PEG-SS-PPS: Reduction-Sensitive Disulfide Block Copolymer Vesicles for Intracellular Drug Delivery

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Under appropriate conditions, block copolymeric macroamphiphiles will self-assemble in water to form vesicles, referred to as polymersomes. We report here polymersomes that can protect biomolecules in the extracellular environment, are taken up by endocytosis, and then suddenly burst within the early endosome, releasing their contents prior to exposure to the harsh conditions encountered after lysosomal fusion. Specifically, block copolymers of the hydrophile poly(ethylene glycol) (PEG) and the hydrophobe poly(propylene sulfide) (PPS) were synthesized with an intervening disulfide, PEG_{17}-SS-PPS_{30}. Polymersomes formed from this block copolymer were demonstrated to disrupt in the presence of intracellular concentrations of cysteine. In cellular experiments, uptake, disruption, and release were observed within 10 min of exposure to cells, well within the time frame of the early endosome of endolysosomal processing. This system may be useful in cytoplasmic delivery of biomolecular drugs such as peptides, proteins, oligonucleotides, and DNA.

Introduction

Arguably, the most exciting drugs of the present day are biological macromolecules, namely, peptides and proteins, oligonucleotides, and DNA. Since these drugs are hydrophilic and macromolecular, their penetration into the cell's interior is rather limited. For oligonucleotide and DNA therapy access of the molecules to the cell's interior, where they must go to exert their intended effects, is highly inefficient in the absence of a viral carrier. For peptide and protein drugs limited membrane permeation means that most of the corresponding molecular targets are extracellular, thereby limiting the most sensitive parts of the cell's regulatory machinery from consideration. Generally, these biological macromolecules enter the cell by passive or receptor-mediated endocytosis and are thus accumulated inside the endosomes.[1] The majority of such compartments ultimately fuse with lysosomes,[2] where pH and oxidative conditions are too harsh for the delivered drugs to keep their activity. Therefore, for the successful use of such drugs, delivery systems must be developed for triggering release of the drugs from their carriers within the endosome, prior to lysosomal fusion, as well as triggering disruption of the endosomal membrane.

Smart polymers have been explored to encapsulate drugs and protect them during circulation and cellular uptake, thereafter targeting them for endolysosomal release and escape. Among such nonviral delivery systems many examples can be found in the literature: the most studied triggers are changes of pH,[3−5] oxidative conditions,[6,7] temperature,[8] specific enzymatic cleavage,[9] and external application of ultrasound[10] or light.[11,12] Once within the endolysosome the drug must overcome the endolysosomal membrane barrier to finally reach its cytoplasmic target. For this reason, drug delivery systems often need to be formulated together with fusogenic molecules (e.g., dioleoylphosphatidylethanolamine, DOPE),[13] inactivated viral components,[14] or lysosomotropic agents (e.g., chloroquine),[15] the common effect of which is disruption of the endolysosomal membrane and consequent drug escape into the cytosol. However, due to high toxicity (e.g., as observed with chloroquine) and a variety of undesirable side effects, use of such agents can be problematic both in vitro and in vivo. Some polymers themselves have been designed to be fusogenic, such as copolymers comprising poly(propyl acrylic acid).[16,17] The key feature of such copolymers is that they are converted from being hydrophilic to hydrophobic when they are protonated within the acidic environment of the endolysosomal compartment. This probably causes their partition into the endolysosomal membrane and triggers its disruption. On the basis of such approaches, pH-responsive membrane-disrupting polymers and copolymers have been designed that are able to bind and deliver different kinds of drug in a highly specific way.[18]

One exciting approach that has been explored for cellular targeting and cytoplasmic delivery is encapsulation of drugs within vesicular mesophases, referred to herein as polymersomes,[19−21] of block copolymeric macroamphiphiles. Self-assembly is driven by association of the hydrophobic blocks to form the vesicular membrane, and shape determination of the assembly is related to the relative sizes of the hydrophile and the hydrophobe; under certain ratios, the assemblies form vesicles with a watery core contained within the hydrophobic membrane, which itself has a hydrophilic corona within and without. With regard to triggered release after cellular uptake, polymersomes formed from poly(ethylene glycol) (PEG) as a hydrophile and poly(lactic acid) or poly(ϵ-caprolactone) as a hydrolytically sensitive hydrophobe self-assemble with PEG block copolymers with poly(butadiene), also a hydrophobe, into vesicles and have been shown to hydrolyze under the acidic conditions experienced within the endolysosomal compartment and release their contents.[5,22,23] It would even seem that the

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macroamphiphiles themselves or their degradation products are somewhat membrane fusogenic because release from the endolysosomal compartment into the cytoplasm has been observed.[5] Yet one potential limitation in the use of pH-triggered hydrolysis lies within the rate of pH change and hydrolysis—substantially low pHs are encountered only within the lysosomal compartment of the endosomal−lysosomal processing pathway and hydrolysis is not entirely fast; as such, release can be somewhat late for sensitive biological macromolecules, i.e., within the less desirable lysosome, where biomolecular drugs are exposed to very harsh conditions, rather than the more desirable endosome.

