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REVIEW

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Polymerization-induced self-assembly for drug delivery: A critical appraisal

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Abstract

Polymerization-induced self-assembly (PISA) with (in situ) encapsulation of (therapeutic) cargo has proven to be an efficient preparation method for loaded polymeric micelles and polymersomes, thereby presenting significant opportunities in the field of drug delivery. However, despite extensive research efforts, no significant advances toward systematic in vivo studies or clinical applications have been achieved to date. In this Review, we outline the current state-of-the-art of cargo encapsulation via PISA with a specific focus on developments achieved in the past 5 years. Considering the general requirements for functional drug delivery systems, we identify the major hurdles that still need to be overcome in order to push PISA-derived systems from a promising academic exercise to viable candidates for clinical translation.

KEYWORDS

amphiphilic block copolymers, drug delivery, polymeric micelles, polymerization-induced self-assembly (PISA), polymersomes

1 | INTRODUCTION

Polymerization-induced self-assembly (PISA) is a preparation method for self-assembled polymeric nanoparticles (NPs) that combines the formation of amphiphilic block copolymers (BCPs) and their self-assembly (SA) in a single step (Figure 1A). First reported in 2002,[1] PISA has since proved to be a versatile, efficient, and reproducible addition to the conventional preparation methods for polymeric NPs, such as spherical micelles, worm-like micelles and polymersomes (Figure 1B).[2,3] Such NPs are of great interest due to their core-shell structure: Compartmentalization on the nano-scale allows for encapsulation of (hydrophobic) cargo in the core and/or shell, thereby enhancing its solubility in aqueous media and protecting it from degradation. In the case of polymersomes, encapsulation of hydrophilic cargo in the water-filled lumen is an additional option. Using the toolbox of organic and polymer chemistry, NP properties such as composition, size, shape, and stability can be finely tuned. This versatile platform finds (potential) application in biomedicine, but is also of interest to other fields such as colloidal templates,[4,5] organic–inorganic hybrid nanomaterials,[6] antimicrobial materials,[7,8] Pickering emulsifiers,[9–11] nanoreactors,[4,12] sensing,[13] electrochemistry,[14,15] and others.[16]

The conventional approach to prepare micellar NPs is to first synthesize the amphiphilic BCPs (Figure 1B). After isolation and purification, the BCPs are generally dissolved in a good (organic) solvent. Then, (gradual) replacement of the good solvent with a non-solvent (typically water) reduces the solubility of the core-forming block, which triggers SA if the BCP concentration is above the critical micelle concentration (CMC).[17] To encapsulate

