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Polymersomes

Cellular Integration of an Enzyme-Loaded Polymersome Nanoreactor**

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Protein therapy aims to use in vitro produced proteins to intracellularly replace or complement faulty ones,[1] making it a promising strategy to fight protein-deficiency diseases.[2] Unfortunately, the hurdles a protein must take to reach its therapeutic effect have hampered clinical applications. Many proteins suffer from poor in vivo stability, and cellular uptake and directed intracellular trafficking are hard to achieve.[3] Owing to these issues, the success of protein therapy is limited to treatment of a small set of lysosomal diseases,[3,4] for which delivery efficiency is still low, costs are high, and long-term efficiency has not yet been established.

A way to overcome some of these limitations is to couple therapeutic proteins to cell-penetrating peptides (CPPs) which promote the cellular uptake of their linked cargoes.[4] It has become clear, however, that most CPP-mediated uptake of proteins occurs through endocytosis. Poor release from the endosome and proteolytic breakdown have been identified as major factors limiting the biological activity of delivered molecules.[2] Protection may be achieved by encapsulation of unmodified proteins inside delivery vehicles such as liposomes that display CPPs.[5] Once inside the cell, the enzyme is released. However, inside the cytoplasm, the lifetime of an enzyme may be limited by denaturation or degradation.

Cells often use compartmentalization to organize, isolate, or protect enzymes, and this provides optimal conditions for specific cellular reactions. In compartments, for example organelles, reactants are exchanged by diffusion, through channels, or by means of transporter molecules. Therefore, it stands to reason that the introduction of new protein functionality to a cell may be best achieved by delivering the biomacromolecule encapsulated in a porous shell,[6] thus mimicking an organelle. To be suited for protein therapy, this artificial compartment should be stable in the bloodstream and capable of cellular uptake. Furthermore, it should offer protection against proteases but be permeable to the substrates and products of its cargo.

Herein, we describe the preparation and cellular uptake of enzyme-loaded polymersome capsules, and we report on the intracellular routing and activity of these nano-objects in mammalian cells. Cellular internalization of the capsules is mediated by the CPP tat linked to their surfaces.[7]

Polymersomes are self-assembled vesicles made from block copolymers and may be regarded as stable alternatives to liposomes.[8] They have dimensions in the nanometer range, a size considered to be suitable for in vivo applications.[9] Their properties can be engineered by changing their constituent polymers. As illustrated in Scheme 1, we present here a polymersome with a semiporous membrane based on polystyrene$_{40}$-*block*-poly[L-isocyanoalanine(2-thiophen-3-yl-ethyl)amide]$_{50}$ (PS-PIAT, **1**).[10] Enzyme-filled PS-PIAT polymersomes have been reported as efficient nanoreactors,[11] capable of protecting their contents from proteolytic degradation.[12] To promote the cellular uptake of such a nanoreactor, an azide-containing version of tat was covalently linked to a polystyrene-*block*-poly(ethylene glycol)-oxanorbornadiene (PS-PEG-crDA, **2**) using a Cu-free tandem cycloaddition/retro-Diels–Alder (crDA) "click" reaction.[13] Coassembly of a 10 wt% solution of PS-PEG-tat (**3**) with PS-PIAT thus constructed a tat-presenting nanocapsule, hereafter referred to as a tat-polymersome (Scheme 1).

Figure 1 shows transmission electron microscopy (TEM) images of polymersomes prepared in hepes-buffered saline (HBS) containing either green fluorescent protein (GFP, Figure 1a) or horseradish peroxidase (HRP, Figure 1b,c). Neither the handling, the aggregation behavior, nor the spherical morphology of the polymersomes was influenced by the admixture of **3**, which is in line with previous results for PS-PEG-enzyme conjugates.[14] The tat-polymersomes had an average diameter of (114 ± 28) nm (Figure S1 in the Supporting Information), with no obvious size variation between the polymersomes with different protein contents. This demonstrates that the tat-polymersomes provide a modular platform for protein and enzyme encapsulation.