We report here the design and characterization of a vesicle-forming macroamphiphile that leads to sudden vesicular burst within a few minutes of endocytosis while the vesicles are within the early endosome. We based our design on AB block copolymers of PEG and the hydrophobe poly(propylene sulfide) (PPS), which our group has previously shown to self-assemble into polymersomes at appropriate relative block sizes.[7,24] We have further shown that the PPS block can be eventually rendered hydrophilic by biological oxidation to poly(propylene sulfone).[6,7,25] However, oxidative conditions are not encountered until the vesicles are processed within the lysosome. Here, we placed a reduction-sensitive disulfide link between the two blocks (PEG_{17}-SS-PPS_{30}), which, we show, triggers vesicular disruption within the endosome. As the endosome enters its process of maturation, its pH is somewhat lowered (to ca. 6.5) and its interior becomes more reductive due, for example, to the influx of cysteine.[1] The strategic presence of a disulfide bond between the hydrophilic and hydrophobic blocks of the copolymer makes it sensitive to this reductive environment. Indeed, this sensitivity is high in that a single reduction per polymer chain triggers cleavage of the macroamphiphile to a hydrophobic and a hydrophilic homopolymer; we do not know what fraction of PEG_{17}-SS-PPS_{30} chains must be cleaved to affect disruption of the parent polymersome, but it is presumably far less than 100%.

Due to its stability under certain conditions and its reversibility under others, the disulfide bond occupies a special place among both biological systems and bioengineering systems for controlled release. Disulfides have been used in cationic liposomes and polycation-based DNA delivery systems, for example.[26−30]

Experimental Section

Polymer Synthesis. PEG_{17}-SS-PPS_{30} was synthesized (see Scheme 1). Synthesis and purification of PEG-thioacetate as a protected hydrophilic block was accomplished as described previously using monomethoxy-PEG_{17} (Fluka).[31] Propylene sulfide (650 mg) was ring-opening polymerized using benzyl mercaptan (38 mg) as an initiator in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 55 mg) as a base in dry THF (about 5 mL) in a Schlenk tube under an Ar atmosphere. Simultaneously, in another Schlenk tube the PEG thioacetate (1.5 g) was deprotected by reaction with sodium methoxide (4.5 mL of a solution 0.5 M in methanol) in dry THF (about 15 mL) under an Ar atmosphere. After stirring for about 1.5 h at rt, the two reactive thiolated polymers were formed. At this point the PEG thiolate solution was transferred under Ar to the PPS tube. When completely transferred, the mixture was exposed to atmospheric oxygen and a molecular halogen was added as an oxidant to favor disulfide formation as described,[32] however using iodine (1 g of molecular iodine in about 5 mL of dichloromethane) instead of bromine solution. Due to the use of the PEG thioacetate in 5-fold molar excess, disulfide-PEG homopolymer is the main byproduct of the reaction, but due to its water solubility, it could be easily purified away by means of column chromatograph on Sephadex G75 and subsequent dialysis in water. The other side product, present at only very low levels due to the stoichiometric excess of the PEG thioacetate, is the disulfide PPS homopolymer, which precipitates away when the block copolymer is put in water. Polymer compositional analysis was performed by NMR. ^{1}H NMR ($CDCl_3$): $\delta = 1.30-1.40$ (d, CH_3 in PPS chain), 1.75−1.85 (br q, 2H, $-OCH_2CH_2CH_2S-$), 2.5−2.6 (m, CH in PPS chain), 2.75−2.90 (m, CH_2 in PPS chain), 3.35 (s, 3H, $-OCH_3$), 3.5−3.55 (m, 2H, $-OCH_2CH_2CH_2S$), 3.55−3.65 ppm (br, PEG chain protons), 3.73 ppm (s, 2H, $Ar-CH_2$), 7.25, 7.27 ppm (br s, 4H, ArH), 7.18−7.2 ppm (m, 1H, ArH). The PEG_{17}-SS-PPS_{30} block copolymer has a polydispersity of 1.14, as measured by GPC in THF, using PEG standards. All reagents were from Aldrich unless specified.

