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Polymer Nanocontainers for Intracellular Delivery

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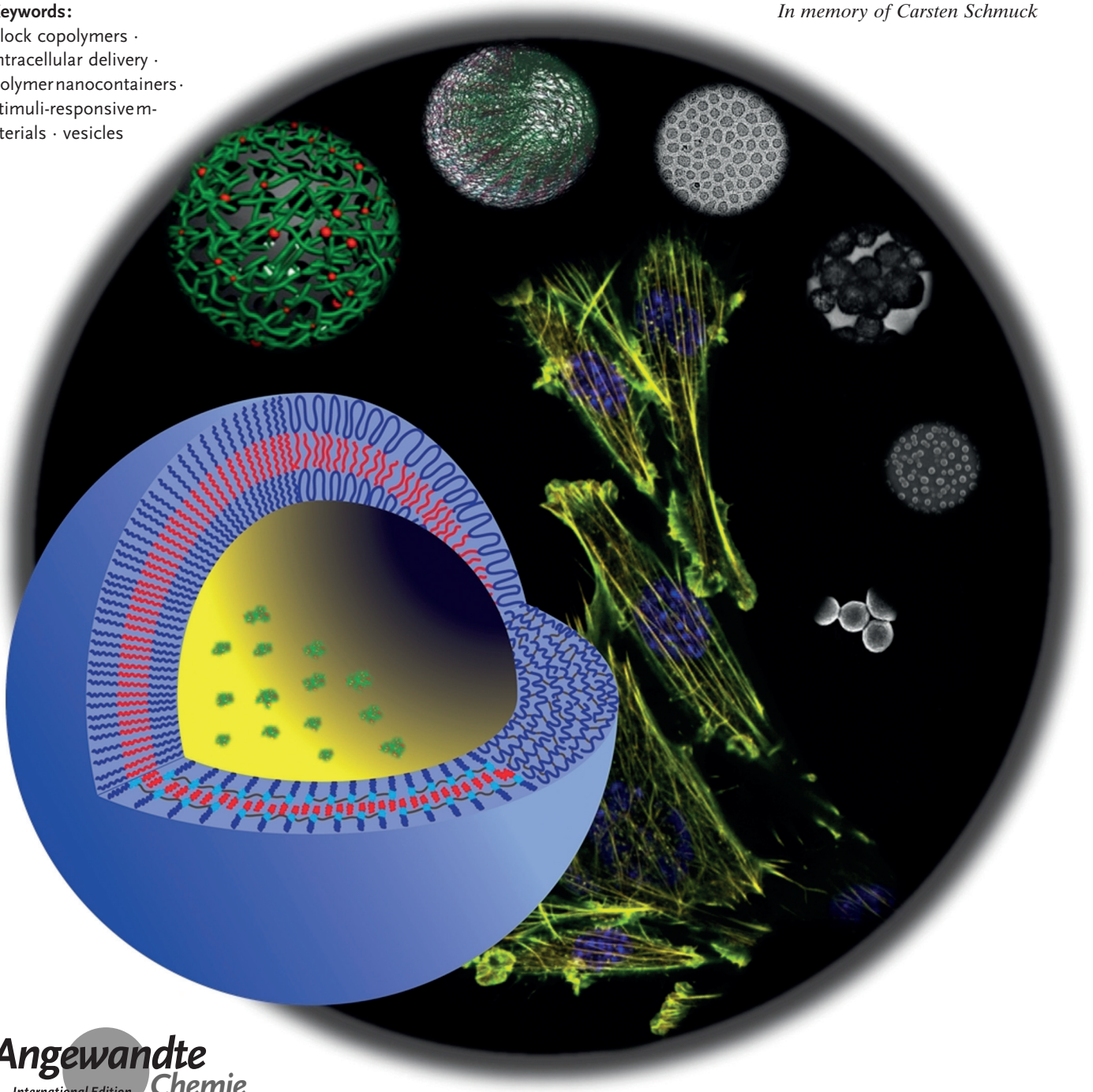
In memory of Carsten Schmuck



Carriers for intracellular delivery are required to overcome limitations of therapeutic agents such as low specificity, systemic toxicity, high clearance rate, and low therapeutic index. Nanocontainers comprised of an aqueous core and a polymer shell have received increasing attention because they readily combine stimuli response to improve intracellular payload release and surface modification to enhance selectivity towards the desired region of action. This Minireview summarizes the design and properties of polymer nanocontainers for intracellular delivery, classified according to the polymer architecture.

1. Introduction

The intracellular delivery of therapeutic agents and other biomolecules by using nanosized carriers has been widely studied in the past few decades. Even if the ultimate goal of a "magic bullet" for drug delivery remains elusive, many research groups across the globe have investigated increasingly sophisticated nanocontainers for the intracellular delivery of therapeutic payloads.[1] Nanocarriers have been employed to overcome various hurdles faced by therapeutic agents before they reach their site of action.

Nanocarriers protect the therapeutic agents from binding to plasma proteins, increase their half-life in the blood stream, suppress the clearance rate, and hence increase their efficiency. They also help to increase biodistribution and tissue uptake and control the dosage of the therapeutic agents through controlled release. Moreover, they can overcome the barrier of the lipid bilayer of the cell membrane by different endocytosis pathways. In the design of nanocarriers for intracellular delivery, the optimal combination of size, shape, surface properties, colloidal stability, biocompatibility, minimal toxicity, payload release, etc. continues to pose a phenomenal scientific and technological challenge.[2]

In many respects, viruses are the source of inspiration for all synthetic nanocontainers. During the course of millions of years of evolution, viruses have optimised their ability to enter host cells to deliver their genetic material in order to proliferate. Various nanosized intracellular delivery carriers are known, including micelles, liposomes, synthetic vesicles, and polymersomes.[3] Micelles are highly dynamic assemblies of amphiphiles composed of a hydrophobic core and hydrophilic shell.[4] Their potential for intracellular delivery is limited since they can only encapsulate hydrophobic payloads, their critical micelle concentration is typically rather high, and many amphiphiles damage cell membranes (lysis). Phospholipids self-assemble into liposomes that mimic the membrane of eukaryotic cells by having a large inner aqueous compartment and a very thin outer bilayer. Such nanostructures are capable of encapsulating a large amount of hydrophilic cargo as well as small amounts of hydrophobic or amphiphilic payloads. Synthetic vesicles are similar to liposomes but made from synthetic amphiphiles.[5] For liposomes and synthetic vesicles, the critical aggregation concentration (CAC) limits their delivery applications because of disassembly upon dilution, while their thin membrane and low colloidal stability lead to premature release of the payloads. Polymersomes are similar to liposomes but formed from amphiphilic polymers with lower CAC values and thicker membranes.[6]

In this Minireview, we focus on intracellular delivery by polymer nanocontainers. We define polymer nanocontainers as containers that:

1) are prepared from synthetic macromolecules,
2) have a size from 10 nm to 500 nm,
3) have a water-filled compartment, and
4) can encapsulate hydrophilic cargo in the aqueous compartment and hydrophobic payload in the polymer shell.

According to this definition, related carriers such as polymer micelles,[7] polymer nanogels,[8] and inorganic nanoparticles[9–11] are outside the scope of this Minireview.