To investigate whether tat would induce efficient cellular uptake, unmodified polymersomes and tat-polymersomes, both loaded with GFP, were incubated with a variety of cell

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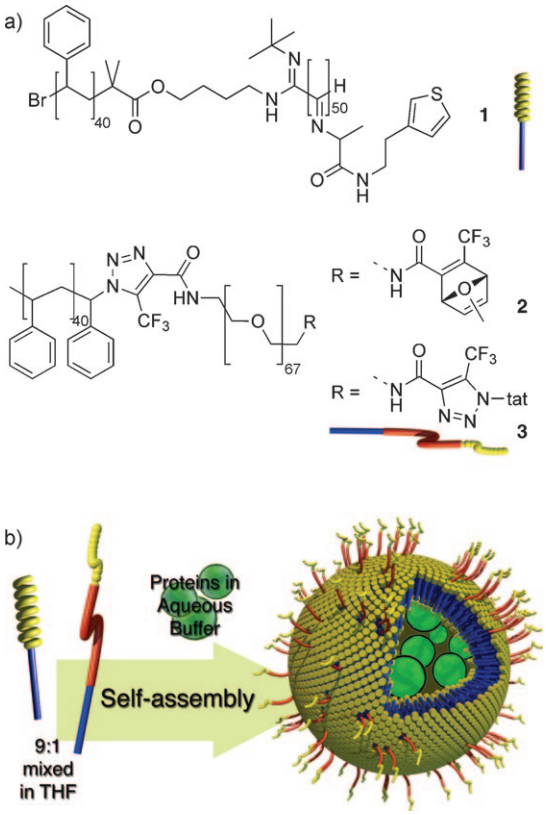

Scheme 1. a) Structures and representations of the polymers used; b) In our strategy, a 10 wt% solution of **3** was mixed with **1** to produce porous tat-functionalized polymersomes loaded with protein.

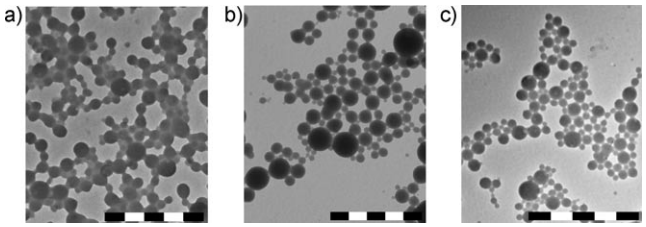

Figure 1. TEM images of PS-PIAT polymersomes prepared in HBS. a) GFP-loaded, with 10 wt% **3**; b) HRP-loaded; c) HRP-loaded, with 10 wt% **3**. Scale bars: 1 μm.

lines (HeLa, Jurkat, and HEK 293). At this point, directed cellular uptake of polymersomes was restricted to cells with high phagocytic activity, in other words, cells with the intrinsic ability to engulf large particles.[15] As shown in Figure 2, tat-polymersomes were taken up by all three cell types. No internalization of unmodified polymersomes was detected. These results were corroborated by flow cytometry (Figure S2 in the Supporting Information). Residual fluorescence could be attributed to extracellular polymersomes that had not been washed away. Having established their efficient internalization, we set out to investigate the route of uptake.

Arginine-rich peptides like tat are a class of CPPs for which endocytosis is known to be important, and macropinocytosis is the endocytic pathway that has been most

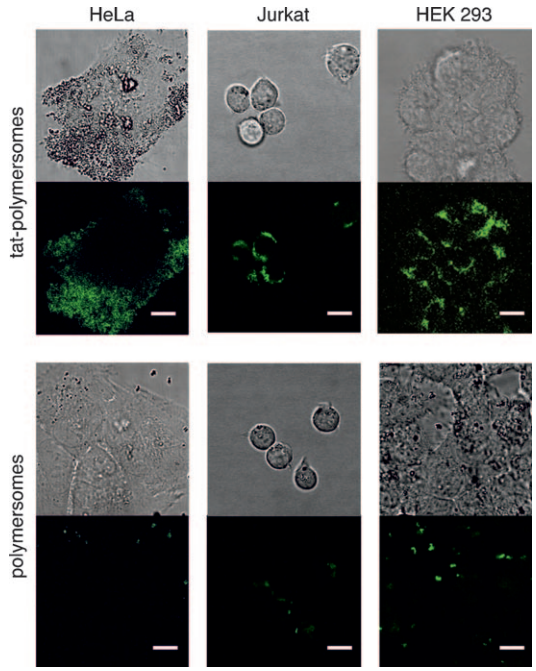

Figure 2. Confocal micrographs of different cell types incubated with GFP-loaded polymersomes with or without tat. Scale bars: 10 μm.

regularly associated with their cellular uptake.[16] Also, given the average size of the present polymersomes (114 nm), macropinocytosis can be expected to be the most prominent mechanism for their uptake, as size restrictions are typically ascribed to other pathways.[17]