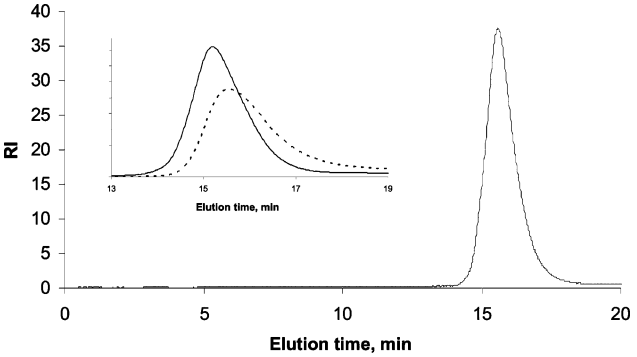


Figure 1. Gel permeation chromatography (GPC) of PEG_{17}-SS-PPS_{30} after purification and after reduction. The block copolymer PEG_{17}-SS-PPS_{30}. (Inset) Analysis before (solid line) and after (dashed line) reduction of the block copolymer to form PEG_{17}-SH and PPS_{30}-SH.

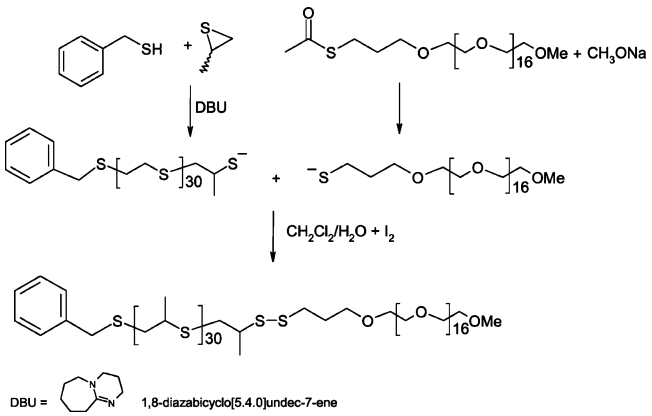
PEG_{17}-PPS_{60}-PEG_{17} was synthesized as described elsewhere.[7,31] This copolymer, of similar fraction of the hydrophile PEG, has been previously demonstrated to form polymersomes.

GPC Analyses of Degradation. Degradation analyses by GPC to establish the sensitivity of the block copolymer to the presence of a reducing agent were done before and after addition of dithiothreitol (DTT) as a reducing agent (at the amounts listed below) in the presence of trace amounts of an organic base (sodium methoxide solution in methanol) and under N_2 atmosphere. When trace amounts of the base were added to the mixture of copolymer and DTT, some precipitation was observed, which was filtered away before analysis. Formation of such precipitate is due to the low solubility of the freed PEG chains in THF cooled by N_2 bubbling and formation of THF-insoluble sodium salts. Analyses were performed on a Waters Styragel GPC system.

Vesicle Formation. A desired amount of the copolymer was weighed in a 10 mL round-bottom flask and then solubilized in a minimal amount of CH_2Cl_2. The solvent was then evaporated under rotation to yield a thin layer of polymer coating the walls of the flask. Then, either 10 mM phosphate-buffered saline (PBS, in double-distilled water, pH 7.4) or 0.1 M calcein in PBS was added to give a final concentration of polymer of 1 wt %. The mixture was then stirred well to favor polymer hydration. The multilamellar vesicles formed were then extruded multiple times through a 100 nm polycarbonate membrane (Avanti Polar Lipids Inc.) to reduce lamellarity and size polydispersity (while the size of the hydrophobic block determines the wall thickness, the actual size of the polymersome is primarily determined by the pore size of the extrusion membrane). In the case of hydration with calcein solution, a further purification step to remove free calcein by gel filtration on Sephadex G75 and subsequent dialysis for several days (MWCO 12−14000, SpectraPor) was performed. Vesicles were then analyzed by cryo-TEM and DLS (Brookhaven BI-9000AT with a 514 nm laser source).

Fluorescence Measurement To Determine Destabilization. A 20−50 μL amount of the vesicle suspension after purification was added

Scheme 1. Copolymer Synthesis[a]



[a] (Top left) Living ring-opening polymerization was used to synthesize a thiolate-terminated PPS, PPS_{30}-S^-. (Top right) Monomethoxy-PEG_{17} was functionalized with a protected thiol (thioacetate group), which was deprotected to form a thiolate-terminated PEG, PEG_{17}-S^-. (Bottom) The two polymers were mixed, and oxidation was carried out by adding a molecular halogen to form the block copolymeric macroamphiphile PEG_{17}-SS-PPS_{30}.

to 2 or 3 mL of PBS in a cuvette. The fluorescence emission was read before and after addition of different reducing agents (cysteine, DTT, GSH) at different concentrations in the range from 4×10^{-7} to 2×10^{-2} M over a period of 60−70 min (Perkin-Elmer LS50B luminescence spectrometer). $\lambda_{ex} = 490$ nm, and $\lambda_{em} = 524$ nm. One hundred percent release from the vesicles was finally obtained by addition of Triton X-100 surfactant at 5×10^{-3} M. Release percent due to the reducing agent was calculated from $(I_t - I_0)/(I_{tot} - I_0) \times 100\%$, where I_t is the fluorescence emission at each time point t, I_0 is the emission before addition of the reducing agent, and I_{tot} is the fluorescence after addition of Triton X-100 surfactant.

