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HPMA-based block copolymers promote differential drug delivery kinetics for hydrophobic and amphiphilic molecules

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ABSTRACT

We describe a method how polymeric nanoparticles stabilized with (2-hydroxypropyl)methacrylamide (HPMA)-based block copolymers are used as drug delivery systems for a fast release of hydrophobic and a controlled release of an amphiphilic molecule. The versatile method of the miniemulsion solvent-evaporation technique was used to prepare polystyrene (PS) as well as poly-d/l-lactide (PDLLA) nanoparticles. Covalently bound or physically adsorbed fluorescent dyes labeled the particles' core and their block copolymer corona. Confocal laser scanning microscopy (CLSM) in combination with flow cytometry measurements were applied to demonstrate the burst release of a fluorescent hydrophobic drug model without the necessity of nanoparticle uptake. In addition, CLSM studies and quantitative calculations using the image processing program Volocity® show the intracellular detachment of the amphiphilic block copolymer from the particles' core after uptake. Our findings offer the possibility to combine the advantages of a fast release for hydrophobic and a controlled release for an amphiphilic molecule therefore pointing to the possibility to a 'multi-step and multi-site' targeting by one nanocarrier.

Statement of Significance

We describe thoroughly how different components of a nanocarrier end up in cells. This enables different cargos of a nanocarrier having a consecutive release and delivery of distinct components. Most interestingly we demonstrate individual kinetics of distinct components of such a system: first the release of a fluorescent hydrophobic drug model at contact with the cell membrane without the necessity of nanoparticle uptake. Secondly, the intracellular detachment of the amphiphilic block copolymer from the particles' core after uptake occurs. This offers the possibility to combine the advantages of a fast release for a hydrophobic substance at the time of interaction of the nanoparticle with the cell surface and a controlled release for an amphiphilic molecule later on therefore pointing to the possibility to a 'multi-step and multisite' targeting by one nanocarrier. We therefore feel that this could be used for many cellular systems where the combined and orchestrated delivery of components is prerequisite in order to obtain the highest efficiency.

1. Introduction

Nanocarriers typically contain several compounds. In the simplest case this would be a higher molecular weight component like a polymer and a payload which is typically of a lower molecular weight. Furthermore other components like surfactants are needed for preparation of nanoparticles. Release of payload is of utmost importance for a drug or gene delivery system applied in cancer treatment, for instance. The fates of the other components like carrier polymer or surfactant are also important as these may cause side effects or toxicological effects. Alternatively two components could serve as therapeutic payloads with different delivery and release mechanisms [1]. Previously we have shown that a short contact of a nanocarrier to a lipophilic surface like a phospholipid bilayer as a model for a cell membrane as well as a mammalian cell itself can release the cargo. So the cargo and the carrier take different paths [2]. While this is an untargeted effect it could be avoided

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by using a shielding of the hydrophobic core carrying the hydrophobic cargo [3]. Alternatively this could be exploited for the first contact a nanocarrier encounters. To date the most well-known mechanism for an enhancement of the delivery of nanocarriers to an area in the body is based on the enhanced permeability and retention effect (EPR effect) [4,5] which gives an imperfect enhancement of accumulation of polymeric drug delivery systems in many tumor vasculatures. One of the ideas to enhance the EPR effect is to use nanocarriers that deliver nitroglycerin as a hydrophobic prodrug [6] to the tumor endothelium as endothelial cells are the first cell type to be contacted when a nanocarrier wants to leave the blood stream. Release of nitroglycerin would increase the influx of a second type of nanocarriers with an anti-tumor agent. While the dilatation of vessels and the increase of the leakiness takes place on a macroscopic and supracellular level we also wanted to investigate the cellular and subcellular fate of a hydrophobic drug load and other components like amphiphilic ones as it may be advantageous to deliver certain drugs directly by uptake [1] or indirectly, e.g. as shown for hydrophobic molecules in nanocarriers by cell surface contact [2].

The aim of this work was to investigate the release and distribution of hydrophobic drugs and the other components of the nanocarrier which are mediated by contact to and uptake into cells. Therefore, polystyrene (PS) and poly-d/l-lactide (PDLLA) nanoparticles containing a hydrophobic fluorescent dye were used as models for drug-mediating systems. The particles were stabilized by amphiphilic HPMA-based block copolymers consisting of poly(N-(2-hydroxypropyl)methacrylamide) and poly(laurylmethacrylate) (P(HPMA)-*b*-P(LMA)) with a molecular weight of 10 KDa and 10 mol% of hydrophobic LMA. These block copolymers were synthesized by reversible addition–fragmentation chain transfer (RAFT) polymerization [7] in combination with the reactive ester approach allowing a facile functionalization of the polymers [8,9]. The advantage of using those block copolymers instead of conventional surfactants like sodium dodecyl sulfate (SDS) lies in their biocompatibility [10]. Thus, tedious and time consuming dialysis steps can be avoided. Furthermore, the synthesized block copolymers could be easily modified by attaching e.g. fluorophores like Texas Red covalently to the hydrophilic block. So, highly functionalized particle systems with specifically labeled and easily traceable components could be prepared and were used for *in vitro* drug release and localization studies. Also active targeting has been achieved with HPMA based systems [11].

