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Imaging of doxorubicin release from theranostic macromolecular prodrugs via fluorescence resonance energy transfer

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ABSTRACT

Herein we present a FRET-based theranostic macromolecular prodrug (TMP) composed of (a) dendritic polyglycerol (PG) as polymeric nanocarrier, (b) Dox linked via a pH-sensitive hydrazone to (c) a tri-functional linker, and (d) an indodicarbocyanine dye (IDCC) attached in close proximity to Doxorubicin (Dox). The drug fluorescence is quenched via intramolecular FRET until the pH-sensitive hydrazone bond between the TMP and Dox is cleaved at acidic pH. By measuring its fluorescence, we characterized the TMP cleavage kinetics at different pH values *in vitro*. The intracellular release of Dox from the carrier was monitored in real time in intact cancer cells, giving more insight into the mode of action of a polymer drug conjugate.

1. Introduction

Theranostic nanomedicine has emerged as a field of research with great potential for the simultaneous detection and treatment of various diseases such as cancer and cardiovascular dysfunctions. [1] The functional combination of drugs and imaging agents within theranostic nanocarriers enables diagnosis, drug delivery, and monitoring of therapeutic response. It has been suggested that nanotheranostics will play an important role on predicting treatment responses, providing highly relevant insights for the improvement and understanding of targeted medicine, emphasizing the relevance of using both drugs and imaging agents within a single formulation. [2,3]

In order to circumvent the limitation of the current medicines, several nanocarrier technologies are currently available or under development. [4] Drugs conjugated to synthetic polymers or serum proteins and drugs encapsulated in liposomes or other micro- or nanoparticles have been extensively explored and collectively termed polymer therapeutics. [5,6] The accumulation of these macromolecules in solid tumors due to a leaky capillary combined with a defective or absent lymphatic drainage system (enhanced permeation and retention effect, EPR effect) forms the rationale for developing polymer-based drug delivery systems. [7] Covalent linkage of a payload to a macromolecule leads to the reduction of drug toxicity, elimination of undesirable side effects, and improvement of the solubility, stability, and prolonged blood half-life. [5,6,8] Such chemical linkages, however, can potentially induce steric hindrances and prevent interaction of the drug with its molecular target and thus render it inactive. Therefore, improved therapeutic efficacy can be realized when the active agent is linked to the carrier through a cleavable linker that is stable in blood and in healthy tissues but readily hydrolyzed upon entry into the target cancer cell or tumor. The over-expression of certain enzymes, an acidic and hypoxic environment in solid tumors, as well as targeting to the endolysosomal system offer several options for designing drug polymer conjugates that are preferentially cleaved within the tumor. [5,6,8–10]

Doxorubicin (Dox) is a cytostatic agent that is currently used as a first line of treatment against several cancers/tumors. It has been used for more than 40 years as a free or liposomal formulation. [11] Great improvements by decreasing the non-specific toxicity and increasing the targeting of doxorubicin have been reported via the conjugation to various kinds of polymer nanoparticles as linear polymers, [12] dendritic polymers, [8,13,14] albumin, [15] bow-tie or star-like structures, [16] among others. Impressive results have been reported regarding the *in vitro* and *in vivo* efficacy of such polymer therapeutic approaches. However, little information is known about the intracellular drug release kinetics. Understanding the drug release mechanism and the intracellular fate of the polymer conjugates is crucial for the rational comprehending and improvement of the next generation of polymeric nanocarriers.

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Different attempts have been developed to monitor intracellular Dox release utilizing quantum dots, [17] fluorescent targeting probes, [18] or magnetic nanoparticles. [19] However, the development of a system that allows one to obtain spatial and temporal information about the cellular uptake and intracellular release kinetics of Dox from a polymer conjugate has not yet been reported. It is therefore necessary to develop functional probes that act as a reporter after Dox linkage and release from any kind of polymer nanoparticle. Such a universal approach would enable the evaluation of the great variety of polymer-Dox conjugates that have been described so far. We hypothesize that the native fluorescence of Dox could allow developing such a ubiquitous system using the fluorescence resonance energy transfer (FRET) phenomena.