Cell Uptake and Release Experiments. A mouse macrophage cell line (J774A-1, ATCC) was used. Cells were cultivated as a monolayer until 80% confluence in a 75 cm^3 cell flask in the presence of culture medium DMEM, 10% fetal calf serum, 1% antibiotic antimycotic 100× (penicillin G sodium, streptomycin sulfate and amphotericin B as fungizone, all from Gibco Invitrogen Corporation). Then, they were gently scraped off in 3 mL of fresh medium and seeded in special chambered borosilicate cover glasses (Lab-Tek) at 100 000 cells/chamber and incubated overnight before addition of the vesicles. Confocal images (Carl Zeiss LSM 510) were taken at different time points on live cells, as indicated in the text.

Cell Viability Tests. J774A-1 cells were grown into a 96-well plate to about 70−80% confluence. A 1 wt % polymersome suspension was prepared, as explained above, in PBS. The vesicle suspension was diluted 1:100 in sterile PBS. Five different amounts of such stock polymersome suspension ranging from 5 to 500 μL were added to different wells and incubated overnight.

Cell viability tests were performed the next day using the cell proliferation reagent WST1 (Roche Diagnostics GmbH) diluted in 90% cell medium. Before addition of the WST1 reagent, the wells were washed twice with fresh medium, 100 μL of the medium containing 10% of the WST1 reagent was added, and they were incubated for 1 h. The wells were then read at a microplate reader at 460 nm (Tecan). Results showed no effect of toxicity at any of the concentrations of polymer tested with respect to nontreaded cells taken as the control.

Results and Discussion

PEG_{17}-ss-PPS_{30} was synthesized, as shown in Scheme 1, by oxidatively joining a PEG-monothiolate and a PPS-monothiolate under conditions such that a disulfide PPS homopolymer was disfavored, yielding the desired copolymer and as a major side product the PEG homopolymer PEG_{17}-ss-PEG_{17}, which could be readily removed by dialysis; synthesis and purification methods are described in detail further below. Because of the living nature of the propylene sulfide polymerization,[31] the polydispersity of the resulting product was very low as measured by gel permeation chromatography (GPC, polydispersity index = 1.14).

To characterize the sensitivity of PEG_{17}-SS-PPS_{30} to reduction, the block copolymer was dissolved in THF, a good solvent for both domains of the copolymer, and exposed to dithiothreitol (DTT) as a reducing agent. GPC analyses were performed before and after addition of the reducing agent. Figure 1 shows a shift in elution time toward lower weight-average molecular weight (M_w) of the copolymer soon after addition of the reducing agent. This result was in agreement with our anticipation, consistent with scission of the link between the PEG and the PPS chains to yield two chains of lower M_w due to reduction of the central disulfide.

Due to the presence of the hydrophilic PEG and hydrophobic PPS domains, PEG-PPS block copolymers spontaneously aggregate into lyotropic mesophases in water, a good solvent only for the PEG block.[7,24] The degree of polymerization of the blocks was selected on the base of well-established findings,[19,20,33] which indicate the optimal hydrophilic fraction (f) of the total molecular weight for the formation of polymer vesicles being equal to or less than 30%. Indeed, three main factors have been demonstrated to influence the aggregation behavior of amphiphilic block copolymers: condensation of the hydrophobic chains, interfacial energy, and repulsion between the hydrophilic groups.[34,35] Vesicles were in fact formed from PEG_{17}-SS-PPS_{30} ($f = 27\%$) in phosphate-buffered saline (PBS) at 1 wt % polymer by simple hydration of a dry thin layer of the block copolymer in a round-bottom flask. The spontaneously forming vesicles were then extruded through a 100 nm porous membrane multiple times to reduce the vesicles in size and decrease their polydispersity. Cryo-transmission electron microscopy (cryo-TEM) measurements, performed as indicated in previous work,[36] and dynamic light scattering (DLS) showed the presence of mostly monolamellar polymersomes with a narrow distribution of sizes and an average diameter of about 120 nm, consistent with the pore diameter of the membrane used for the extrusion, as shown in Figure 2. The vesicles showed a wall thickness of about 9 nm, consistent with previous studies from our laboratory on similar PEG-PPS block copolymer architectures.[7]