Based on a thorough review of the literature, polymer nanocontainers can be broadly classified into two classes: cross-linked and non-cross-linked nanocontainers. Cross-linking of polymers in nanocontainers often enhances the colloidal stability, reduces permeability and premature release of the payload, and enables a response to a stimulus such as a pH change or a redox process. Non-cross-linked nanocontainers are further divided in two subgroups depending on the polymer used: 1) polymersomes formed from amphiphilic block copolymers,[12] and 2) polymersomes formed from other amphiphilic polymers. On the other hand, cross-linked nanocontainers can be classified according to their mode of preparation, that is, without or with a template. The first group consists of cross-linked nanocontainers formed from amphiphilic polymers that are cross-linked in situ. In this case, the hollow core is often altered by the cross-linking of the polymer shell. The template-mediated method overcomes this problem, since the polymer is deposited on a template and then cross-linked. In the case of sacrificial templates, the template is removed to create the hollow compartment, while other templates are retained inside the hollow core.[13–15] Layer-by-layer (LBL) deposition is a versatile strategy to prepare template-based polymer nanocontainers.[16] The architectures of polymer nanocontainers discussed in this Minireview are shown schematically in Figure 1.

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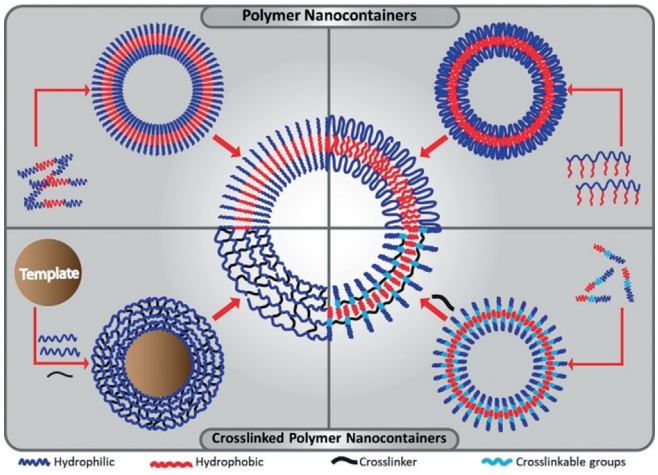

Figure 1. Polymer nanocontainers for intracellular delivery discussed in this Minireview. Polymer nanocontainers contain a polymer shell that encapsulates an aqueous interior and can be prepared without and with cross-linking of the polymer shell. Polymer nanocontainers can be assembled from amphiphilic block copolymers (top left) or from other types of amphiphilic polymers (top right). Cross-linked polymer nanocontainers can be prepared by in situ cross-linking of amphiphilic polymers (bottom right). Alternatively, cross-linked polymer nanocontainers can be prepared using (sacrificial) templates (bottom left).

Selective and controlled intracellular delivery of the nanocontainers is essential for therapeutic applications. Stimuli-responsive bonds or moieties are introduced in the polymers or the cross-linkers to achieve the selective and controlled intracellular release of the payloads. The response is either induced autonomously upon intracellular entry (typically: endocytosis) or it is induced externally using a physical stimulus such as temperature or light. Since the intracellular glutathione (GSH) concentration is much higher (2–10 mM) than the extracellular concentration (2–20 μM), GSH can be used to cleave disulfides upon uptake by the cell. Similarly, the lysosome is acidic (pH 5), while the cytosol and blood stream are neutral (pH 7.4). Whereas imines, acetals, and hydrazines are cleavable at acidic pH values, moieties such as amines and acids change the amphiphilicity of the polymers when the pH value is varied. Different pathological conditions are associated with several enzymes being overexpressed. Esterase and protease enzymes cleave esters and peptides, respectively, while there are several other enzymes that can cleave specific peptide sequences. Elevated temperature (40–42 °C) is associated with conditions such as inflammation or tumour growth, and polymers such as polyethylene glycol (PEG) or poly(*N*-isopropylacrylamide) (PNIPAM) can become insoluble in water at these temperatures because of the loss of hydrogen bonding (lower critical solvation temperature, LCST). Light is another stimulus often considered, and light-reactive units such as nitrobenzenes can affect the amphiphilicity of the polymers.[17–19] To further increase the selectivity, the surface of the nanocontainers are modified with targeting ligands which can enhance receptor-mediated endocytosis.[20]

In this Minireview, we discuss selected examples of polymer nanocontainers that have been investigated for the purpose of intracellular delivery. We have organised the Minireview according to the architecture of the polymer nanocontainers as outlined above and illustrated in Figure 1. We have selected examples on the basis of the combination of innovative polymer chemistry, detailed characterisation of the nanocontainer structure, and demonstrated intracellular delivery.

2. Polymer Nanocontainers

2.1. Polymer Vesicles Assembled from Amphiphilic Block Copolymers

Advances in living polymerisation techniques, such as ring-opening polymerisation (ROP), radical polymerisation (atom-transfer radical polymerisation (ATRP), and reversible addition-fragmentation chain-transfer polymerisation (RAFT)), as well as anionic polymerisation, have paved the way for the design of increasingly complex block copolymer structures. The synthesis of block copolymers allows the preparation of amphiphilic polymers with well-defined hydrophilic and hydrophobic blocks. Stimuli-responsive bonds and moieties can be introduced in the polymer backbone or the side chains, while targeting ligands and imaging molecules can be easily attached to specific blocks. In this section we discuss a selection of responsive polymersomes formed from diblock and triblock copolymers that are well-suited for the intracellular delivery of hydrophilic and hydrophobic payloads.

In view of the increased concentration of GSH in the intracellular environment, redox-responsive polymeric vesicles have received much attention. A disulfide-linked triblock copolymer of polyethylene glycol and poly ε-benzyloxycarbonyl-L-lysine (PzLL-SS-PEG-SS-PzLL; Figure 2a) self-as-

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Bart Jan Ravoo received his MSc and PhD from the University of Groningen with Prof. Engberts. After a postdoctoral fellowship at University College Dublin (Ireland) he became assistant professor at the University of Twente (The Netherlands). Currently, he is full professor at WWU Münster (Germany). The focus of his research is self-assembly and, together with his group, he develops responsive soft materials and functional surfaces by the bottom-up self-assembly of molecular and nanoscale building blocks.

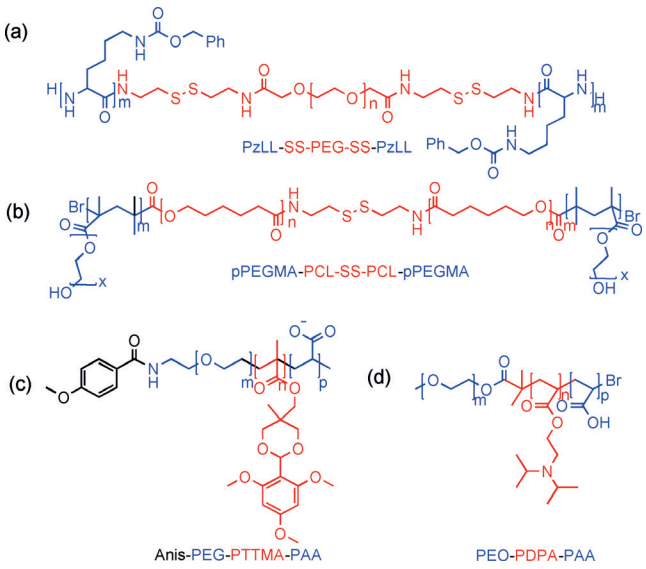

Figure 2. Amphiphilic block copolymers used for the preparation of stimuli-responsive polymersomes (red: hydrophobic block, blue: hydrophilic block).

sembled into polymeric vesicles that were able to encapsulate hydrophilic and hydrophobic molecules and could be cleaved by reduction.[21] Transmission electron microscopy (TEM) images and confocal laser scanning microscopy (CLSM) showed the formation of nanocontainers with a diameter of about 350 nm that were composed of a hollow inner compartment protected by a polymeric outer layer (Figure 3). These vesicles can be taken up by cells through endocytosis. CLSM images of HeLa cervical cancer cells treated with vesicles containing doxorubicin (DOX) showed an increased uptake and colocalisation of DOX in the nucleus after release by reductive cleavage of the polymers by GSH. Moreover, vesicles loaded with the anticancer drug gemcitabine hydrochloride could reverse the drug resistance observed in a resistant breast-cancer cell line.