The drug release and its distribution over time were investigated by confocal laser scanning microscopy (CLSM). The distribution of particle components was analyzed in the same manner. Moreover, quantitative analysis of the uptake behavior and the distribution of particle components within CLSM images were realized using Volocity® software.

2. Results and discussion

2.1. Drug-mediating model system

Polystyrene (PS) and poly-d/l-lactide (PDLLA) nanoparticles were obtained using the miniemulsion process in combination with the solvent evaporation technique (Fig. 1) as already shown by Kelsch et al. [12]. An amphiphilic HPMA-based block copolymer consisting of poly(*N*-(2-hydroxypropyl)methacrylamide) and poly(laurylmethacrylate) (P(HPMA)-*b*-P(LMA)) was synthesized by RAFT polymerization and used for particle stabilization. The block copolymer had an over-all number molecular weight M_n of 9.5 kg/mol, resulting from a M_n of 8.5 for the P(HPMA) block and 1.0 for the P(LMA) block with a polydispersity of 1,2. *N*-(2,6-diisopropylphenyl)perylen-3,4-dicarbonacidimide (PMI, see Table S1 in Supporting Information) was applied as a hydrophobic drug model and incorporated in the polymeric matrix during nanoparticle preparation, as indicated in Fig. 1. Fig. 2 shows the SEM micrographs of the synthesized poly-d/l-lactide (Fig. 2(A)) or polystyrene (Fig. 2(B)) nanoparticles respectively synthesized via miniemulsion–solvent-evaporation technique. The obtained nanoparticles show a narrow size distribution in agreement with Ref. [12]. Dynamic light scattering experiments of comparable not-fluorescently labeled colloids show a μ_2-value of 0.06–0.1. Here in total five fluorescently labeled nanoparticle systems of different composition were prepared. It varied –depending on the system (PS or PDLLA as core material and the dye)-between a diameter of 250 and 300 nm (Table 3). In total five nanoparticle systems of different composition were prepared. The particle core was labeled either covalently with Bodipy (structure see Table S1 in Supporting Information) or with the dissolved hydrophobic dye PMI as mentioned previously. Further, the stabilizing block copolymer was also labeled with fluorescent dyes (either Texas Red or Oregon Green) by covalent linkage (see Table S2). For a better understanding the structure of a representative nanoparticle is depicted in Fig. 2(C). The important characteristics of the synthesized particle systems are listed in Table 3. For more information concerning labeling of nanoparticles' core and stabilizing block copolymer see Supporting Information Tables S1 and S2.

2.2. In vitro release studies

Former experiments revealed that PS as well as PDLLA nanoparticles stabilized with HPMA-based copolymers were taken up by HeLa cells and no influence of cell viability was observed up to high concentrations of 1200 μg/mL [12]. Referring to these findings release kinetics of PMI from PDLLA nanoparticles **NP4** stabilized by Texas Red-labeled P(HPMA)-*b*-P(LMA) were performed. Thus, HeLa cells were incubated with **NP4** for different incubation times (1/4, 1/2, 1, 2, 4, 8 h). After the respective incubation time CLSM imaging of live cells was conducted subsequently after staining cell membranes with CellMask™ Deep Red (CDR). The merged images for each incubation time shown in Fig. 3 demonstrate a very fast release of PMI before uptake of nanoparticles occurred. Even after an incubation time of 15 min fluorescence signals of PMI were already detected indicating a very fast influx of the hydrophobic drug model. Signals of Texas Red which is covalently attached to the HPMA-based block copolymer could be clearly observed after 2 h incubation. The signal intensity as well as the amount of Texas Red spots increased with increasing incubation time. In contrast, the fluorescence signals of PMI were also increasing in their intensity over time but the number of spots did not seem to change. The CLSM images (Fig. 3) indicate that PMI was released from nanoparticles as they came in close proximity and contact to the cells. However, nanoparticles had not necessarily to be taken up to initiate that burst release. Obviously, no intracellular colocalization of PMI and Texas Red signals could be detected although there is colocalization in the surrounding medium (red arrows in Fig. 3) indicating that PMI was effectively incorporated into the polymer matrix of the nanoparticles. The results of the conducted experiments resemble a first hint of an independent uptake mechanism of the incorporated drug-model and the nanoparticles themselves which has also been shown for other nanocarrier systems [2].

To fortify the previous statement that there was an independency between uptake of nanoparticle and the burst release of PMI, the cellular uptake was investigated by flow cytometry measurements. For the quantification of nanoparticle and PMI uptake HeLa cells were incubated for 1 and 8 h with **NP4**. The graph in Fig. 4 shows that after 1 h of incubation a vast amount of PMI was detected compared with the weak signal of Texas Red. An influence of differences in fluorescence intensity of both fluorophores could be excluded since normalized values were used for calculation of the diagram. Because of the fact that there were