As described above, PEG_{17}-SS-PPS_{30} was demonstrated to be reductively sensitive in solution, but this does not guarantee the accessibility of the disulfide site to hydrophilic reducing agents in aqueous solution since it might be shielded within the hydrophobic domains of the polymersome membrane. To probe this, calcein, a hydrophilic fluorescent molecule, was loaded in the polymersome's watery core by hydrating the copolymer thin film in a 0.1 M calcein solution. At this concentration calcein fluorescence is autoquenched. In the case of vesicle rupture, the release of the entrapped dye can then be followed by measuring the increase in fluorescence at 524 nm due to dilution of the dye in the surrounding fluid and its subsequent dequenching. After formation of the vesicles by film hydration and membrane extrusion for homogenization of polymersome sizes, the free dye was removed by means of gel filtration on sephadex G75 and dialysis. As previously shown,[24] the stability of similar PEG-PPS polymersomes is at least in the months range. Moreover, calcein-loaded polymersomes were stored for at least 2 weeks in PBS at rt, and for months a 4 °C in PBS and showed no significant leakage of calcein (data not

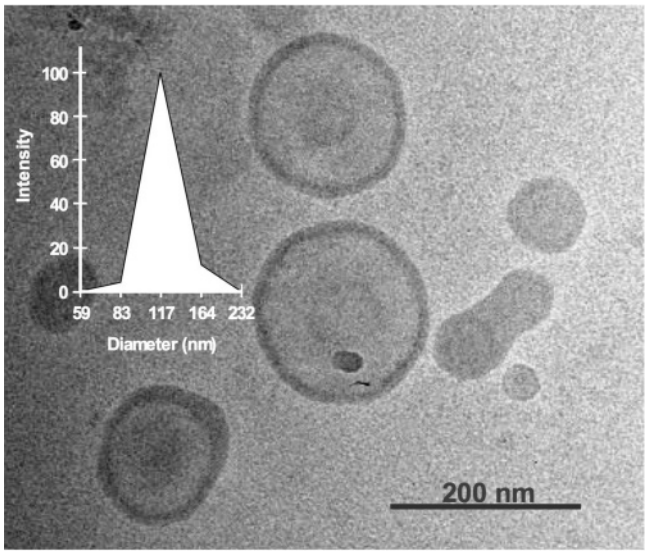


Figure 2. Cryo-transmission electron microscopy (cryo-TEM) analysis of the polymersomes. Cryo-TEM clearly demonstrated mostly monolamellar polymersomes with wall thicknesses around 9 nm. (Inset) Dynamic light scattering (DLS) analysis of the polymersomes, which gave an average size of about 120 nm and a quite narrow polydispersity.

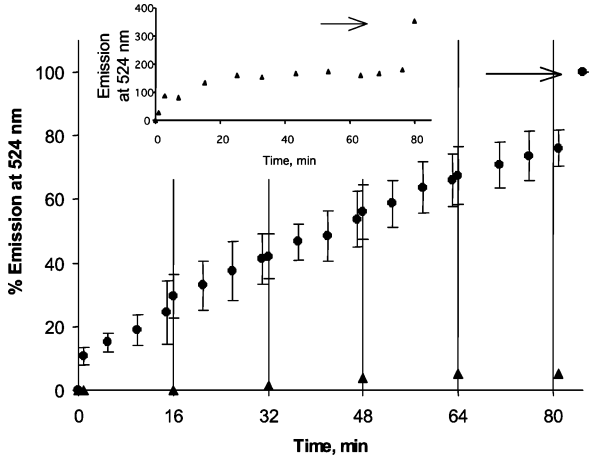


Figure 3. Fluorescence test of calcein release from reduction-sensitive polymersomes after multiple additions of cysteine as the reducing agent. Calcein was loaded within the polymersomes at autoquenching concentrations. Fluorescence demonstrating release of calcein from calcein-loaded polymersomes obtained after multiple additions (5×, shown by the lines) of cysteine. The signal is given by the dequenching of the calcein due to breakdown of the polymersomes and dilution in the cuvette below autoquenching concentrations. Final cysteine concentration is 0.7 mM. One hundred percent release is obtained by addition of Triton X-100 (arrow). (Inset) Release behavior over time after a single addition of the same total amount of cysteine to the reduction-sensitive polymersomes. It can be observed that polymersome membrane rupture is very rapid.

shown). The PEG_{17}-SS-PPS_{30} block copolymer aggregated to form vesicles, as demonstrated by cryo-TEM and by the capability to retain calcein, even after a few months from formation of the polymersomes.

