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Research paper

Impact of covalently Nile Red and covalently Rhodamine labeled fluorescent polymer micelles for the improved imaging of the respective drug delivery system

Gregory Trubitsyn[1], Van Nga Nguyen[1], Claudia Di Tommaso, Gerrit Borchard*, Robert Gurny, Michael Möller

School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 1 Rue Michel Servet, CH-1206 Geneva, Switzerland

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ABSTRACT

Novel fluorescently labeled poly(ethylene glycol)-poly(hydroxyoctanoic acid) (MPEG-PHOA) block-copolymers were synthesized for the improved visualization of the deriving polymeric micelle drug delivery system. Albeit commonly used, one has to be aware that by simple incorporation of Nile Red (hydrophobic) or Rhodamine B (hydrophilic) as fluorescent compounds in nanocarriers (e.g., nanoparticles, liposomes or micelles) for imaging applications, these fluorescent probes can diffuse out of the carrier system and lead to artefacts due to the concomitant fluorescence loss or areal distribution. In order to inhibit such an uncontrolled diffusion, the Nile Red derivative 2-((9-(diethylamino)-5-oxo-5H-benzo[*a*]phenoxazin-2-yl)oxy)acetic acid was synthesized and covalently attached to the MPEG-PHOA block-copolymer via a mild Mitsunobu reaction to yield the desired MPEG-PHOA-Nile Red polymer for micelle preparations. Rhodamine B was coupled via its native carboxylic acid group with the copolymer MPEG-PHOA under mild conditions using DMAP, EDC, and NHS. For the proof of concept, aqueous solutions of composite micelles made of 0.5% (w/w fluorescence dye) MPEG-PHOA-dye and MPEG-PHOA copolymers were prepared ("spiking" of the non-labeled base MPEG-PHOA micelles) and characterized by transmission electron microscopy (TEM), dialysis and fluorescence spectrometry. The fluorescence intensity of the Nile Red in the solutions was followed up at physiological temperatures and pH values (37 °C, pH = 7.4 PBS buffer 0.01 M) over a period of 8 weeks. The labeled and non-labeled micelle formulations were tested *in vitro* in cells (Rhodamine-micelle formulations), then *in vivo* in a case study of an ophthalmic application (Nile Red micelle formulations). Both *in vitro* and *in vivo* experiments revealed a significant improvement of fluorescence stability of the MPEG-PHOA-dye formulations, facilitating the investigations on tracing the micelles and their stability.

The results clearly demonstrate the value of the novel Nile Red and Rhodamine derivatives, whose simple synthesis and covalent attachment may easily be transferred to other nanosized polymeric drug delivery systems, e.g., MPEGylated or non-MPEGylated PLA/PLGA nanoparticles and be envisioned for novel theranostic systems.

1. Introduction

A challenge in pharmaceutical technology is the formulation of poorly water soluble drugs as well as targeting of these drugs to the pathological site [1]. A promising strategy is the use of nanosized drug carriers such as polymeric micelles based on amphiphilic biodegradable and biocompatible co-polymers [2–6]. Particularly co-polymers based on poly(lactide) (PLA) and poly(ethylene glycol) (PEG) are widely used, due to their excellent biocompatibility [7–11]. These polymers and co-polymers showed neither systemic, nor genetic toxicity, or pyrogenic reactions [12]. In the present work, the novel methoxy-poly(ethylene glycol)-poly(hydroxyoctanoic acid) (MPEG-PHOA) block-copolymer was used [13]. The advantage of the PHOA moiety (a hexyl substituted PLA derivative) is the increased hydrophobicity of the micelle core that stabilizes the micelles and its pharmaceutical formulations, and increases its capacity for the incorporation of hydrophobic drugs. Moreover, initial studies have proven its good biocompatibility [14].

It is preferable that such drug loaded nanocarriers target right to the pathological site to reduce systemic side effects and increase the efficacy of the treatment. Therefore, it is of special interest to be able to

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* Corresponding author.
E-mail address: gerrit.borchard@unige.ch (G. Borchard).
[1] Both authors contributed equally to this work.

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trace and image the MPEG-PHOA micelles after application in order to verify the specific targeting of micelles. For staining micelles, fluorescent markers like fluorescein, pyrene, Rhodamine B or Nile Red are commonly used. Practically, fluorescein has the advantage of being already approved by the FDA for clinical use [15]. However, the disadvantage for labelling micelles with fluorescein or pyrene is the non-discrimination between incorporated and non-incorporated compound upon release or diffusion out of the micelles. Moreover, pyrene is not visible by common confocal laser scanning microscopy due to its low excitation wavelength (335 nm after encapsulation in MPEG-PHOA micelles). In contrast, Nile Red may be used due to its property to be fluorescent only under hydrophobic conditions such as present in the micelle core [16–18]. Incorporated Nile Red thus visualizes intact micelles, and their distribution can be studied by fluorescence microscopy. Importantly, Nile Red at concentrations giving a sufficient fluorescence signal is non-toxic for human cells [19–22]. Nevertheless, simply incorporated Nile Red can also diffuse out of the micelles and lose its fluorescence in contact with the surrounding hydrophilic environment. Consequently, the measured fluorescence intensities do not any longer correspond to micelles in number or concentration at the studied location. To prevent Nile Red diffusion out of the micelles we followed the strategy to covalently attach this dye to the MPEG-PHOA copolymer micelle core. For this purpose the novel Nile Red derivative 2-((9-(diethylamino)-5-oxo-5H-benzo[*a*]phenoxazin-2-yl)oxy)acetic acid **5** was synthesized. Moreover, in order to have a larger choice of fluorophores, the hydrophilic dye – Rhodamine B – was also covalently attached to the copolymer. Rhodamine B is one of the most commonly used biomarkers [23,24].