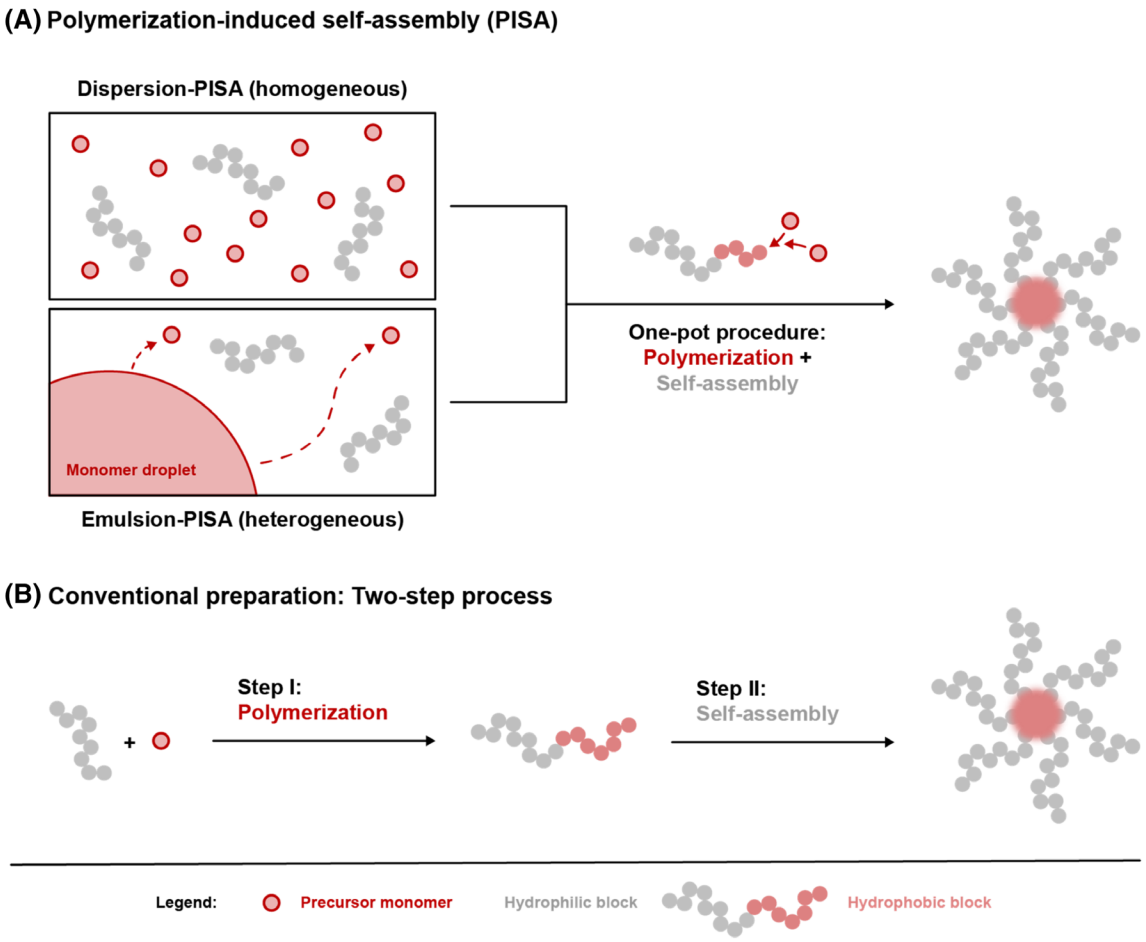

FIGURE 1 Preparation of polymeric nanoparticles (NPs) (A) via polymerization-induced self-assembly (PISA), starting from a dispersion or an emulsion, and (B) conventionally in a two-step process.

cargo, the SA step is simply performed in the presence of the desired cargo or it is loaded via post-SA approaches, such as latex swelling.[18] Over the years, several different SA strategies based on direct and indirect dissolution have been developed to accommodate different polymer and cargo chemistries. An overview of the most relevant methods is provided in Table 1. However, the presence and difficulty associated with the removal of organic solvents can be problematic in for example, biomedical applications.[17,19] Other drawbacks are that the SA process can be time-consuming and is usually conducted at low solid content (often <1 wt%). These aspects limit options for economically favorable upscaling. Finally, the formation of higher-order morphologies remains challenging with conventional SA.[20]

PISA on the other hand conveniently combines polymerization and self-assembly in a one-pot procedure that can be conducted at higher solid content (usually 10–50 wt%).[35–37] In a typical PISA process, a solvophilic polymer block is chain-extended with an either soluble (dispersion PISA) or poorly soluble (emulsion PISA) monomer (Figure 1A). During chain extension, the solvophobic blocks become increasingly insoluble. Upon reaching a critical block length, the onset of SA is triggered. Further polymerization of unreacted monomer continues beyond this onset, often with an increased reaction rate due to monomer accumulation in the hydrophobic domains of the newly formed micelles/nuclei.[35] Due to the continuous chain growth, the volumetric aspect ratio of the polymer blocks, reflected by the micelle packing parameter p (Figure 2), evolves as a function of the monomer conversion. It is generally accepted that $p \leq 1/3$ leads to spherical micelles, $1/3 \leq p \leq 1/2$ worm-like micelles, and $1/2 \leq p \leq 1$ polymersomes. $p > 1$ results in inverted morphologies.[38] Hence, initially formed spherical micelles can in principle undergo morphological transitions toward higher-order morphologies, such as worm-like micelles and polymersomes during PISA.[34]

For PISA to occur, a suitable solvophilic stabilizing block needs to be matched with a monomer that forms a polymer block that will gradually become insoluble to drive SA in the respective solvent. Living step-growth

TABLE 1 Self-assembly strategies for block copolymer (BCP)-based nanoparticles.[21]

Method	General concept	Advantages (+) and disadvantages (−)	Ref.
Direct dissolution	BCP and cargo are dissolved at or above the CMC in an aqueous solution, may be facilitated by heating (for thermosensitive BCPs SA upon heating above the CMT = Fast heating)	+ Simple, no organic solvent, feasible for upscaling − BCP needs to be sufficiently soluble in aqueous solution, low cargo loading	22,23
Nanoprecipitation	A water-miscible organic solution of BCP and cargo is rapidly mixed with water, inducing precipitation and self-assembly	+ Simple, fast, particle size controlled by BCP concentration and mixing time, high cargo loading, feasible for upscaling − Kinetic trapping very likely	24–27
Thin film hydration	BCP and cargo are casted onto a surface by evaporating the organic solvent, rehydration with (heated) aqueous solvent	+ High drug loading capacity, feasible for upscaling − Time-consuming, requires expensive equipment	23
Direct freeze-drying	A freeze-dried BCP-cargo cake is reconstituted with aqueous solution	+ Suitable for thermolabile cargo, solvents recyclable, feasible for upscaling − Freeze-dryable solvent required, time-consuming, requires expensive equipment	28
Solvent exchange, e.g., via dialysis or evaporation	BCP and cargo are dissolved in a water-miscible organic solvent, which is gradually replaced by water	+ Slow exchange reduces chance of kinetic trapping, high drug loading capacity − Time-consuming, large volumes of water necessary for dialysis, not feasible for upscaling	17,29
Oil-in-water (O/W) emulsification	Cargo is solubilized in water-immiscible solvent droplets, BCP partitions at the solvent/water interface, cargo is incorporated upon solvent evaporation; Variation of the method: Salting-out[30]	+ High drug loading capacity − Often requires chlorinated solvents, not feasible for upscaling	17
Crystallization-driven self-assembly (CDSA)	BCP with a semicrystalline block crystallizes upon solvent exchange	+ Non-spherical and more complex, hierarchical assemblies accessible − Requires semicrystalline core-forming block	31–33
Polymerization-induced self-assembly (PISA)	Simultaneous BCP synthesis and particle assembly in one pot, concurrent cargo encapsulation	+ Efficient one-pot procedure, higher-order morphologies accessible − Suitable monomer/polymer combination required, many parameters influencing NP morphology require tuning of reaction conditions	34