Reductive sensitivity was determined within a cuvette of a fluorimeter after exposure to cysteine (shown) as well as gluthathione and dithiothreitol (not shown). Gluthathione and cysteine are the main reducing agents that maintain the redox equilibrium intracellularly (slightly reducing) and extracellularly within tissues (slightly oxidizing). The concentration of gluthathione is strictly correlated to the cysteine concentration, as cysteine is directly responsible for maintaining the proper level of redox potential in the intracellular space.[37] Cysteine efflux/afflux is controlled by a Na^+-dependent system and influences the redox state of several other metabolites normally found in the blood. For this reason, cysteine was the focus of our experimental investigations, although results with glutathione were similar (data not shown).

A rapid increase of fluorescence emission was observed always in the first 15−20 min after addition of 0.7 mM cysteine (Figure 3, inset). At a later time the amount of fluorescence associated with complete release was determined by addition of Triton X-100 surfactant. The burst release observed in the first 20 min was always 40−50% of the total entrapped calcein for each of the reducing agents analyzed. We then hypothesized that a stronger effect could be obtained in the presence of a more constant level of reduction potential. Therefore, another test of reductive sensitivity was carried out by multiple additions of the same total amount of reducing agent to the polymersomes in the cuvette. Indeed, when 0.7 mM cysteine total was added in five small additions, almost total release of the calcein from the polymersomes could be achieved, as shown in Figure 3. The response to DTT and glutathione was similar (data not shown).

For the reduction-sensitive system that we propose to be useful, its response must be very fast, on the same time scale as that of endosomal processing; likewise, sensitivity must be at cysteine concentrations that are found physiologically within the endosome.[38,39] Indeed, it has been proposed that in vivo the endosomal redox potential is modulated by the presence of a specific cysteine/cystine transporter.[1] Also, a specific reducing enzyme (GILT) has been recently identified,[40] being present in the endosomal pathway, its optimal pH being lower than neutrality and having as a primary function the unfolding of proteins destined for endolysosomal degradation. It should be understood that the endosomal concentrations of reducing agents are very high relative to extracellular concentrations: the micromolar level of plasma concentration of glutathione (GSH), the main molecule responsible for controlling the redox system of tissues, is much lower than the millimolar concentrations of reducing agent found intracellularly.[41,42] Moreover, serum concentrations of cysteine are also quite low,[37] and indeed, high concentrations of the amino acid are known to be toxic.[43] On the basis of the above discussion, it is clear that the PEG_{17}-SS-PPS_{30} polymersomes can be expected to be stable extracellularly but highly unstable intracellularly, including within the early endosome.

Following the chemical demonstrations of reductive destabilization of PEG_{17}-SS-PPS_{30} polymersomes, we proceeded to determine the uptake and intracellular fate of the vesicles after endocytosis using a mouse macrophage cell line as an example of a cell that is highly active in endocytosis. To cultured J774A-1 cells was added a few microliters of a PBS suspension of the polymersomes loaded with calcein at autoquenching concentration (0.1 M), as prepared for the fluorescence tests described above. Fluorescence within the cells was then monitored by confocal scanning laser microscopy after 10, 60, and 120 min incubation. After the predetermined time, each well was washed twice to prevent any interference with potentially rupturing extracellular polymersomes. As a reference, the same experiment was conducted with reduction-insensitive polymersomes made of a PEG_{17}-PPS_{60}-PEG_{17} triblock copolymer, which also forms polymersomes of similar shape and dimension. PEG_{17}-PPS_{60}-PEG_{17} polymersomes do not respond to reduction since the polymer contains no disulfide; rather, this polymer responds only to oxidation by conversion of the sulfides to sulfones,