After the covalent labeling, the MPEG-PHOA-Nile Red micelle solutions were characterized and investigated for their *in vitro* stability under physiological conditions in comparison to the corresponding micelle solutions of pure MPEG-PHOA with equal amounts of incorporated Nile Red. With the same aim, the MPEG-PHOA-Rhodamine micelle formulations were dialyzed to demonstrate the successful covalent labeling of Rhodamine B to the copolymer and its stability in aqueous solution. The formulations were then investigated *in vitro* with respect to their cell uptake and tracing feasibility.

To evaluate whether these micelles could be traced and imaged after an application as a pharmaceutical formulation, and if the covalent labeling would improve their visualization, *in vitro* studies were carried out in regard to a topical application in ophthalmic diseases, for which the micelles were additionally loaded with cyclosporin A (CsA) [25]. In a previous study, the compatibility of such formulations was proven on rabbit eyes *in vivo* [14]. A future application of such formulations in the eye would open the possibility of easy tracing and imaging the drug delivery system in patients by clinical ocular fluorometry.

As the *in vitro* studies revealed here, the covalent labeling of the micelles significantly improved the fluorescence intensity stability throughout the measurements over time, proving the successful inhibition of “out-diffusion” of Nile Red and Rhodamine B, respectively. Since physiological *in vitro* conditions are still a very simple model, a more realistic model in rats was chosen for a proof of concept *in vivo*. The micelle formulations were topically applied onto rat eyes and afterwards the flat-mounted corneas were imaged by confocal fluorescence microscopy to localize the micelles in the corneal tissues. The results of these investigations have been published recently [25]. We would like to share here the synthesis details together with the *in vitro* and *in vivo* proof of concept.

2. Materials and methods

2.1. Materials

All used solvents were dried by common procedures described in literature. 1,6-Dihydroxynaphtalene, benzyl bromoacetate, Rhodamine B, methanol, and chloroform were purchased from Sigma-Aldrich (Germany), N,N-diethylaminophenol-3, N,N-dimethylformamide, dimethylsulfoxide, hexane, triethylamine from Acros Organics (Switzerland), and diisopropyl azadicarboxylate, 4-Dimethylaminopyridine (DMAP), N-Hydroxysuccinimide (NHS), 1-Ethyl-3-[3-dimethylaminopropyl]carbodi-imide hydrochloride (EDC) from Fluka (Germany). All products used in cell culture experiments were purchased from Gibco Life Technologies (UK).

The MPEG-PHOA copolymer was synthesized by the method described by Trimaille et al. [13]. 5-Diethylamino-2-nitrosophenol hydrochloride **2** was synthesized as described by Elslager et al. [26] and 9-Diethylamino-1-hydroxy-5H-benzo[*a*]phenoxazin-5-one **3** as described by Briggs et al. [27].

The products were characterized by ^{1}H and ^{13}C NMR (Varian, 300 MHz). Co-polymer molecular weights and polydispersity indexes (PdI) were determined by gel permeation chromatography (GPC) equipment composed of Waters Styragel HR1-3 columns and a Waters 410 differential refractometer (Milford, USA). For the calibration curve polystyrene standards of different molecular weights (PSS Polymer Standards Service GmbH, Mainz, Germany) were used.

2.2. Methods

2.2.1. Synthesis of benzyl-2-((9-(diethylamino)-5-oxo-5H-benzo[a] phenoxazin-2-yl)oxy)acetate (4)

A mixture of 0.33 g (1 mmol) 9-diethylamino-1-hydroxy-5H-benzo[*a*]phenoxazin-5-one **3**, 0.40 g (3 mmol) potassium carbonate and 0.40 g (15 mmol) benzyl 2-bromoacetate in 100 mL dimethylformamide was stirred at room temperature for 12 h. The excess of potassium carbonate was filtered off and the filtrate concentrated by evaporation. The crude residue was dissolved in 30 mL dichloromethane and washed three times with water. The organic phase was dried with anhydrous Na_2SO_4, filtered, and then poured into 50 mL hexane under vigorous stirring. The precipitate was collected by filtration and dried under vacuum for 12 h to yield a dark red crystalline solid (0.25 g, 52%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.04 (d, $J = 8.7$ Hz, 1H), 7.84 (d, $J = 1.8$ Hz, 1H), 7.37 (d, $J = 9.0$ Hz, 1H), 7.03 (dd, $J = 8.7, 2.0$ Hz, 1H), 6.46 (dd, $J = 9.1, 1.4$ Hz, 1H), 6.27 (s, 1H), 6.12 (s, 1H), 5.09 (s, 2H), 4.69 (s, 2H), 3.28 (q, $J = 7.0$ Hz, 4H), 1.08 (t, $J = 7.0$ Hz, 6H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 183.07 (s), 168.35 (s), 160.24 (s), 152.16 (s), 150.85 (s), 146.92 (s), 135.09 (s), 134.08 (s), 131.22 (s), 128.6 (s), 128.02 (s), 126.50 (s), 124.77 (s), 118.43 (s), 109.63 (s), 106.71 (s), 105.32 (s), 96.32 (s), 76.64 (s), 67.24 (s), 65.42 (s), 45.15 (s), 12.67 (s).

2.2.2. Synthesis of 2-((9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl)oxy)acetic acid (5)

The solution of 0.7 g (1.5 mmol) benzyl-2-((9-(diethylamino)-5-oxo-5H-benzo[*a*]phenoxazin-2-yl)oxy)acetate **4** and 50 mg 10% Pd/C in 20 mL absolute methanol was stirred under a hydrogen atmosphere for 12 h. Afterwards the methanol was removed under reduced pressure, the mixture dissolved in 20 mL DMSO and filtered through a thin layer of silica gel. The solution was poured into the equivalent volume of water and the precipitants filtered off, dried in vacuum and washed three times with diethylether. After final drying 0.5 g (85%) of pure product were obtained.