Abbreviations: CMC, critical micelle concentration; CMT, critical micelle temperature.

polymerizations, such as reversible deactivation radical polymerization (RDRP), are best suited for PISA, as they enable good control over the architecture, for example, the blocking efficiency, and the length of the formed amphiphilic BCPs. Reversible addition-fragmentation chain-transfer (RAFT) polymerization is most commonly employed due to its broad monomer scope, compatibility with water as solvent, and possibilities to conduct polymerizations under very mild conditions.[39,40] However, atom transfer radical polymerization (ATRP), nitroxide-mediated radical polymerization (NMP), ring-opening (metathesis) polymerization (RO(M)P), as well as more exotic techniques, such as organotellurium-mediated living radical polymerization (TERP), anionic polymerization, addition-fragmentation chain transfer (AFCT) polymerization, reversible complexation-mediated polymerization (RCMP), and cobalt-mediated radical polymerization (CMRP), have been successfully applied for PISA as well.[41–43] As such, PISA is compatible with a wide range of monomers, solvents, additives, and initiation mechanisms that go beyond thermal initiation, for example, photo-, redox/oscillatory reaction-, enzyme-, and ultrasound wave-initiation.[44]

Given this versatility, PISA has the potential of being a synthetic workhorse for biomedical and pharmaceutical applications, such as bioimaging,[45–47] biocatalysis,[48] tissue culture,[49] and cell preservation.[50,51] One of the most envisioned applications of PISA is, however, the preparation of polymeric NPs for drug delivery.[43,52–55] Incorporating therapeutic agents, such as small (hydrophobic)

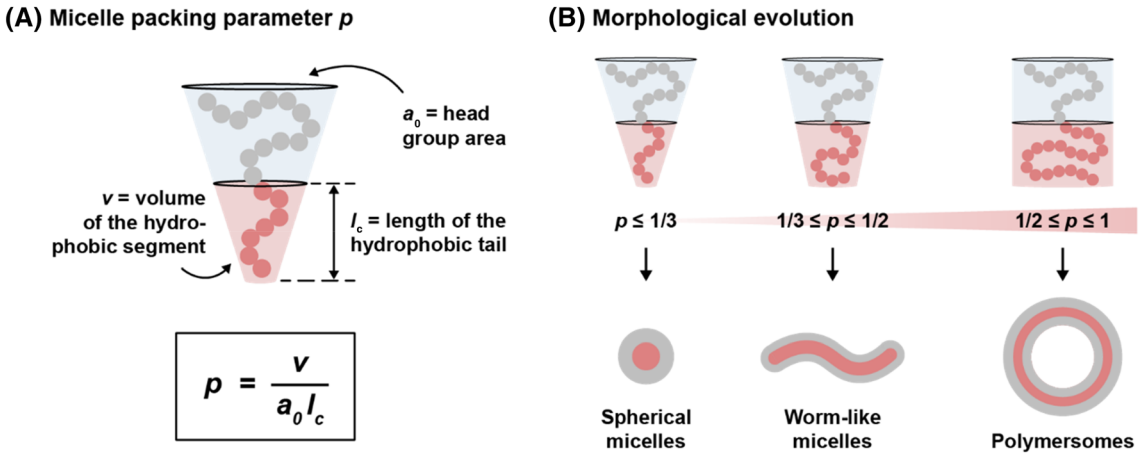

FIGURE 2 (A) The micelle packing parameter p is based on the volumetric aspect ratio of the polymer blocks. (B) p indicates the preferred nanoparticle morphology based on geometrical considerations for ideal chain packing.

drug molecules, peptides, proteins, and nucleic acids, as cargo in BCP-based NPs may improve their solubility and stability in biological media, as well as prolong their in vivo circulation. Furthermore, the physical–chemical properties of the encapsulating NPs may enable tissue- or organ-specific drug delivery and controllable drug release profiles to decrease adverse off-target effects on healthy tissue.