A reduction cleavable polymersome designed from triblock copolymer poly(polyethylene glycol methacrylate)-poly(caprolactone)-SS-poly(caprolactone)-poly-(polyethylene glycol methacrylate) (pPEGMA-PCL-SS-PCL-pPEGMA) (Figure 2b) was modified to a dual cancer-targeting nanocontainer using folate and trastuzumab ligands, and was used to deliver DOX to breast cancer cells.[22] In vivo studies on mouse models showed an increased effect with this nanocontainer compared to free DOX, as well as eliminating the possible cardiotoxic effects of DOX.

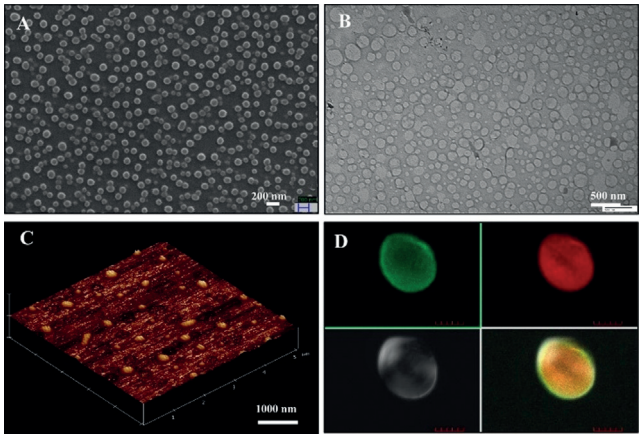

Figure 3. A) SEM, B) TEM, and C) AFM images of DOX-loaded polymersomes. D) CLSM images of polymersomes with encapsulated fluorescent dyes in the core and in the shell. Reproduced with permission.[21] Copyright 2015, American Chemical Society.

Whereas GSH enables a response to reduction, reactive oxygen species (ROS), such as H_2O_2, which are associated with pathological conditions, such as cancer, provide opportunities for the design of oxidation-responsive nanocontainers. Oxidation-responsive polymersomes were made from a diblock copolymer containing self-immolative pendant linkages capped by arylboronate ester groups, and then surface-functionalised using mitochondria-targeting peptides.[23] The oxidation by H_2O_2 leads to removal of the arylboronate ester caps, followed by a series of cascade decaging reactions leading to the cross-linking of the vesicle bilayer by the generated amine groups and, hence, inducing permeability for the transported cargo.

The difference in the pH value of lysosomal compartments (pH 5.5) and cytoplasm (pH 7.4) has also been exploited for the controlled release of cargo from polymer vesicles. Lu et al. presented their studies on anisamide-decorated pH-responsive polymersomes for protein delivery to treat lung cancer cells.[24] Self-assembly of the triblock copolymers anisamide-poly(ethylene glycol)-*b*-poly(2,4,6-trimethoxybenzylidene-1,1,1-tris(hydroxymethyl)ethane methacrylate)-*b*-poly(acrylic acid) (Anis-PEG-PTTMA-PAA; Figure 2c) and PEG-PTTMA-PAA in water gives anisamide-decorated polymersomes that are able to load the apoptotic protein granzyme B. Anisamide guides the way to the intracellular environment by receptor-mediated endocytosis, with high specificity towards cancer cells because of the overexpression of a membrane-bound protein (sigma receptor) which has a high affinity towards anisamide. The acid-cleavable acetal moieties on the pendant groups of the polymer release the proteins upon endocytosis.

In the study discussed above, the pH sensitivity is due to acetals that are cleaved at acidic pH values. Similarly, pH-sensitive bonds such as imine and hydrazine can be introduced in block copolymers. Alternatively, a pH response can be introduced through moieties such as tertiary amines that are (de)protonated to modulate electrostatic interactions. Polymers having a charge are used to stabilise negatively charged biomolecules such as siRNA. Polymer vesicles formed from pH-responsive poly(ethylene oxide)-*b*-poly(2-(diisopropylamino)ethyl methacrylate)-*b*-poly(acrylic acid) (PEO_{43}-$PDPA_{76}$-PAA_{17}; Figure 2d) were labelled with anti-EpCAM (epithelial cell-adhesion molecule) monoclonal antibodies, which target cancer stem cells, and used for the delivery of DOX and siRNA.[25] Cancer stem cells are resistant to anticancer drugs and are responsible for sustaining tumour growth and are capable of initiating tumours. While the delivered siRNA helped to overcome the resistance by silencing the expression of oncogene, DOX destroyed the DNA of these cells.

Enzyme-responsive nanocontainers are another emerging class for intracellular delivery. Many enzymes such as esterases and proteases are overexpressed in pathological cells. Ge and co-workers reported enzyme-responsive asymmetric vesicles for the delivery of the anticancer drug paclitaxel. These vesicles were made of a triblock copolymer poly(ethylene glycol)-GPLGVRG-*b*-poly(ε-caprolactone)-*b*-poly(3-guanidinopropyl methacrylamide) (PEG-GPLGVRG-PCL-PGPMA). The peptide GPLGVRG is cleaved by matrix metalloproteinase (MMP) enzyme, which is overexpressed in tumour tissues. This leads to dePEGylation and a morphological transformation to multicavity vesicles with the cell-penetrating PGPMA segments exposed to the outside, thereby enhancing cellular uptake (Figure 4(i)).[26] TEM images of the vesicles before and after treatment with MMP are shown in Figure 4(ii), and clearly shows the formation of multicavity vesicles after treatment with MMP.

The elevated temperature in tumours (40–42 °C) as a result of their higher metabolism was exploited to make temperature-responsive nanocontainers. The temperature-responsive triblock copolymer poly(*N*-vinylcaprolactam)$_n$-poly(dimethylsiloxane)$_{65}$-poly(*N*-vinylcaprolactam)$_n$ (PVCL$_n$-PDMS$_{65}$-PVCL$_n$) was synthesised using RAFT polymerisation and self-assembled into polymersomes capable of encapsulating DOX.[27] The nanocontainers were stable at room temperature, but controlled release was obtained over a temperature range of 37–42 °C, depending on the length of the PVCL block. The same group developed another temperature-sensitive polymersome from the diblock copolymer poly(*N*-vinylcaprolactam)$_n$-*b*-poly(*N*-vinylpyrrolidone)$_m$ (PVCL$_n$-PVPON$_m$), which self-assembles at temperatures above the LCST of the polymer as a result of dehydration of the PVCL block.[28] The addition of tannic acid at $T >$ LCST leads to polymersomes that are stabilised at $T <$ LCST. These polymersomes delivered DOX and higher molecular weight fluorescein isothiocyanate-dextran (FITC-dextran) to alveolar adenocarcinoma cells; release was achieved by enzymatic degradation of tannic acid.

