+) calc. for [M+H]\textsuperscript{+} \((\text{C}_{35}\text{H}_{58}\text{N}_{6}\text{O}_{10})\): 723.4287, found 723.4271

**General procedure for the synthesis of photoactive carbamates with amine 13**

Compound 13 was dissolved in dry DCM and the solution was added to a solution of triphosgene (0.33 eq) in dry DCM. The mixture was stirred at room temperature for 1 to 3 h, followed by addition of the alcohol (4 eq), which was pre-treated with NaH (4 eq) in DCM. The resulting mixture (final concentration of 13 ca. 35 mM) was stirred for another hour and the conversion was assessed by UPLC-MS (compounds 17-19) or TLC (compound 14). Compound 14 was purified by flash column chromatography (DCM/MeOH, 1:0 – 9:1 v/v) to afford a blue solid. HRMS (ESI+) calc. for [M+H]\textsuperscript{+} \((\text{C}_{81}\text{H}_{126}\text{BF}_{2}\text{N}_{7}\text{O}_{10})\): 1406.9700, found 1406.9709.
Fig. 4.11: UPLC MS analysis of the reaction mixtures for the synthesis of compounds 17, 18 and 19. The substrate (13) and product peaks are indicated in the chromatogram (TIC) and the retention times of the product peaks highlighted with a red circle. The detected mass spectra (m/z) of the product peaks are shown on the right.

Fig. 4.12: HRMS spectrum of compound 14.
**PREPARATION OF LIPOSOMES**

The liposomes were prepared by mixing equimolar amounts of compound 1 with DOPC in chloroform. After evaporation of the organic solvent, TBS buffer (0.9 mL, 50 mM Tris, 150 mM NaCl, pH 7.5) was added to the dry lipid film to yield a concentration of 2.78 mM for both components. Five cycles of freezing, thawing and ultrasound sonication afforded the final liposomes. A solution of gadolinium trichloride in TBS buffer (2.5 mM GdCl₃, 0.1 mL) was added to the liposomes and incubated for 2 h during gentle stirring. Afterwards, the liposomes were purified from free Gd³⁺ salts via dialysis (benzoylated dialysis tubing, MWCO: 2000, Sigma Aldrich).

**CRYO-TEM AND EDX ANALYSIS**

Cryo-TEM was performed on a Tecnai T20 electron microscope (FEI) operated at 200 keV using a Gatan model 626 cryo-stage. Samples were vitrified with a vitrobot (FEI) on quantifoil 3.5/1 grids. Images were recorded on a slow-scan CCD camera under low-dose conditions. EDX analysis was done on the same microscope using a X-max 80T SDD detector (Oxford instruments). The ratio of phosphorus to gadolinium signal was analyzed in two selected areas and determined to be 1: 1.76 (Gd/P).

**NMRD MEASUREMENTS**

The liposomes were prepared as described above. The relaxation rates were determined over a frequency range of 0.01 – 10 MHz at 37 °C with 20 data points collected. The samples were irradiated in the NMRD vessel with λ = 400 nm for 60 min and NMRD profiles were recorded at the indicated time points. For the stability test, the sample was stored for up to four weeks in the dark at +4 °C.

![Fig. 4.13: a) NMRD profiles of three independently prepared samples of liposomes containing Gd-1 (sample 1-3); b) NMRD profiles of liposomes containing Gd-1 after preparation, after storage for one and four weeks.](image)
SPECTROPHOTOMETRIC ANALYSIS

The absorbance was analyzed on a UV/Vis spectrophotometer. The liposomal formulation was irradiated as described above and the samples diluted with DMSO for analysis of the UV-Vis absorbance.

**Fig. 4.14:** a) UV-Vis absorption spectra of liposomes containing Gd-1 before irradiation and after irradiation with λ = 400 nm for the indicated time points; b) visible change of the sample color upon irradiation

DETERMINATION OF RELAXATION RATES AT 4.7 T

The relaxation rate of the liposomal formulation (prepared as described above) was determined on a Varian MercuryPlus spectrometer (4.7 T) at 37 °C using an inverse recovery experiment. The sample was irradiated in the NMR tube with λ = 400 nm light for 60 min and the relaxation rates were determined at the indicated time points.
**A LIGHT-RESPONSIVE LIPOSOMAL AGENT FOR MRI CONTRAST ENHANCEMENT AND CARGO DELIVERY**

**Fig. 4.15** Relaxation rate at 4.7 T in the dark and after irradiation for the indicated times

**DETERMINATION OF FREE Gd\textsuperscript{III} CONCENTRATION**

**Fig. 4.16**: a) Calibration curve showing the ratio of absorbance intensity at $\lambda = 573$ nm and $\lambda = 433$ nm for increasing Gd\textsuperscript{III} concentration in the presence of xylenol orange (0.60 mM); b) concentration of free Gd\textsuperscript{III} before and after irradiation with $\lambda = 400$ nm for 1 h and total gadolinium concentration (determined by ICP OES).