To address the involvement of macropinocytosis in uptake, HeLa cells were incubated with GFP-loaded polymersomes and fluorescently labeled dextran, a polysaccharide that is a marker for macropinocytosis. Incubation was limited to 25 min to prevent endosomal mixing and ensure that colocalization did indeed result from endocytosis along a shared uptake route. Cells co-incubated with both GFP-loaded tat-polymersomes and Texas Red labeled dextran (70 kDa) showed a prominent Texas Red fluorescence. The GFP signal that was detected was low, but colocalized with the dextran signal (Figure 3). Next to the colocalization, uptake through a common route was supported by two further observations. First, with tat-free polymersomes, Texas Red fluorescence was greatly reduced, indicating that tat-polymersomes induced the uptake of dextran (Figure 3c). Second, dextran inhibited the uptake of polymersomes, as can be concluded from comparison of GFP fluorescence in Figure 3a and b, and from the flow cytometry results (Figure S2 in the Supporting Information).

Next, we investigated the fate of tat-polymersomes after their uptake into cells. A frequently observed trafficking route for cell-penetrating peptides leads to late endosomes and lysosomes.[18] These compartments have an acidic pH and can be labeled by Lysotracker Red. After incubation of HeLa cells with tat-polymersomes for 4 hours, a considerable fraction of the GFP signal colocalized with the acidic vesicles (Figure 4). An almost equally large population of GFP-containing punctuate structures retained a neutral pH, as

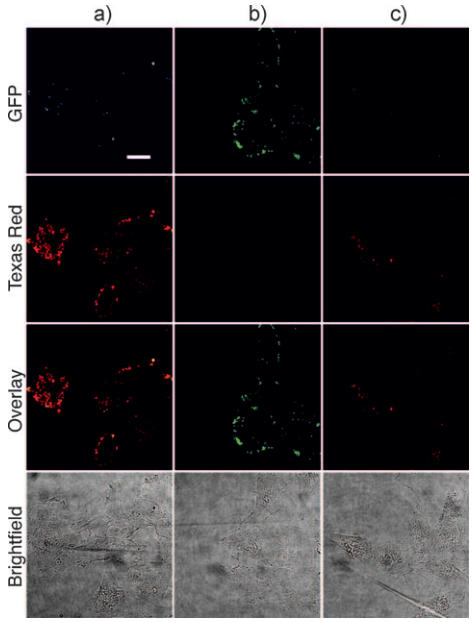

Figure 3. Confocal micrographs of HeLa cells incubated with a) GFP-loaded tat-polymersomes and Texas Red labeled dextran (70 kDa); b) GFP-loaded tat-polymersomes; c) GFP-loaded polymersomes and Texas Red labeled dextran (70 kDa). Scale bar: 20 μm. Larger copies of the brightfield image can be found in Figure S3 in the Supporting Information.

indicated by the lack of Lysotracker staining. It is unclear whether this indicates that polymersomes were retained in non-acidic vesicles, as reported for tat-cargo constructs,[19] or whether the absence of colocalization indicates cytosolic delivery. Both options would be advantageous for the use of polymersomes in protein therapy, as acidification is avoided.

The efficient uptake of tat-polymersomes through macropinocytosis and their merely partial colocalization with acidic vesicles are promising starting points for the introduction of enzyme-loaded nanoreactors into cells. We previously reported a variety of nanoreactors containing different enzymes, one of which was horseradish peroxidase (HRP).[12,20] A substrate that is neutral under physiological conditions both prior to and after oxidation by HRP is 3,3′,5,5′-tetramethylbenzidine (TMB, **4**).[21] It can diffuse through most lipid-based membranes, making TMB in the culture medium intracellularly available. The oxidation product of TMB is easily detected by measuring its absorbance ($\lambda = 370$ nm).

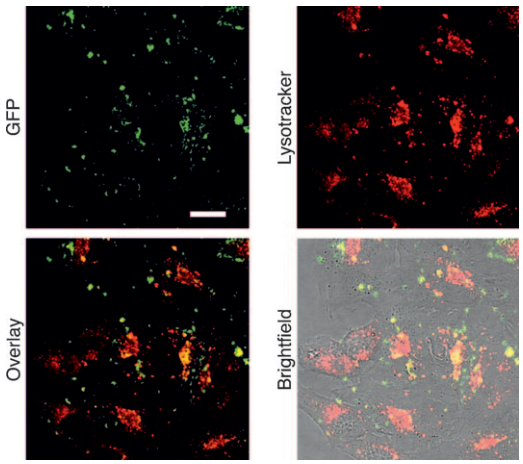

Figure 4. Confocal micrographs of HeLa cells incubated with GFP-loaded tat-polymersomes and Lysotracker Red. Scale bar: 20 μm.