2.2. Polymer Vesicles Assembled from Hydrophobically Modified Polymers

In addition to block copolymers, other types of amphiphilic polymers form functional polymer vesicles. Hydrophilic polysaccharides such as dextran and chitosan can be modified into amphiphilic polymers by conjugation with hydrophobic substituents such as cholesterol that aggregate to form the hydrophobic layer of the polymer vesicles. As in the case of block copolymers discussed in the previous section, a stimulus response, such as to reduction, pH, and light, can be easily induced in these polymers by incorporating reactive bonds or moieties.

Dong and co-workers reported a lipopolysaccharide amine from modified alginate containing two oppositely charged hydrophilic parts, an anionic oxidised alginate and a cationic polyethyleneimine, as well as hydrophobic cholesterol substituents (Figure 5a). This polymer self-assembled in water to form polymersomes.[29] The cationic polyethylene imine helps in the endosomal escape (proton sponge effect) and hence the cytosolic delivery of the payloads, as shown by TEM imaging. Polymersomes trapped in the endosomes after endocytosis and then released by rupture of the endosomal membranes were observed in the TEM images.

The increased metabolism in tumours results in a large amount of lactic acid being produced, which decreases the pH value in cancer tissues. Chang and co-workers exploited this fact and designed a dual-responsive PEG-coated polymeric lipid vesicle (PPLV) based on dextran (Figure 5b).[30] The PEG that is covalently linked to hydrazine is removed in acidic conditions, thereby exposing the positively charged lysine to the outside and thus increasing the cellular uptake. The encapsulated drugs are released upon reductive cleavage of the alkyl chain linked through a disulfide linkage (Figure 6). Similarly, dextran modified with acid groups and hydrophobic side chains (Figure 5c) have been used for cancer therapy.[31] The polymer was conjugated with biotin, which targets cancer cells, and the disulfide-containing hydrophobic side chains were linked through an ester bond

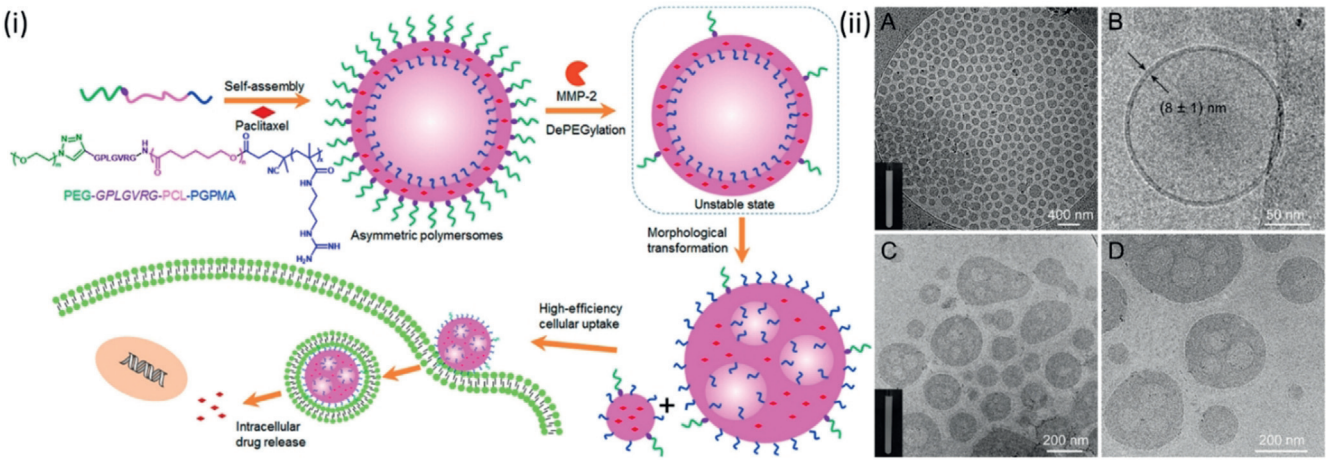

Figure 4. i) Asymmetric polymer vesicles, their morphological transformation upon dePEGylation by MMP enzymes, and high-efficiency cellular uptake on exposure of the cell-penetrating peptides. ii) Cryo-TEM images of vesicles before (A,B) and after (C,D) treatment with MMP enzymes. Reproduced with permission.[26] Copyright 2017, American Chemical Society.

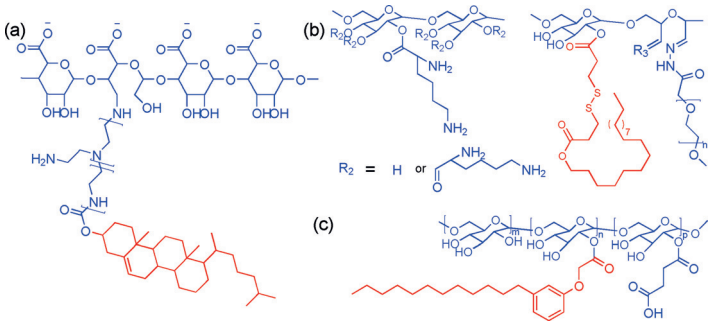

Figure 5. Modified polysaccharides used for the preparation of nanocontainers.

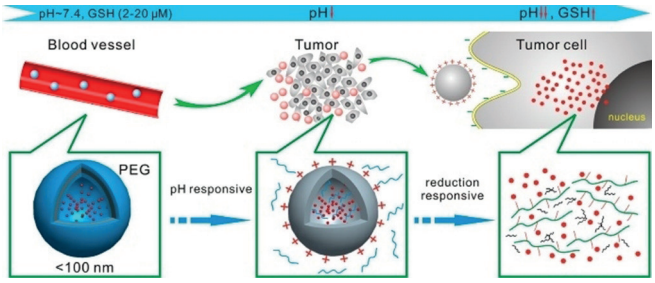

Figure 6. Mode of action of PPLV: PEG removal at acidic pH values in the tumour, enhanced uptake of positively charged vesicles, and reduction-responsive release. Reproduced with permission.[30] Copyright 2014, American Chemical Society.

(Figure 7(i)). DOX-loaded vesicles were taken up by receptor-mediated endocytosis and DOX was released by the esterase cleaving the ester bonds as well as by GSH reducing the disulfide linkages. CLSM images (Figure 7(ii)) show enhanced uptake of the DOX-loaded targeted polymer vesicles ($V_{BIOTIN+DOX\cdot HCl}$) into HeLa cancer cells compared to wild-type MEF cells.

In a related study, the star-shaped terpolymer poly(ethyleneglycol)-poly(ε-caprolactone)-poly(*N*-isopropylacrylamide) (PEG-PCL-PNIPAM) with an acetal and a disulfide bond self-assembled into pH- and reduction-responsive polymer vesicles for imaging and thermo-chemotherapy.[32] Upon irradiation, the encapsulated near-infrared fluorescence imaging agent indocyanine dye (ICG) increased the temperature from 30 °C to 42 °C, thereby inducing hyperthermia and releasing ROS. The second payload, DOX, was released from the lysosomes by ROS-mediated lysosomal rupture.