2. Materials and methods

2.1. General methods

Chemicals, MPLC gradient solvents as well as deuterated solvents, and all reagents were used as purchased from commercial suppliers without any prior purification. Solvents were purified by conventional methods prior to use. All chemicals were of analytical grade and purchased from Fluka (Germany), Aldrich (Germany), and Merck (Germany), respectively. Polyglycerol (average MW 200 kDa, PDI = 1.6, Fig. S1) was prepared according to a slightly modified protocol in emulsion as described earlier. [20] Polyglycerol azide with 1% of the total hydroxyl groups bearing azido groups was prepared as previously described. [21] Briefly, polyglycerol azide was prepared by a two-step protocol starting from hyperbranched polyglycerol, conversion of OH groups into mesyl (Ms) groups followed by transformation of Ms groups into azide (N_3) functionalities (Scheme S2). The indocarbocyanine IDCC dyes (Fig. S1) have been purchased from mivenion GmbH and were synthesized following literature procedures. [22,23] The (6-maleimidocaproyl) hydrazone derivative of doxorubicin (aldoxorubicin, Fig. S1) was prepared as described previously. [24] Water of Millipore quality (resistivity ~18 MΩ cm^{-1}, pH 5.6 ± 0.2) was used in all experiments and for preparation of all samples. All measurements were carried out with freshly prepared solutions at 25 °C. pH values were measured with a Scott instruments handylab pH meter at 25 °C. All reactions that involved air or water sensitive compounds were carried out in dried flasks under an argon atmosphere and dried solvents from the solvent purification system MB SPS 800, M. Braun Inertgas-Systeme GmbH, Garching, Germany. Column chromatography was conducted with RediSepRf Reversed-phase C18 Columns (average particle size: 40–63 μm, mesh: 230–400, average pore size: 60 Å) on a CombiFlashR_f, Teledyne Isco, Inc., Lincoln, NE, USA. Thin layer chromatography (TLC) was conducted on Merck silica gel 60 F-254 and TLC silica gel 60 RP18 F-254s plates. Spots were visualized by UV light. ^{1}H NMR and ^{13}C spectra were recorded on a Jeol ECX-400 400 MHz spectrometer or a BrukerBioSpin (700 MHz) instrument at room temperature, and chemical shift values (δ) are given in ppm relative to internal standard MeOD-d4 (3.31 ppm). MS ESI-TOF analyses were performed on an Agilent 6210 ESI-TOF, Agilent Technologies, Santa Clara, CA, USA. The fluorescence spectra were recorded on a Jasco FP-6500 spectrofluorometer. UV/Vis spectra were recorded on a Scinco S-3100 spectrometer. Ultrafiltration was performed in solvent-resistant stirred cells from Millipore (Billerica, MA, USA) with Ultracel regenerated cellulose membranes (MWCO 5000 g mol^{-1}). CENTRIPREP-10-concentrators from Amicon, FRG were used for ultracentrifugation. GPC analysis was performed by analytical size-exclusion HPLC using a Agilent-HPLC system with WinGPC Unity software from PSS; column: three 300 × 7.8 mm Polymer Laboratories PFgel mixed C; particle size: 5 μm; flow: 1.0 mL/min; isocratic; injection: 20 μL; mobile phase: water (0.05% NaN_3); UV detection at 237 and 600 nm. Preparative column chromatography was performed on silica gel 60 (0.040–0.063 mm, 230–400 mesh ASTM). Detection was accomplished by UV irradiation (254 and 366 nm) or aqueous solutions of $KMnO_4$. For SEC purification, Sephadex™ G-25 superfine and Sephacryl™S-100 HR were used. Human plasma was purchased from Merck KGaA, Darmstadt.