To assess the ability of polymersome nanoreactors to function inside cells, we incubated HeLa cells with HRP-loaded tat-polymersomes for 4 hours to allow their internalization. The cells were then washed and incubated with TMB (120 μg mL^{-1}) and H_2O_2 (250 μM). Visual inspection of the samples revealed the intracellular activity of HRP by the appearance of a blue color. Microscopy showed that the stain emanated from the cells, leading to the formation of precipitates (Figure 5 and movie in the Supporting Information). The rate at which TMB was converted was linearly dependent on the administered dosage of polymersomes (Figure S5 in the Supporting Information).

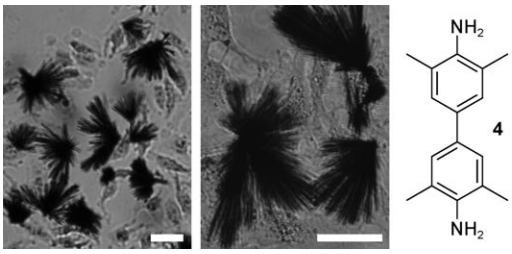

Figure 5. Representative transmission micrographs of HeLa cells containing HRP-loaded tat-polymersomes after 30 min of TMB (**4**) conversion. The scale bars approximate 30 μm.

This demonstrated that the assay could be used to quantitatively assess cellular HRP activity. Therefore, we next investigated how long the intracellular activity of HRP-loaded tat-polymersomes persists over time. Four hours after internalization, 75% of the original activity was still present. After 16 h, 42% of the original activity was preserved (Figure 6a). These results show that the polymersome-based approach maintained activity to a much higher degree than what was reported for free HRP trafficked to lysosomes, which achieved a lysosomal half-life of roughly 1 hour.[22] The half-life of HRP encapsulated in PS-PIAT polymersomes in buffer is 15 days.[12]

To identify why the HRP activity decreased over time, we tested the effects of chloroquine and nordihydroguaiaretic acid (NDGA) on our system. Both compounds can induce the release of endosomal contents into the cytoplasm.[18,23] If the observed decrease was due to degradation of the enzyme molecules in an acidic environment or endosomal recycling and cellular release, then these compounds should increase HRP activity. However, none of them exhibited a pronounced effect (Figure 6b). The reason for the decrease in HRP activity is therefore unknown at present.

In summary, we have designed a polymersome nanoreactor that is capable of entering cells, were it can induce intracellular catalysis. To this point, the cellular delivery of

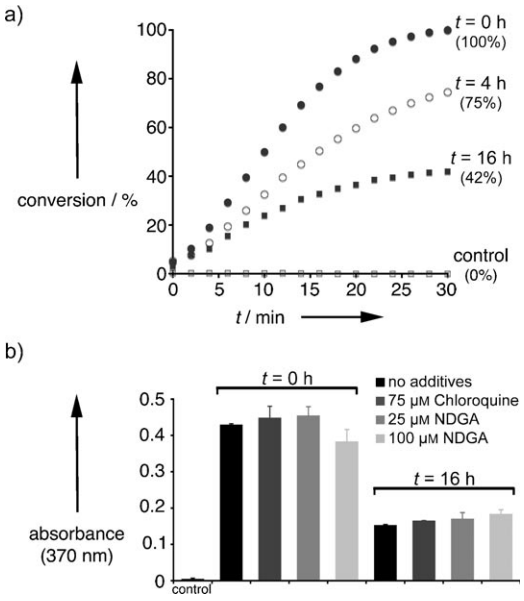

Figure 6. Activity of intracellular HRP-loaded tat-polymersomes over time ("control" denotes HRP-loaded polymersomes without **3**). a) TMB conversion at various points in time after cell uptake. b) The effect of chloroquine and NDGA; cells were pulsed with chloroquine or NDGA during the final hour of incubation.

polymersomes was restricted to cells with an intrinsically high phagocytic activity. The catalytic activity conferred to the cells was maintained at levels that were significantly higher than those reported for soluble enzymes. The results therefore represent a significant step towards a functional artificial organelle.

Experimental Section

See the Supporting Information for detailed procedures and complete references.

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