Polymer nanocontainers can also be functionalised with metal nanoparticles to enable surface-enhanced Raman scattering (SERS) plasmonic imaging of tumours. For this purpose, an amphiphilic gold nanoparticle modified with polymers PEG and polymethyl methacrylate vinylpyridine (PMMAVP) was prepared. Polymersomes formed from this amphiphile contained encapsulated DOX and a Raman reporter dye and the surface was decorated with cancer-targeting HER2 antibody.[33] The closely packed gold nanoparticles enhanced the SERS intensity through strong interparticle plasmonic coupling. As a result, the SERS intensity decreases when the polymersomes disassemble at lower pH values, and this was used to track the release of the payload. The same team developed a photo-responsive polymer vesicle similar to the above system for the targeted delivery of anticancer drugs, with monitoring by plasmonic imaging.[34] Amphiphilic gold nanoparticles comprised of hydrophilic PEG and hydrophobic poly(2-nitrobenzyl acrylate) (PNBA) formed the plasmonic vesicles, with gold nanoparticles arranged in the hydrophobic PNBA layer. SERS-active vesicles were taken up by endocytosis mediated by the folate receptor. Irradiation by UV light resulted in PNBA losing its hydrophobicity and the polymer

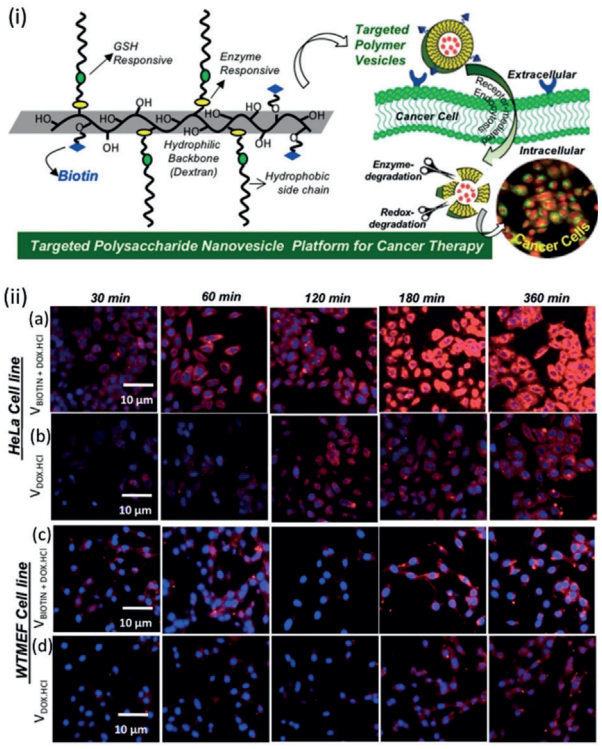

Figure 7. i) Formation of biotin-decorated polysaccharide vesicles and their receptor-mediated endocytosis. ii) CLSM images of the a, c) $V_{BIOTIN+DOX\cdot HCl}$-treated and b, d) $V_{DOX\cdot HCl}$-treated HELA cell line (a, b) and wild-type MEF cell line (c, d). Reproduced with permission.[31] Copyright 2018, American Chemical Society.

vesicles falling apart with dispersal of the gold nanoparticles, hence destroying their plasmonic coupling and switching off the SERS signal (Figure 8).

3. Cross-Linked Polymer Nanocontainers

3.1. Template-Free Assembly of Cross-Linked Polymer Nanocontainers

Similar to liposomes composed of shorter amphiphiles, polymersomes are also dynamic in nature, that is, the polymersomes are in equilibrium with the polymers in

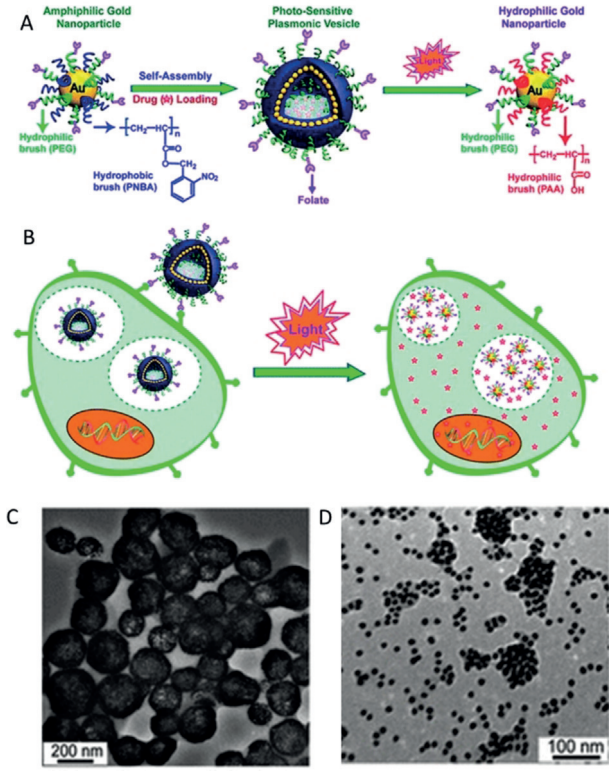

Figure 8. A) Self-assembly of plasmonic vesicles composed of amphiphilic gold nanoparticles and their photoresponsive destruction. B) Receptor-mediated endocytosis and light-responsive release of the payloads. TEM images C) before and D) after photo-irradiation of the vesicles. Reproduced with permission.[34] Copyright 2013, Royal Society of Chemistry.

solution, and dilution below their CAC leads to disassembly of the polymersome and premature release of encapsulated payloads. Cross-linking the polymers in a polymer nanocontainer is a powerful strategy to overcome this limitation. Various factors should be considered when cross-linking a polymer nanocontainer: Cross-linking should retain the size and shape of the nanocontainer and its hollow compartment, avoid interparticle cross-linking and aggregation, and not affect the payload. Polymersomes containing functional groups such as carboxylic acids and amines can be cross-linked using bifunctional linkers. Moreover, cross-linkers can be used to introduce a stimulus-responsive moiety. For example, cysteamine can cross-link poly(carboxylic acids) with redox-responsive disulfide bonds. Alternatively, thiol- or disulfide-containing polymers are exploited to make self-cross-linkable nanocontainers by thiol–disulfide exchange. Selected examples of cross-linked polymer nanocontainers are illustrated in this section.

The linear brush diblock copolymer poly(ONBAn$_n$-*b*-ONB-PEG$_m$) (ONBAn$_n$ = oxanorbornenyl anhydride, ONB = ω-oxanorbornenyl) synthesised by ring-opening metathesis polymerisation self-assembled into polymeric vesicles. The polymer bilayer was cross-linked using an acid-sensitive diamino ketal cross-linker, and the anticancer drug cisplatin was conjugated in the bilayer.[35] The cross-linked vesicle protected the drug at physiological pH 7.4 and efficiently released the payload in the acidic lysosome with pH 5.5 (Figure 9). The triblock copolymer poly(ethylene-glycol)-*b*-poly(acrylic acid)-*b*-poly(*N*-isopropylacrylamide) (PEG-PAA-PNIPAM) self-assembled into dual-responsive polymersomes capable of loading proteins such as lysozyme, bovine serum albumin, cytochrome *c*, and ovalbumin.[36] Proteins were labelled with FITC for imaging and to track the delivery of the proteins. Further stabilisation of the polymersomes was obtained by cross-linking using cysteamine to connect the acid functionalities in the PAA blocks. Whereas the disulfide cross-linker imparted the reduction response, the PNIPAM provides an additional temperature sensitivity.