^{1}H NMR (300 MHz, DMSO-d_6): δ 7.81 (d, $J = 8.7$ Hz, 1H), 7.64 (s, 1H), 7.33 (d, $J = 9.1$ Hz, 1H), 7.04 (d, $J = 8.7$ Hz, 1H), 6.54 (d, $J = 9.2$ Hz, 1H), 6.36 (s, 1H), 5.93 (s, 1H), 4.67 (s, 2H), 3.36–3.20 (m, 4H), 0.95 (t, $J = 6.8$ Hz, 6H).

^{13}C NMR (75 MHz, DMSO-d_6): δ 181.75 (d, $J = 1.5$ Hz), 170.30 (d, $J = 1.7$ Hz), 160.71 (d, $J = 1.9$ Hz), 152.17 (d, $J = 1.6$ Hz), 151.26 (d, $J = 1.8$ Hz), 146.87 (d, $J = 1.8$ Hz), 138.48 (d, $J = 2.2$ Hz), 133.88 (d, $J = 2.0$ Hz), 131.37 (d, $J = 4.1$ Hz), 127.68 (d, $J = 4.3$ Hz), 125.75 (d, $J = 1.8$ Hz), 124.36 (d, $J = 1.8$ Hz), 118.28 (d, $J = 6.4$ Hz), 110.47 (d, $J = 7.5$ Hz), 106.88 (d, $J = 10.4$ Hz), 104.47 (d, $J = 11.3$ Hz), 96.34 (d, $J = 9.9$ Hz), 65.10 (d, $J = 16.1$ Hz), 44.93 (s), 12.96 (s).

2.2.3. Synthesis of MPEG-PHOA-Nile Red (*6*)

40 mg (0.02 mmol) diisopropylazadicarboxylate were added drop wise under vigorous stirring to the solution of 0.69 g (0.014 mmol) MPEG-PHOA, 78 mg (0.2 mmol) 2-((9-(diethylamino)-5-oxo-5H-benzo[*a*]phenoxazin-2-yl)oxy)acetic acid **5** and 52 mg (0.02 mmol) triphenylphosphine in 20 mL of absolute acetone under an argon atmosphere, and the reaction mixture was stirred at room temperature for 4 days. Afterwards, the mixture was filtered and the solvent removed in vacuum at 40 °C. The crude product was dissolved in diethylether and centrifuged at 3000 rpm for 10 min. The supernatant was carefully collected with a pipette and the solvent removed under vacuum at 40 °C to yield 0.72 g (57%) of the product.

The structure of the compound was determined by ^{1}H NMR and ^{13}C NMR, and identified with the appearance of the carboxylic ester group signal at $\delta = 208.7$ versus the previous Nile Red acid derivative **5** carboxylic acid group signal at $\delta = 170.3$.

^{1}H NMR (300 MHz, DMSO-d_6): δ 8.70 (d, $J = 3.6$ Hz, 1H), 7.86 (d, $J = 8.6$ Hz, 1H), 7.72 (s, 1H), 7.08 (d, $J = 14.6$ Hz, 1H), 6.47 (d, $J = 5.1$ Hz, 1H), 6.01 (d, $J = 4.9$ Hz, 1H), 5.55 (t, $J = 3.9$ Hz, 1H), 5.02–4.93 (m, 2H), 4.61–4.50 (m, $J = 12.5$, 6.2 Hz, 1H), 3.30 (s, 54H), 3.15 (s, 34H), 2.30 (s, 2H), 1.16 (t, $J = 11.1$, 5.6 Hz, 23H), 1.08–0.89 (m, 45H), 0.63 (d, $J = 3.9$ Hz, 19H).

^{13}C NMR (75 MHz, Acetone-d_6): δ 208.71 (s), 204.22 (s), 174.92 (s), 169.20 (s), 156.50 (s), 148.59 (s), 132.76 (s), 129.86 (s), 118.10 (s), 95.85 (s), 84.10 (s), 80.4 (s), 76.42 (s), 71.09 (s), 61.76 (s), 54.52 (s), 51.73 (s), 48,10 (s), 30.81 (s), 29.63 (s), 25.02 (s), 22.42 (s), 21.01 (s), 19.37 (s), 17.88 (s), 15.76 (m), 14.24 (s), 12.06 (s) 8.08 (s).

2.3. Synthesis of MPEG-PHOA-Rhodamine (*7*)

A round bottom flask containing a stir bar was flame-dried under vacuum and then filled with argon. NHS (0.12 mmol, 14 mg), EDC (0.12 mmol, 23 mg) and MPEG-PHOA with a Mw of 5000 g/mol (0.1 mmol, 500 mg) were solubilized in 3 mL of dry CH_2Cl_2. From a second reaction flask, also flame-dried and filled with argon, the solution of DMAP (0.05 mmol, 6 mg) and Rhodamine B (0.1 mmol, 48 mg) in 2 mL of dry CH_2Cl_2 was added drop wise into the first flask. After 24 h of reflux under argon atmosphere, the obtained mixture was washed with 10 mL of respectively HCl 0.5 N, saturated $NaHCO_3$ and saturated NaCl solutions. The organic phase was dried with anhydrous Na_2SO_4 and then the solvent was evaporated. The red crude product was purified by flash column chromatography with MeOH/CH_2Cl_2 1/9. The pure product was collected by evaporation of the solvent under reduced pressure (yield = 61%).