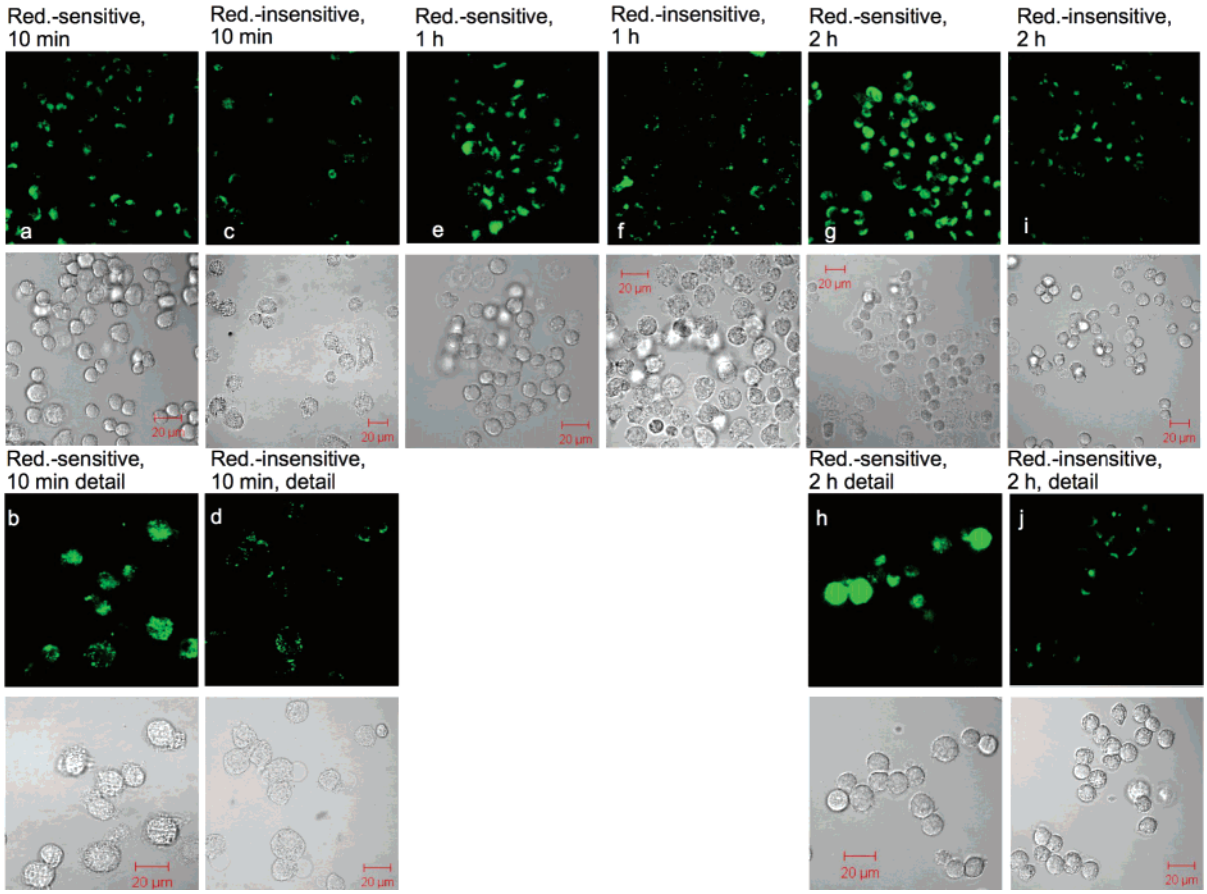


Figure 4. Biological test of intracellular release of the quenched fluor calcein from the reduction-sensitive polymersomes, relative to release from reduction-insensitive polymersomes. Mouse macrophages were incubated with reduction-sensitive PEG_{17}-SS-PPS_{30} polymersomes and reduction-insensitive control polymersomes (PEG_{17}-PPS_{60}-PEG_{17}) loaded with calcein at autoquenching concentration. Confocal scanning laser microscopy analyses were done at 10 (panels a−d), 60 (panels e and f), and 120 min (panels g−j) time points on live cells after washing each well and replacing the medium with PBS. As can be seen from panel a, and in more detail in panel b, after 10 min incubation with the reduction-sensitive vesicles most of the cells show extensive punctate fluorescence. This is the early delivery event where the calcein is released from the intraendosomal polymersomes due to block copolymer reduction but remains at least partially localized inside the endosomes. The control experiment, as shown in panels c and d, reveals also the presence of some, but much less, punctate fluorescence, indicating that most of the reduction-insensitive polymersomes remain intact within the endosomes. Over time, at 60 (panel e) and 120 min (panels g and h), the number of cells from those treated with reduction-sensitive polymersomes that show more diffuse and bright fluorescence increase remarkably. In these cells with diffuse fluorescence the calcein was able to overcome the endosomal barrier (late delivery event) and freely diffuse in the cytoplasm (see detail in panel h), giving an intense and diffuse signal of fluorescence. On the contrary, the intense diffuse fluorescence in the cytoplasm was not observed in any of the cells treated with the control polymersomes at any time point (panels f, i, j). Here, most of the calcein remains entrapped inside the reduction-insensitive vesicles at autoquenching concentration, and the small amount that is release remains entrapped within the endolysosome.

presumably within the lysosome.[7] In both cases, detection of vesicle internalization started within 10 min after incubation (see Figure 4, panels a−d). Such fast internalization of particles has been shown for this cell type.[44] In the case of the reduction-sensitive vesicles (Figure 4, panels a and b), already after 10 min most of the cells exhibited punctate intracellular fluorescence, consistent with intraendosomal release but not endosomal rupture. At the longer time points of 60 and 120 min (Figure 4, panels e and g, and in more detail in h), the amount of fluorescence within the cell could be observed to substantially increase. At these longer time points fluorescence was much more diffuse, indicating calcein escape from the endosome into the cytoplasm by some form of endosomal rupture (Figure 4, contrasting panels d and h).