2.2. Synthesis of the theranostic macromolecular prodrug and controls

Following the synthetic route described in Scheme 1, the theranostic macromolecular prodrug (TMP) was prepared in three consecutive steps: (a) synthesis of dye-labeled tri-functional linker, (b) coupling of the fluorescent linker to the polymer nanocarrier, and (c) coupling of the pH-sensitive Dox prodrug. As a highly orthogonal tri-functional linker, the di-protected fluorenylmethyloxycarbonyl (Fmoc) and tert-butyloxycarbonyl (Boc) protected amino acid lysine **1** was chosen. After aminolysis of propargyl amine and the carboxyl group of the lysine using benzotriazol-1-yl-oxy-tris-dimethylamino-phosphoniumhexafluorophosphate (BOP) and *N,N′*-diisopropylethylamine (DIPEA) with 95% yield, a Fmoc deprotection according to literature procedures was performed yielding 81% of product **2**. [25] The IDCC dye was introduced via amide bond formation by an amino acid coupling strategy using *N,N,N′,N′*-Tetramethyl-*O*-(benzotriazol-1-yl)uroniumtetrafluoroborate (TBTU) and triethylamine (TEA) with 86% yield. Boc deprotection using trifluoroacetic acid (TFA) and slightly modified literature procedures gave 85% of product **3** (Scheme 1, a). [26] The IDCC dye-labeled linker derivatives were purified by reversed phase column chromatography and characterized by ^{1}H-NMR spectroscopy (700 MHz), ^{13}C-NMR spectroscopy (176 MHz), MS ESI-ToF, and UV-Vis. The next step was to conjugate the alkyne and dye containing linker construct **3** to the polymeric carrier PG (average MW 200 kDa) having a 1% azide functionalization by Huisgen 1,3-dipolar cycloaddition (Scheme 1b). The reaction was catalyzed by copper sulfate and sodium ascorbate as the reducing agent to form copper(I) *in situ*. [27] After 12 h of reaction in a mixture of water/MeOH (1/1 v/v) and DIPEA, product **4** was purified by ultrafiltration using water as solvent with one running-cycle with EDTA solution to remove all copper content yielding 84% of product. The dye conjugate **4** was characterized by UV-Vis spectroscopy, fluorescence spectroscopy, and gel permeation chromatography (GPC). The third and last step was the conjugation of the Dox prodrug aldoxorubicin via thiol-ene chemistry (Scheme 1c). Initial thiolation of the primary amine groups of **4** *in situ* was achieved by activation with 2-iminothiolane in 50 mM PB solution (pH 7.4) for 20 min, followed by a selective Michael addition between the maleimide group of aldoxorubicin (Fig. S1, SI) and the sulfhydryl groups of the thiolated PG, yielding the TMP (**5**). The thiol group was added to the double bond of the maleimide group in a fast and selective reaction at room temperature forming a stable thioether bond. [14] After 2 h, the reaction solution was concentrated with a Centriprep® and purified by size exclusion chromatography (SEC) using Sephacryl S-100 gel. The conjugate was characterized by UV-Vis, GPC, and fluorescence spectroscopy. Non-cleavable and non-quenching conjugates were synthesized as control molecules **6** and **7** following a similar synthetic strategy (see details in SI section, Scheme S1). Molecule **6** bore Dox coupled via a more stable amide linker, whereas molecule **7** contained IDCC and aldoxorubicin randomly linked to the PG surface (Fig. 1).

2.3. Fluorescence analysis

The different conjugates **5**, **6**, and **7** (10 nM, concentration stated in Dox equivalents) were incubated in buffers of various pH (4, 5, 6, and 7.4) at 25 °C. For pH 7.4, 6, and 5 with 50 mM PB buffer and in the case of pH 4, 50 mM acetate buffer. The fluorescence was recorded exciting the samples at 500 nm, in the absorption range of the Dox, over time. The fluorescence emission peaked at 588 nm and increased over time. The lower the buffer pH, the faster the increase of the fluorescence intensity. The donor fluorescence was used to determine kinetic parameters. The concentration of TMP in the reaction mixture was determined by changes of the fluorescence at 588 nm.

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