^{1}H NMR (300 MHz, $CDCl_3$): Aromatic signals of Rhodamine B: δ 8.28 (d, $J = 7.5$ Hz), 7.61–7.83 (m), 7.19–7.33 (m), 7.07 (d, $J = 9.9$ Hz), 6.85–6.91 (m); MPEG-PHOA specific signals: δ 5.05–5.15 (m, 23x1H), 4.21–4.32 (m, 2H), 3.63 (s, 46x4H), 3.37 (s, 3H), 1.87 (m, 23x2H), 0.87–1.27 (m, 23x11H).

^{1}H NMR (300 MHz, CD_3OD): Aromatic signals of Rhodamine B: δ 8.30 (dd, $J = 7.5$, 1.5 Hz), 7.77–7.89 (m), 7.43 (dd, $J = 6.9$, 1.5 Hz), 6.85–7.14 (m); MPEG-PHOA specific signals: δ 5.05–5.15 (m, 26x1H), 4.21–4.32 (m, 2H), 3.63 (s, 51x4H), 3.37 (s, 3H), 1.87 (m, 26x2H), 0.87–1.27 (m, 26x11H).

^{13}C NMR (75 MHz, $CDCl_3$): δ 175.47 (s), 174.90 (s), 169.11(m), 157.88 (s), 131.36 (s), 114.24 (s), 96.68 (s), 72.70 (m), 70.68 (s), 68.95 (s), 64.39 (s), 59.18 (s), 46.27 (s), 31.70 (m), 31.11 (m), 28.93 (m), 25.07 (m), 22.68 (s), 14.16 (s).

The NMR spectra revealed the specific aromatic C—H signals of the Rhodamine B part with good integral proportion in $CDCl_3$, CD_3OD, Acetoned$_6$ and DMSOd$_6$. However, the integration of the spectra does not show the total amount of protons due to the formation of micelles with the amphiphilic copolymer.

Table 1
Composition of micelle formulations.

Solution	Polymer	Polymer mass	Conc. encapsulated fluo. marker	Conc. CSA
A	MPEG-PHOA	20 mg	0.01% Rhodamine B	–
B	MPEG-PHOA MPEG-PHOA-Rhodamine	19.8 mg 0.02 mg	–	–
C	MPEG-PHOA	20 mg	0.05% Nile Red	–
D	MPEG-PHOA MPEG-PHOA-Nile Red	19.9 mg 0.1 mg	–	–
E[*]	MPEG-PHOA	20 mg	3% Nile Red	0.5%
F[*]	MPEG-PHOA MPEG-PHOA-Nile Red	19.4 mg 0.6 mg	–	0.5%

[*] In 10 mM phosphate buffer/10% sucrose.

2.4. Preparation of the micelle formulations

Stock solutions of MPEG-PHOA and MPEG-PHOA-dye copolymers in acetone were first prepared. The micelle solutions were formulated by a co-solvent evaporation method. Briefly, 2 mL of the polymer stock solution(s) in acetone (and in case of micelle loading additionally the corresponding amount of dye dissolved in acetone) were added drop wise under probe sonication (Digital Sonifier 450, Branson, USA) to 4 mL of water and 10 mM phosphate buffer, respectively. Afterwards the acetone was evaporated under reduced pressure. The final copolymer concentration in the aqueous phase was 5 mg/mL for the solutions A and B; and 30 mg/mL for the C, D, E and F formulations. Composition of micellar formulations is listed in table 1.

The size of the micelles was determined with a Zetasizer 3000HSa (Malvern Instruments, UK), revealing micelle sizes smaller than 30 nm in diameter. The shape of the micelles was analyzed by Transmission Electron Microscopy (TEM) (FEI Tecnai™ G2 Sphera, USA); samples were deposited on copper grids, stained with 1% uranyl acetate and analyzed after drying (Fig. 1).

2.4.1. MPEG-PHOA-Rhodamine micelle formulations for dialysis and cell culture experiments

Solution (A)
MPEG-PHOA micelle solution with 0.01% w/w incorporated Rhodamine B (20 mg of MPEG-PHOA and 0.02 mg Rhodamine B).
Solution (B)
MPEG-PHOA micelle solution with 0.01% w/w equivalent of Rhodamine B (19.8 mg of MPEG-PHOA and 0.2 mg MPEG-PHOA -Rhodamine).

2.4.2. Ophthalmic formulations for in vitro and in vivo studies

2.4.2.1. Micelle solutions for in vitro studies

Solution (C)
MPEG-PHOA micelle solution with 0.05% w/w incorporated Nile Red.
Solution (D)
An aqueous solution of composite micelles made of 0.5% w/w (in respect to Nile Red) MPEG-PHOA-Nile Red and MPEG-PHOA ("spiking" of the non-labeled MPEG-PHOA micelles).

2.4.2.2. Micelle solutions for in vivo studies

Formulation (E)
MPEG-PHOA micelle solution (10 mM phosphate buffer/10% sucrose) with 3% w/w incorporated Nile Red and 0.5% incorporated CsA.

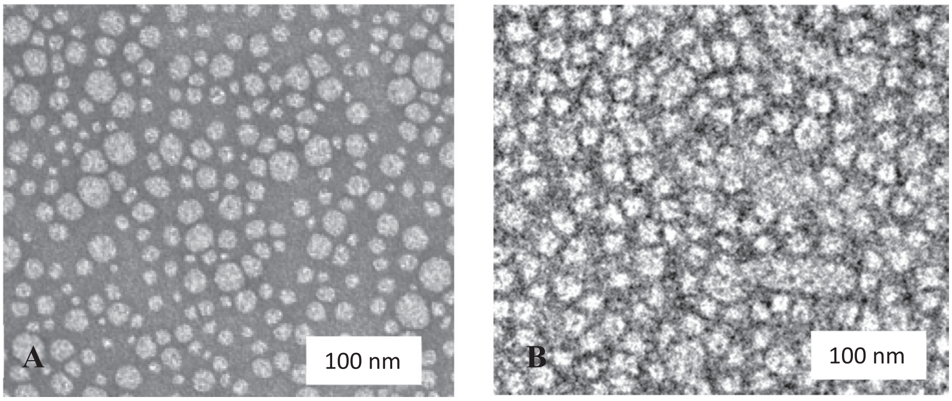


Fig. 1. Transmission Electron Microscopy pictures of the MPEG-PHOA micelle solution (A) and the MPEG-PHOA-Nile Red micelle solution (B).