The triblock copolymer poly(ethylene glycol)-poly(acrylic acid)-poly(2-(diethylamino)ethyl methacrylate) (PEG-PAA-PDEA), synthesised by RAFT polymerisation, was modified post-polymerisation to thiol-containing PEG-PAA(SH)-PDEA.[37] This polymer was soluble in acidic water and formed polymersomes at a physiological pH value by oxidative cross-linking through disulfides. The PDEA block with tertiary amines resulted in acid sensitivity and a proton sponge effect for endosomal escape. These dual reduction- and pH-responsive cross-linked vesicles efficiently encapsulated apoptotic proteins such as bovine serum albumin and cytochrome *c* and delivered the payloads into cancer cells (Figure 10).

Another interesting example of a self-cross-linking polymersome by thiol–disulfide exchange was obtained by the self-assembly of a diblock copolymer of PEG and dithiolane-functionalised poly(trimethylene carbonate) (PTMC). A fraction of the polymers were end-functionalised with PHSCN peptide (ATN-161), which targets melanoma cancer

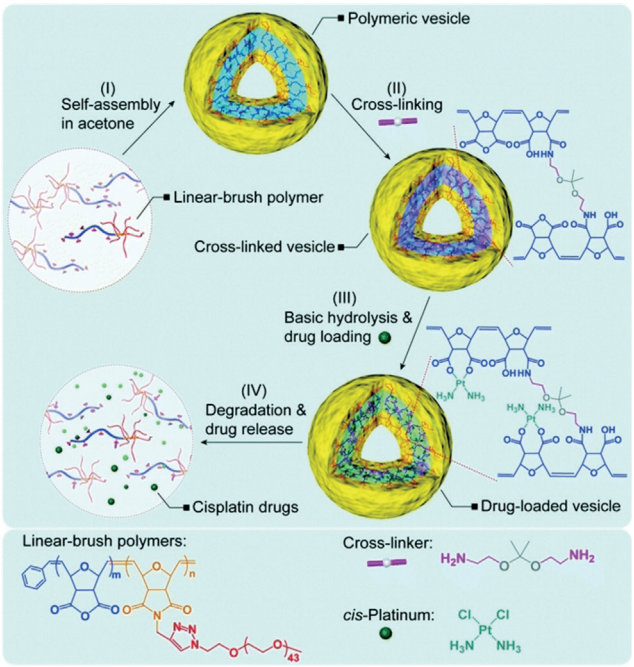

Figure 9. Preparation of cross-linked cisplatin-conjugated vesicles. Reproduced with permission.[35] Copyright 2015, Royal Society of Chemistry.

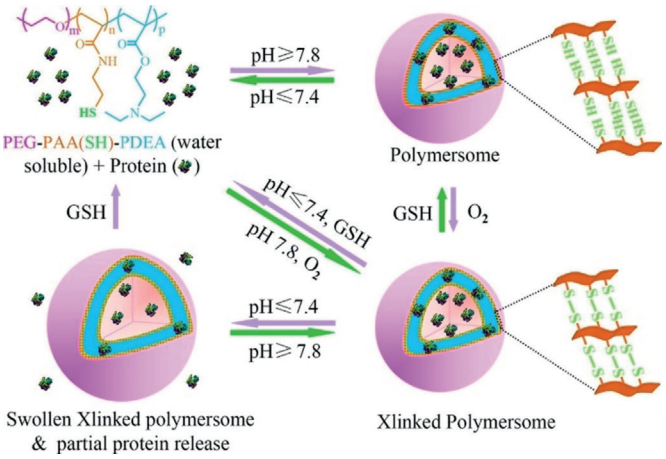

Figure 10. Redox and pH response of disulfide-cross-linked polymersomes. Reproduced with permission.[37] Copyright 2014, Elsevier.

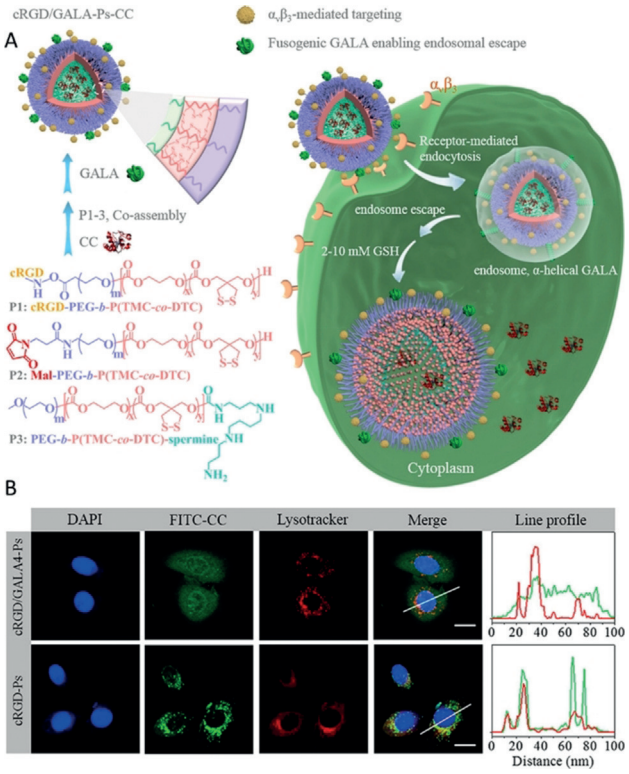

Figure 11. A) Formation of cRGD and GALA peptide dual-functionalised polymersomes, receptor-mediated endocytosis followed by endosomal escape, and efficient cytosolic delivery after GSH action. B) CLSM images of FITC-CC (green) encapsulated in cRGD-Ps- or cRGD/GALA4-Ps-treated A539 cells stained by lysotracker (red) and DAPI (blue). Reproduced with permission.[39] Copyright 2019, American Chemical Society.

cells.[38] DOX-encapsulated ATN-161-decorated polymersomes with disulfide cross-links showed more efficient intracellular internalisation and cytoplasmic DOX release in B16F10 melanoma cancer cells compared to nontargeted polymersomes and clinically used pegylated liposomal DOX.

Since it is important that a nanocontainer delivers its cargo to a specific region inside cells, many therapeutic agents suffer from the critical issue of endosomal entrapment. There are different ways to overcome this barrier, such as using fusogenic peptides and the proton sponge effect. Zhong and co-workers reported the preparation of a self-cross-linking, reduction-responsive polymersome decorated with cRGD and fusogenic GALA peptide by the coassembly of the three triblock copolymers PEG-*b*-poly(trimethylene carbonate-*co*-dithiolane trimethylene carbonate)spermine (PEG-*b*-P(TMC-*co*-DTC)-spermine), cRGD-PEG-*b*-P(TMC-*co*-DTC), and maleimide-PEG-*b*-P(TMC-*co*-DTC) for the delivery of a model protein, cytochrome *c*. cRGD plays the role of the targeting moiety, while GALA assists in the endosomal escape to deliver the proteins to the cytoplasm (Figure 11).[39]