The concentration of free Gd\textsuperscript{III} was quantified by determination of the ratio of absorbance intensity at $\lambda = 573$ nm and $\lambda = 433$ nm of a Gd\textsuperscript{III}-xylenol orange complex\textsuperscript{50} in ammonium acetate buffer (100 mM, pH 5.8, 0.60 mM Xylenol Orange) using a microplate reader. The liposome sample was prepared as described above and diluted 1:20 with ammonium acetate buffer before analysis. The concentration
of free Gd$^{3+}$ before irradiation was determined as 0.029 mM. After irradiation for 60 min with $\lambda = 400$ nm the free Gd$^{3+}$ concentration was determined as 0.033 mM.

**Cargo release from liposomes**

![Fig 4.17: Fluorescence intensity ($\lambda_{\text{exc}} = 490$ nm, $\lambda_{\text{em}} = 520$ nm) of calcein-loaded liposomes containing compound 1 in the dark, upon irradiation with $\lambda = 400$ nm and after addition of Triton X 100.

The liposomes were prepared by adaptation of the method described above. A solution of calcein (0.1 M calcein, 0.1 M Tris buffer, pH 7.5) was used for hydration of the dry lipid film. The concentration of DOPC and compound 1 was 7 mM. The liposomes were purified from free calcein by size exclusion chromatography on a HiTrap desalting Sephadex G25 column using TBS buffer (0.1 M Tris, 0.15 M NaCl, pH 7.5) as elution buffer. The fluorescence ($\lambda_{\text{exc}} = 490$ nm, $\lambda_{\text{em}} = 520$ nm), indicating the amount of free calcein was analyzed on a microplate reader. At first the sample was kept in the dark for 60 min in order to assess the light-independent leakage of calcein. Subsequently, the sample was irradiated for 70 min with $\lambda = 400$ nm and the increase in fluorescence was checked every 10 min. At the end of the experiment, Triton X 100 (1% v/v) was added to determine the maximum calcein concentration.
Liposomes consisting of only DOPC were prepared in the same way and analyzed as a control. Due to photobleaching of the fluorescence of the released calcein, it was not possible to determine the exact fraction of cargo release.

**Dynamic Light Scattering**

The size distribution of the liposomes was analyzed by Dynamic Light Scattering (DLS) using the Dynapro Nanostar apparatus, and the results were analyzed with Dynamics software, version 7. Before the analysis, the samples were diluted and centrifuged for 1 min at 10000 g to remove potential aggregates. As the irradiation progressed, the average size of the liposomes shifted towards smaller objects with a broader size distribution.

![Graph of liposome size distribution](image)

*Fig. 4.18: Size of liposomes containing Gd-1 in the dark and after irradiation for 60 min.*

**In Vitro Analysis of Liposome Cytotoxicity**

Primary colonic epithelium cells were obtained from Tebu-Bio (The Netherlands, 2–96115). Cells were cultured in RPMI-1640 (Lonza, Swiss), supplemented with 10% fetal calf serum (FCS, Thermo Scientific Waltham, MA) at 37°C in a humidified 5% CO₂ atmosphere.

HUVEC cells were obtained from the UMCG Endothelial Cell Facility and were isolated and cultured by the method previously described. Monocytes: PBMCs were subjected to magnetic-activated cell sorting (MACS) with anti-CD14-beads and MS columns (Miltenyi Biotec). Macrophages: monocytes (1 x 10⁶/mL) were treated with 50 ng/mL M-CSF for 6 d, yielding M0 macrophages. M0 macrophages were subsequently stimulated with 50 ng/mL LPS and 20 ng/mL IFNγ to generate M1 macrophages.
To test liposome induced cytotoxicity HUVEC, normal epithelial cells or M1 macrophages were seeded in 48 wells plate at density of 2,0x10^4 cells per well. The next day, cells were incubated for 48 h (M1 macrophages 24 h) with either medium or 10 µL liposomes solution which was irradiated with \( \lambda = 400 \) nm for the indicated time points. Treatment with high dose (70 µM) taxol (Merck) was added as a positive control for apoptosis measurement. After incubation, cells were harvested and stained with Annexin V-FITC (Immunotools, Germany) and propidium iodide (Merck) according to manufacturer’s protocol and analyzed by flow cytometry (Guava, Merck).

![Graph](image)

**Fig.4.19:** Percentage of Annexin V/PI positive cells (analyzed by flow cytometry) after addition of medium or liposomes containing \textbf{Gd-1} before and after irradiation with \( \lambda = 400 \) nm for the indicated times.
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