Formulation (F)

An aqueous solution (10 mM phosphate buffer/10% sucrose) of composite micelles made of 3% w/w (in respect to Nile Red) MPEG-PHOA-Nile Red and MPEG-PHOA ("spiking" of the non-labeled MPEG-PHOA micelles) and 0.5% incorporated CsA. The sizes of the spherical micelles were between 30 and 50 nm in diameter, thus slightly larger than the non-loaded micelles.

2.5. Dialysis

3 mL of the micelle solutions A and B, respectively, were introduced in dialysis tubings of MWCO 2000 Da (Spectrum Laboratories, USA) and then placed in 500 mL of pure water under mild stirring. The dialysis study was carried out for 5 days. At each time point, an aliquot (5 mL) of the supernatant was sampled for fluorescence analysis, and the 500 mL of water were replaced by fresh pure water. The quantification of released Rhodamine was carried out with a Synergy plate reader (Biotek, USA) using a black 96-well-plate with transparent bottom. The concentration of Rhodamine was calculated with a calibration curve of Rhodamine in water ($\lambda_{em} = 560$ nm, $\lambda_{ex} = 580$ nm).

2.6. Cell culture experiments

A549 human lung carcinoma epithelial cells (ATCC® CCL-185™) were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 using F12-K medium (Kaighn's Modification of Ham's F-12 Medium) complemented with 10% fetal bovine serum (FBS) and 1% of penicillin-streptomycin mixture. Cells were grown in T75 flask and trypsinized at above 80% confluence and used between passage numbers 3 and 12.

2.6.1. Viability assay

The *in vitro* toxicity of the micelles on cells was determined by a WST-1 colorimetric assay. Cells were first seeded in a 96-well-plate at an initial density of 10^4 cells/well. After 24 h of seeding, the culture medium was removed and the cells were washed 3 times with PBS. The formulations (A) and (B) respectively were diluted 10 times in HBSS and the respective controls were added in triplicate (100 µL/well); negative control (F12-K full medium); positive control (SDS 0.02% in HBSS) and a control of medium solution without cells. The formulations were incubated for 1 h and then replaced by 100 µL of a solution of WST-1 10% in F12-K medium. The plate was incubated for an additional 30 min before absorbance measurements were performed using a Synergy plate reader at 2 different wavelengths (450 and 690 nm). The cell viability was calculated by the following equation:

$$Cell\ \ viability\ \ (\%) = \frac{Abs_{450\ \ nm}\ (\text{formulation}) - Abs_{690\ \ nm}\ (\text{formulation})}{Abs_{450\ \ nm}\ (-\text{control}) - Abs_{690\ \ nm}\ (-\text{control})} \times 100 \qquad (1)$$

2.6.2. Cell uptake of Rhodamine coupled micelles

Cells were seeded on cleaned and sterilized 24 × 24 mm coverslips in a 6-well-plate at a density of 10^5 cells/well. The culture medium was removed after 24 h of seeding. The cells were washed 3 times with PBS. The formulations A) and B), respectively, diluted 10 times in HBSS, and the negative control (HBSS) were then incubated for 1 h in duplicate (2 mL/well) followed by another cell washing with PBS (3 times). In order to fix the cells on the coverslips, 2 mL of paraformaldehyde 4% in PBS were added and kept for 20 min followed by 3 more washes with PBS. The coverslips were posed on microscope slides containing a drop of DAPI mounting medium Vectashield® (Vector Laboratories, UK) and fixed by commercial uncolored nail polish. Cell uptake of Rhodamine coupled micelles was observed with Confocal Laser Scanning Microscopy (CLSM) (Zeiss LSM 710 (Zeiss, Germany), equipped with a HeNe Laser (543 nm) and a 63x 1.4NA Oil immersion objective (Zeiss).

2.7. Fluorescence and formulation stability studies

The fluorescence wavelengths of the prepared micelle solutions were determined using a TECAN Safire equipment (Männedorf, Switzerland). The two solutions (C) and (D), respectively, showed a wavelength difference of 14 nm: 625 nm (C) and 611 nm (D). A difference of 1–18 nm is reported for Nile Red derivatives with different substituents [25].