Polymersomes formed by the self-assembly of oppositely charged polymers are also named PICsomes (polyion complex polymersomes). The PEG-containing block aniomer poly(ethylene glycol)$_{45}$-poly(α,β-aspartic acid)$_{75}$ (PEG$_{45}$-(PAsp)$_{75}$) along with homocatiomer poly([5-aminopentyl]-α,β-aspartamide)$_{82}$ (homo-P(Asp-AP)$_{82}$) formed a amphiphilic block copolymer that self-assembled into PICsomes (Figure 12).[40] Formation of a peptide bond between the carboxylic acids on the aniomer and amines on the catiomer leads to the cross-linked PICsomes. The amphiphilic photosensitizer A1^{III}-phthalocyanine chloride disulfonic acid (AlPcS2a) was released by photo-irradiation, probably as a result of photochemical damage of the PIC membranes. Recently, a new class of PICsomes called siRNAsomes with siRNA as the ionic homopolymer was developed by Kataoka and co-workers. Electrostatic interactions between the block catiomer poly(ethylene glycol)-*b*-poly[*N*-(5-aminopentyl]-α,β-aspartamide] (PEG-P(Asp-AP)) and the negatively charged siRNA led to formation of the siRNAsomes, which were cross-linked by glutaraldehyde reacting with the pendant

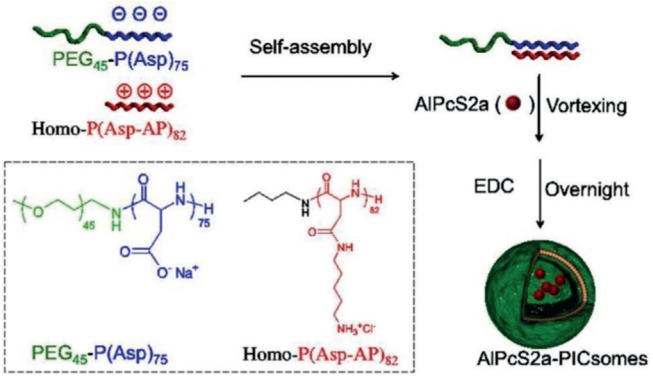

Figure 12. Formation of photosensitiser-loaded cross-linked PICsomes. Reproduced with permission.[41] Copyright 2013, American Chemical Society.

primary amines. This nanocontainer can deliver hydrophilic macromolecular cargoes along with siRNA.[41]

An important feature of any drug delivery carrier is the ease of surface functionalisation, for example, to display targeting ligands. Nanocontainers with host molecules such as cyclodextrin or cucurbituril on the periphery can be readily modified with functional guest molecules. Kim and co-workers developed a reduction-responsive cross-linked polymer

nanocontainer with cucurbituril on the periphery (Figure 13).[42] Carboxyfluorescein, as a model payload, was encapsulated in the nanocontainer and the surface was modified with a targeting galactose ligand, with spermidine used as a guest for cucurbituril. Receptor-mediated endocytosis led to the nanocontainer being efficiently taken up by cancer cells overexpressed with galactose receptors, and a subsequent reductive cleavage of the disulfides released the payload. In a follow-up study, the nanocontainer was decorated with the cancer-targeting ligand RGDyK as well as imaging agents Cyanine 7 and ^{64}Cu-NOTA (NOTA = 1,4,7-triazacyclononane-1,4,7-triacetic acid) complexes for multimodal in vivo imaging.[43]

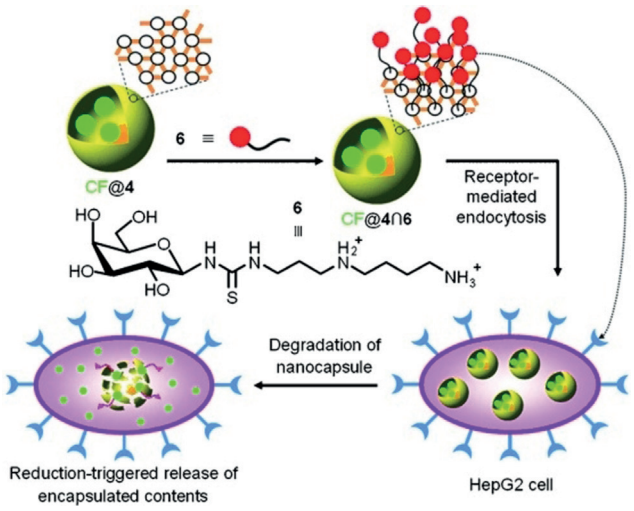

Figure 13. Nanocontainer decorated with galactose through host–guest interactions, receptor-mediated endocytosis, and reduction-triggered release of the cargo. Reproduced with permission.[42] Copyright 2010, Wiley-VCH.

3.2. Template-Mediated Assembly of Cross-Linked Polymer Nanocontainers

The cross-linked nanocontainers discussed in the previous section often alter their size and shape as a result of cross-linking. Templating methods can be used to make cross-linked polymer nanocontainers which maintain their size, shape, and rigidity. Silica nanoparticles constitute a versatile template. Polymers are coated or grown on the surface or allowed to infiltrate into the pores of the template. After cross-linking, the sacrificial templates are removed. Although the templates are usually sacrificial, there are some reports of hollow non-sacrificial templates. The use of methods such as LBL deposition, mesoporous silica templating, and surface polymerisation enable the physiochemical properties of the nanocontainers to be fine-tuned. In general, the size and shape depend on the templates and the rigidity depends on the cross-linking density. The use of biocompatible and biodegradable polymers and the introduction of different stimuli-responsive units on the polymers or cross-linkers make these types of nanocarriers well-suited for intracellular delivery.

Caruso and co-workers reported an LBL deposition method for the silica template mediated formation of enzyme-degradable hybrid polymer nanocapsules with a polymer coating that could be removed by a change in the pH value (Figure 14).[44] Negatively charged poly(methacrylic

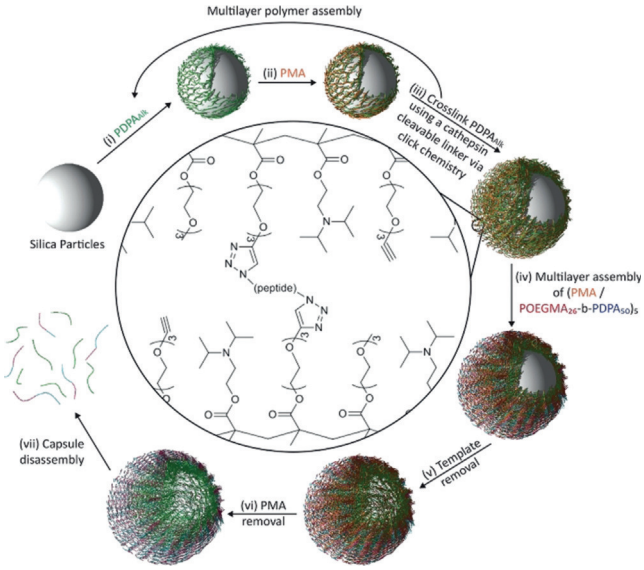

Figure 14. Fabrication of enzyme-degradable cross-linked polymer nanocontainers by LBL deposition. Reproduced with permission.[44] Copyright 2014, Wiley-VCH.

acid) (PMA) and positively charged alkyne-functionalised poly(2-diisopropylaminoethyl methacrylate) (PDPA) were deposited on the silica particles, followed by cross-linking of the PDPA with a bisazide-functionalised enzyme-cleavable peptide linker. Moreover, fluorescent dyes were conjugated to the polymer for imaging. Multilayers of poly(oligo(ethylene glycol) methyl ether methacrylate)-*b*-poly((2-diisopropylaminoethyl) methacrylate) (POEGMA$_{26}$-PDPA$_{50}$) were deposited to suppress premature hydrolysis. The silica core and PMA were removed to obtain the desired nanocarrier. The same team made redox-responsive nanoporous particles of poly(ethylene glycol)-poly(L-lysine) (NPEG-PLL) using a sacrificial mesoporous silica (MS) template (Figure 15).[45] The PLL infiltrates into the pores of the MS and cross-linking was achieved with disulfide linkers. After removal of the core, the nanocontainer was used for the delivery of siRNA, which eliminates the anti-apoptotic factor survivin to treat prostate cancer cells. The redox-responsive cleavage of the cross-linker leads to the release of the siRNA into the cytosol and hence silences the survivin.