Populations of cells were scored and counted as containing fluorescence or not, and for those containing fluorescence, whether fluorescence was punctate or diffuse, performing such scoring at 10, 60, and 120 min incubation (Figure 5). Considering that the calcein is a highly charged molecule, which is not able to diffuse through natural phosopholipid membranes such as the endosomal membrane,[16] release from the endosomes to yield diffuse fluorescence must be correlated with some destabilization activity upon the endosomal membrane due to the separated polymer chains after endosomal reduction. It is apparent from these quantitative studies that the reduction-sensitive polymer yields substantial cellular uptake and very fast release within the endosome with somewhat slower release from the endosome into the cytoplasm. Polymersomes that were not reduction sensitive also yielded intraendosomal fluorescence (although lower in number of cells bearing fluorescence and much lower in amount of fluorescence per cell, vis. Figure 4, panels f and i); however, this fluorescence matured only in a very small number of cells to diffuse cytoplasmic staining. Indeed, only a very weak punctate fluorescence could be observed for cells treated with reduction-insensitive polymersomes, even at the longest time points analyzed (Figure 4, panels c, f, and i and in more detail d and j). Due the high similarity of the macroamphiphile architecture and polymersome morphology between the two systems, a similar uptake was expected for both reduction-sensitive and -insensitive polymersomes, but

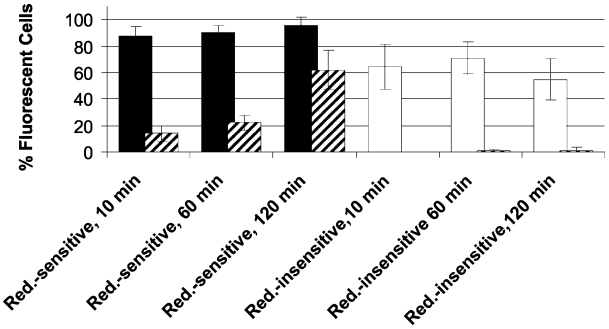


Figure 5. Total release and cytoplasmic release of calcein from reduction-sensitive polymersomes and controls that are reduction insensitive. The *total release* from reduction-sensitive polymersomes is shown as black bars and represents the percentage of all the cells that show fluorescence. This number is the sum of the cells where the release of calcein from the polymersome appeared both in the endosomes (punctuate fluorescence) and in the cytosol (bright, diffuse fluorescence). *Cytoplasmic release* (shown as diagonal striped bars) refers only to that percentage of the total number of cells that show intense diffuse fluorescence due to polymersome breakdown, escape from the endosomes, and calcein dequenching in the cytoplasm. Total release is shown as white bars for the reduction-insensitive control polymersomes and cytoplasmic release as a separate set of striped bars.

little intraendosomal release and very little cytoplasmic delivery from the reduction-insensitive polymersomes could be observed. In addition to endosomal release, the reduction-sensitive block copolymer seemed to be capable of endosomal disruption after reduction, leading to release of the calcein into the cytoplasm; the mechanism of this disruption is unknown. It should be understood that neither polymersome formulation was cytotoxic: viability tests of the mouse macrophages in the presence of different doses of the vesicles, from 5- to 100-fold more concentrated than those used for these experiments and also after overnight incubation, were carried out. In no case could a statistically significant reduction in viability be observed (results not shown).

Conclusions

Polymersomes from a reduction-sensitive block copolymeric macroamphiphile, PEG_{17}-SS-PPS_{30}, were sought to develop nanocarriers that can be endocytosed with designed sensitivity to the endosomal microenvironment. In particular, we sought a system that would yield rupture in the endosome rather than the lysosome, i.e., early within the course of endolysosomal processing. Results with GPC demonstrated sensitivity of the block copolymer to reduction, and results with fluorescence dequenching both in vitro and in cellular systems demonstrated that this takes place under physiological conditions. As hypothesized, due to the existence of a high difference in the redox potential between the mildly oxidizing extracellular space and the reducing intracellular space,[40] the rational design of a disulfide bond in a critical position between the hydrophobic and the hydrophilic blocks of the copolymer resulted in formation of highly sensitive and very rapidly reacting system. The fast response of the vesicles in the presence of a reducing environment may be due to the fact that only one link must be reduced per polymer chain and presumably much less than 100% of the polymer chains must be reduced to destabilize a polymersome. The rapid response observed is compatible with endosomal release within the context of the time course of the endosomal processing prior to lysosomal fusion.

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