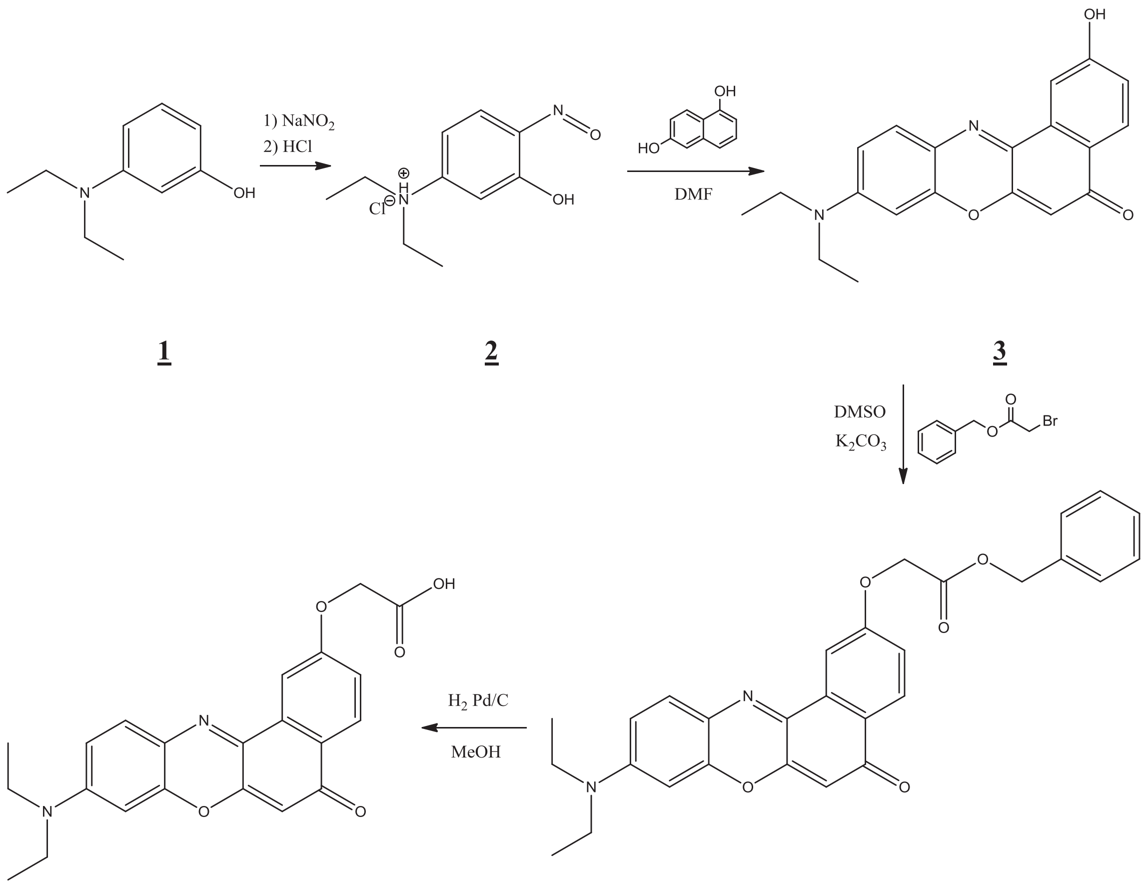
For stability measurements all micelle solutions (C, D, E, and F) were placed in an incubator at 37 °C. Samples for fluorescence measurements were taken at the beginning (t = 0) and after 30 and 60 days.

2.8. Animals

Seven Lewis rats were used for the corneal study, 3 for each formulation (E) and (F), and 1 for a control using a physiological saline solution. All rats were cared for in accordance with the European Committee Directives (Authorization numbers: A 75-06-12 and 75-580) and with the Association for Research in Vision and Ophthalmology resolution concerning the use of animals in ophthalmological research.

2.9. In vivo proof of concept of the drug delivery system

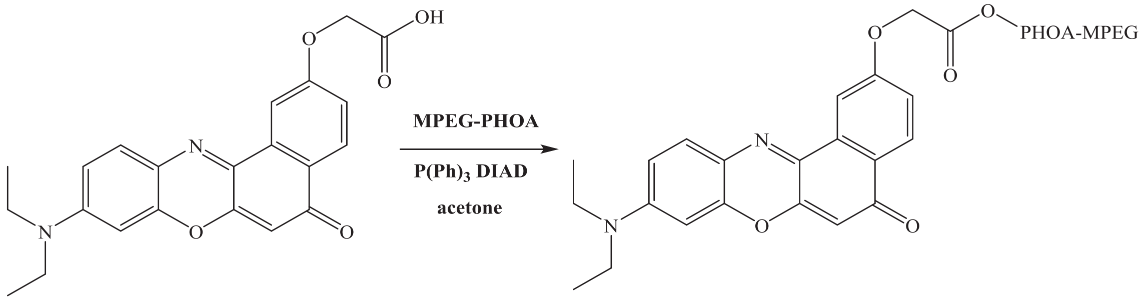
The Nile Red labeled and Nile Red incorporated formulations were tested in a corneal graft model in rats towards their imaging characteristics [1]. The two formulations were instilled onto the rats eyes with a pipette at one drop every 5 min during 30 min. One hour after the last instillation, the rats were sacrificed by a lethal injection of pentobarbital. The corneas were immediately flat-mounted and observed by CLSM.



1 **2** **3**

5 **4**

Scheme 1. Synthesis of the novel Nile Red derivative 2-((9-(diethylamino)-5-oxo-5H-benzo[*a*]phenoxazin-2-yl)oxy)acetic acid **5**.



5 **6**

Scheme 2. Synthesis of the covalently Nile Red labeled MPEG-PHOA copolymer **6**.

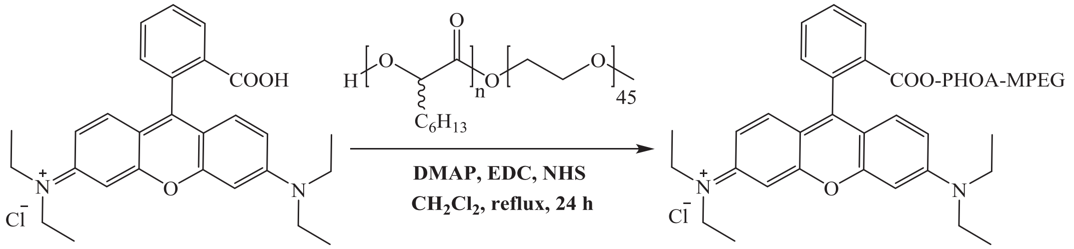
3. Results and discussion

For the desired fluorescently labeled polymeric micelles, Nile Red with its unique property to be fluorescent only under hydrophobic conditions, and Rhodamine B, which is a commonly used hydrophilic fluorophore [20,23] were chosen. They are both non-toxic and therefore widely used for cell staining. Both fluorescent markers were coupled to a novel MPEG-PHOA co-polymer [13].