A related type of multilayered and responsive polymer nanocontainer was designed for cancer therapy.[46] The sacrificial poly(methacrylic acid) (PMAA) core and additional layers were synthesised by distillation/precipitation polymerisation. A pH-sensitive second layer of PMAA imparted the pH response. This was followed by a temperature-responsive poly-((*N*,*N*′-dimethylaminoethyl) methacrylate) (PDMAEMA) polymer layer and finally an outer

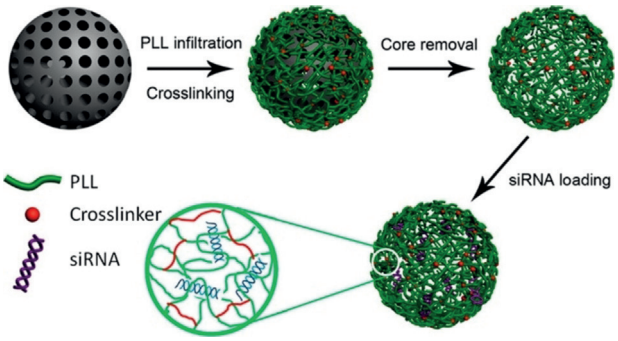

Figure 15. Preparation of siRNA-loaded cross-linked nanoporous particles by templating on mesoporous silica. Reproduced with permission.[45] Copyright 2015, American Chemical Society.

reduction-responsive layer of the disulfide-containing monomer *N*,*N*-bis(acryloyl)cysteamine (BAC), which is also responsible for cross-linking in the outer layer. The PMAA core was dissolved using ethanol/water and the hollow container was filled with the model anticancer drug daunorubicin. The nanocontainers rapidly delivered the drug into cells.

Sacrificial templates are removed under harsh conditions, which can lead to damage of the nanocontainer and, moreover, the encapsulation of cargo becomes difficult once cross-linking is achieved. To overcome this problem, De Vries et al. developed a redox-responsive polymer-shelled nanocontainer with cyclodextrin vesicles acting as a non-sacrificial template (Figure 16).[47] The amphiphilic cyclodextrins self-assembled into vesicles, the adamantane-terminated poly(acrylic acid) formed the polymer shell through host–guest interactions, and cross-linking by reduction-cleavable cysteamine formed the stable polymer nanocontainer. These nanocontainers showed an efficient encapsulation of hydrophilic payloads such as pyranine and phalloidin. Cell imaging revealed intracellular uptake, reduction-responsive release, and cytoplasmic payload delivery, thus making the system suitable for biological applications requiring controlled release.

4. Conclusions and Future Perspectives

Polymer nanocontainers are versatile intracellular delivery carriers with a tunable size, high encapsulation capacity for hydrophilic payloads, and straightforward surface modification. Advances in polymer chemistry enable the introduction of powerful features, such as stimuli responsiveness, in these nanocarriers. Polymer vesicles formed from block copolymers and other amphiphilic polymers have a lower CAC than liposomes and micelles, whereas cross-linked nanocontainers retain their size and shape at any dilution. This overcomes the issues of disassembly and premature release of payload before reaching the intracellular site of action. The presence of targeting units on the surface of the nanocontainers helps them to selectively reach the desired location and stimuli-responsive units provide controlled release.

Despite numerous reports on sophisticated polymer nanocontainers, many improvements are still desirable. Although biocompatibility is the minimum requirement for these nanocarriers, biodegradability is the most desirable quality for long-term applications. Although there are a very large number of different types of nanocontainers with demonstrated proof-of-concept, the number of systems that reach the stage of clinical trials is still very small and (to the best of our knowledge) limited only to polymersomes.[48] The payloads described in the literature are mostly limited to small molecules, whereas nanocontainers would be particularly beneficial for the delivery of biological drugs such as proteins and nucleic acids. In addition, we are not aware of photoresponsive nanocontainers that do not require UV irradiation or the toxic photochemistry of nitro compounds. Furthermore, we expect the development of multipurpose and multi-stimuli-responsive intracellular nanocontainers which release payloads at desired and prolonged time intervals in response to intracellular or external signals. Multicompartment nanocontainers capable of encapsulating different types of payloads and their sequential release would

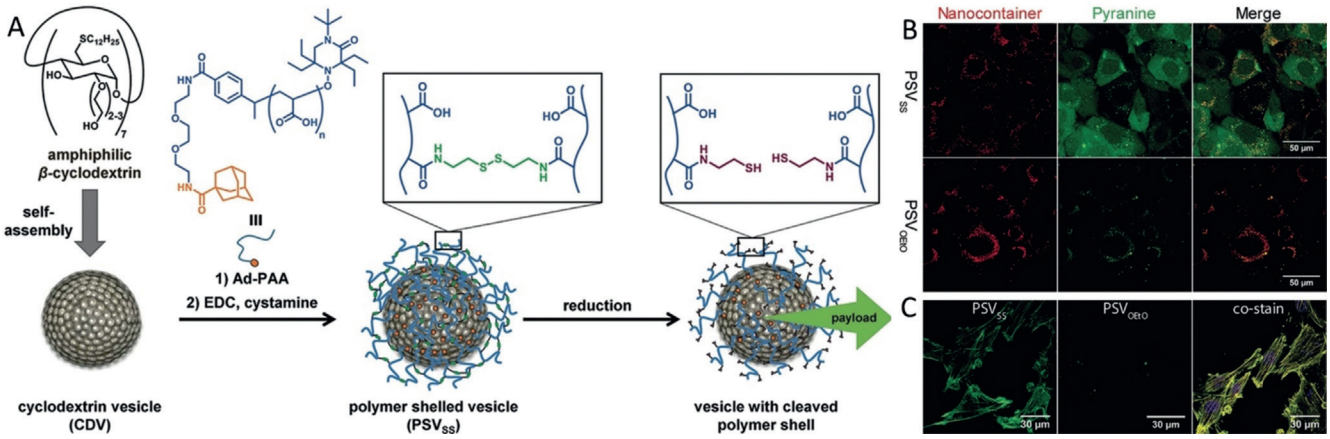

Figure 16. A) Fabrication of polymer-decorated cyclodextrin vesicles and reduction-responsive release of the payload. B) CLSM images of pyranine-loaded rhodamine B labelled PSV_{SS}- and PSV_{OEtO}-treated live 3T3 cells (PSV = polymer-shelled vesicles). C) CLSM images of the same cells treated with FITC-phalloidin-loaded PSV_{SS} and PSV_{OEtO}. In the case of PSV_{SS}, FITC-phalloidin is released by the action of GSH followed by binding to F-actin. In the case of PSV_{OEtO}, the FITC-phalloidin remains trapped. Reproduced with permission.[47] Copyright 2017, Wiley-VCH.

constitute a next generation of polymer nanocontainers that would bring us a step closer to the "magic bullet".

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Conflict of interest

The authors declare no conflict of interest.

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