The hydrophobic Nile Red was coupled to the hydrophobic PHOA chain end and should thus reside and being protected in the core of deriving micelles in an aqueous environment. The polyester backbone of PHOA is easily hydrolyzed under strong acidic or basic conditions and it decomposes at high temperatures. Coupling was therefore carried out by a mild Mitsunobu reaction of the secondary hydroxyl end group of PHOA and a Nile Red derivative bearing a free carboxylic acid group, 2-((9-(diethylamino)-5-oxo-5H-benzo[*a*]phenoxazin-2-yl)oxy)acetic acid **5** (Scheme 1). The latter compound was synthesized as outlined in Scheme 2.

The Mitsunobu reaction on the polymer was run with an extended reaction time of 4 days. The successful coupling was confirmed by the appearance of a new ^{13}C signal of the deriving new ester group at $\delta = 208.7$ ppm.

The coupling of the hydrophilic Rhodamine B, which bears a free carboxylic acid group, was achieved by a mild reaction of MPEG-PHOA



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Scheme 3. Synthesis of the covalently Rhodamine labeled MPEG-PHOA copolymer 7.

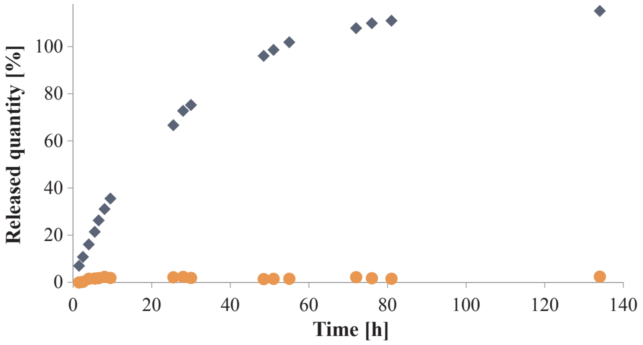

Fig. 2. Release profiles of (●) MPEG-PHOA micelles with covalently coupled Rhodamine, and (◆) MPEG-PHOA micelles with simply incorporated Rhodamine B.

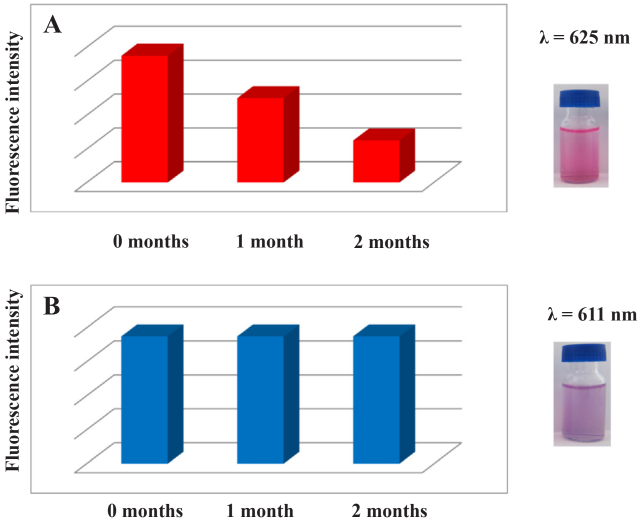

Fig. 3. Comparison of the fluorescence intensity over time of MPEG-PHOA micelle solutions (A) with incorporated Nile Red and (B) with covalently coupled Nile Red. Both solutions were stored under physiological conditions.

with DMAP, EDC and NHS under reflux (scheme 3). A reaction time of 24 h was applied. Due to the micelle formation in solution, the NMR analysis could not absolutely prove and quantify the coupling of Rhodamine B. Therefore a dialysis purification of the product was carried out and compared to the dialysis of simply incorporated Rhodamine B. This experiment showed that no Rhodamine B was released from the covalently labeled polymer over a period of 5 days in contrast to the 100% of incorporated dye released in less than 3 days (Fig. 2). Furthermore, this study revealed the stability of the covalently labeled micelles in water.

A similar *in vitro* stability test on formulations (C) and (D) revealed that covalently labeled Nile Red micelles were stable under the physiological model conditions during the observed period of 8 weeks (Fig. 3). On the contrary, micelle formulations with incorporated Nile Red showed a significant loss of fluorescence intensity under the same conditions, proving a diffusion of the fluorescent dye out of the micelles.

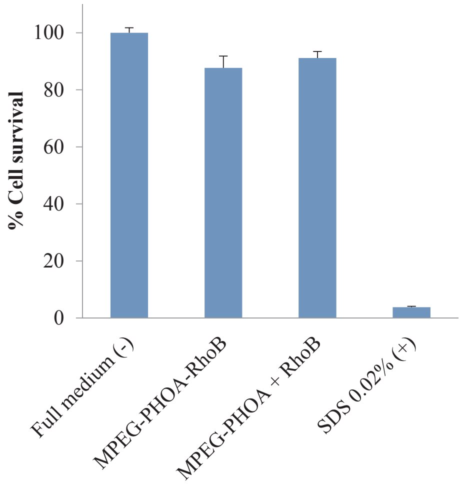

Fig. 4. A549 cell survival after a 24 h seeding time with the Rhodamine B micelle solutions.

To demonstrate the usefulness of covalent coupling towards imaging and drug carrier localization, respectively, *in vitro* cell culture studies and an *in vivo* corneal test on rats were performed.

The cell uptake studies were done with the Rhodamine micelle formulations (A) and (B). In a first step, a cell viability assay on A549 cells was carried out. No significant cell death was observed (Fig. 4). In a second step, the incubated cells were observed by confocal fluorescence microscopy. For formulation (A) in which Rhodamine B was simply incorporated into the micelles, the images did not show any significant number of red dots of Rhodamine B in the cells (Fig. 5A). Most probably, this is due to the fast diffusion of the dye out of the micelles occurring faster under cell culture conditions (HBSS, 37 °C) than during simple dialysis (water, ambient temperature) before a micelle uptake by the cells takes place. In contrast, formulation (B) with coupled Rhodamine showed significant red fluorescent dots around the cell nuclei (Fig. 5B). Considering the stability and integrity of the micelles in an aqueous environment at such short time points, these two examples demonstrate the advantages of the covalently coupled fluorescence marker for visualizing the nanosized drug carrier in cell culture studies.

In vivo experiments on rats' corneas with the application of the Nile Red formulations also revealed the difference between micelles in which Nile Red was simply incorporated (E) and the formulation with covalently labeled micelles (F) (Fig. 6). The observation of the cornea

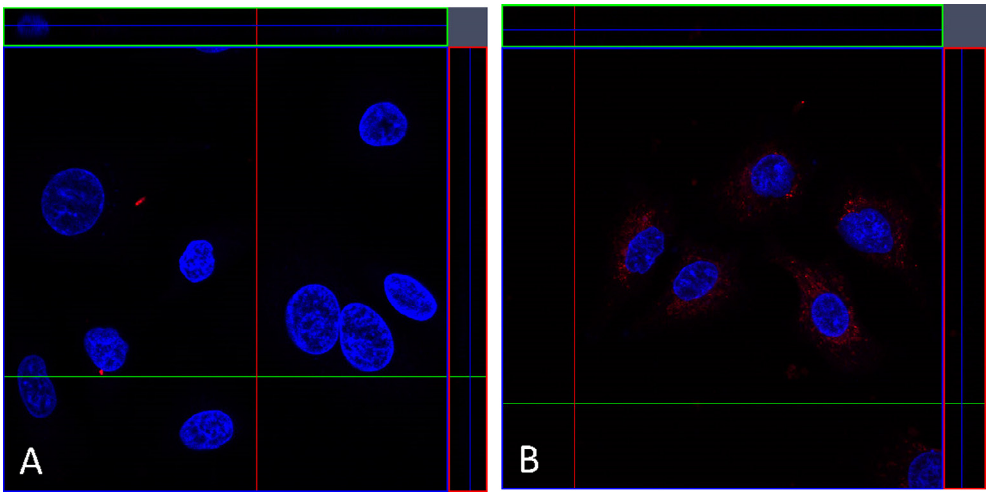

Fig. 5. Confocal fluorescence microscopy visualization of micelles taken up by A549 cells. (A) Rhodamine B incorporated in MPEG-PHOA micelles, (B) Rhodamine covalently coupled to micelles. Cell nuclei, stained by DAPI, reveal in blue. Rhodamine reveals in red color. Both images are the 8th stack from the total 16 stacks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

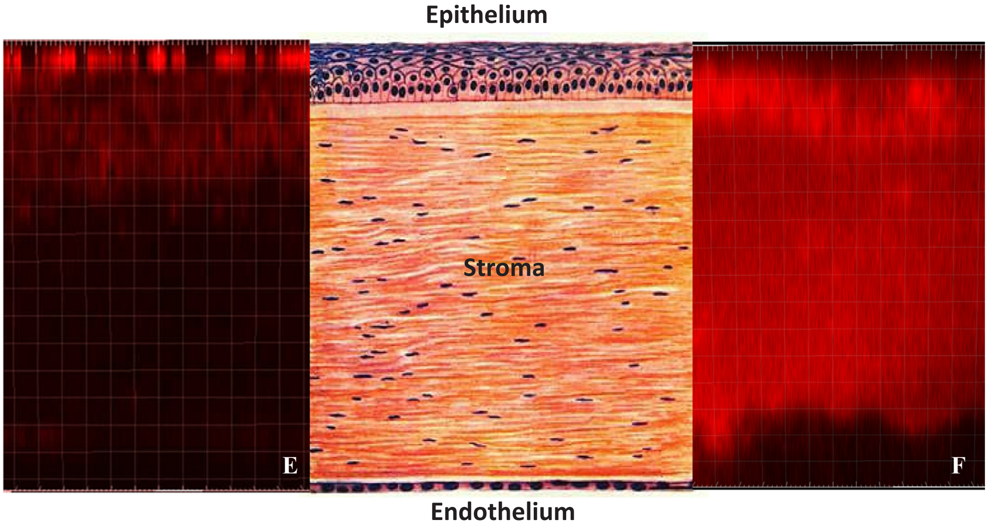

Fig. 6. Visualization of the micelles one hour after instillation of the formulations (E) "incorporated Nile Red" and (F) "covalently labeled Nile Red" on rat corneas.

treated with the nanosized carriers with simply incorporated Nile Red showed the typical red fluorescence mainly on and in the epithelium, and very little in the upper part of the hydrophilic stroma. In contrast, the covalently labeled micelles are clearly visible throughout the whole stroma layer reaching to the endothelium. The results demonstrate that (a) intact micelles are located in the stroma and (b) that the diffusion of free Nile Red into the stroma environment leads to the loss of fluorescence of Nile Red and therefore the micelles are not visible and localizable.

4. Conclusion

The covalently labeled micelles are superior in comparison to non-covalently labeled systems in regard to their imaging and localization because the study results are not influenced by diffusion effects of the fluorescence markers. The Rhodamine B- and Nile Red-derivative coupling reaction, as well as the synthesis of the derivative, can be easily reproduced and transferred to other particulate drug delivery systems and could thus be used in theranostic formulations.

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