Overall, cargo encapsulation aims to increase the efficacy and safety of treatments. However, these goals pose strict requirements for the particle chemistry, structure, and morphology, depending on the cargo, target location and desired particle functionality. The significant advances of the PISA methodology allow addressing many of these aspects, in some cases better than conventional SA methods. However, as promising as the PISA approach appears, to the authors' best knowledge no significant advances toward systematic in vivo studies or clinical application have been reported so far.

In this Review, we first provide an overview of the key parameters in the rational design of polymeric NPs as prospective drug delivery systems (DDS). With this overview in mind, we then discuss the current strategies of (therapeutic) cargo encapsulation via PISA and highlight recent advances in the field. We do not aim to provide an exhaustive overview of publications that address PISA and cargo encapsulation, but focus on the most important developments reported from 2019 to 2023. For previous developments, we refer to preceding reviews covering the field.[34,52–54] Considering the bottlenecks in the development of PISA-derived DDS suitable for in vivo application, we discuss the potential of various other concepts in the general PISA field to improve NP performance and expand the range of cargo types that can be encapsulated via PISA. Remaining challenges in the clinical translation of PISA-derived NPs are identified and suggestions on how to tackle them are made. Pushing PISA-derived DDS to the clinic only has a chance if we develop and design them with the final application and related restrictions in mind right from the early developmental phases.

2 | GENERAL DESIGN CRITERIA FOR POLYMERIC NANOPARTICLES IN DRUG DELIVERY

To prepare functional polymeric NPs, such as micelles and polymersomes, for drug delivery with PISA, we first need to understand the characteristics of DDS that are suitable for specific applications. This section provides a brief overview of the rational design process for such NPs. Careful tuning of a variety of parameters is required to address the treatment- and drug-specific demands and to increase the likelihood of successful translation to the clinic in later stages.[56–59] The main parameters to consider can be clustered into the following categories as shown in Figure 3: (i) Biological constraints, (ii) cargo-specific demands, (iii) polymeric NP design to overcome the challenges posed by the two previous categories, and, lastly, (iv) DDS assessment and regulatory aspects.

2.1 | Biological constraints

The biological constraints are mainly determined by the medical condition that requires treatment: Most commonly, the diseased area is the target location, that is, a specific organ, tissue, cell type or cell compartment. It is defined by its local characteristics (in the pathological

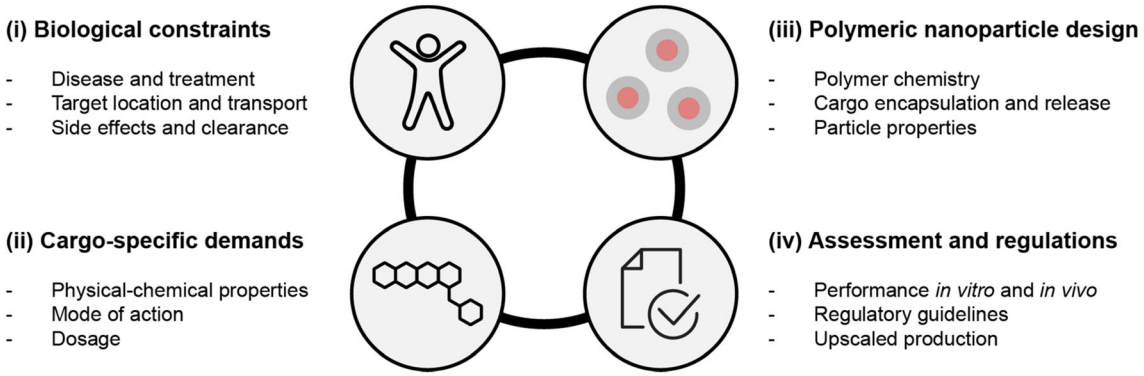

FIGURE 3 Four categories of parameters to consider in the rational design of polymeric nanoparticles (NPs) for drug delivery applications.

state), such as tissue composition and cell types, but also more physical–chemical parameters, for example, slightly acidic pH in tumor microenvironments or inflammation-related concentration increase of reactive oxygen species (ROS).[60–63] The target location combined with the administration route (enteral, parenteral, or others)[21,64–66] dictate the required cargo/particle transport in the body and the number and types of biological barriers the system needs to pass. Along the way, the risks for evoking adverse off-target effects, an immune response, and undesired premature cargo/particle clearance have to be evaluated.

2.2 | Cargo-specific demands

The cargo, that is, biologically active ingredients, such as small (hydrophobic) drug molecules, or larger therapeutics, such as proteins, peptides, inorganic particles, and oligonucleotides, has inherent physical–chemical properties defined by its solubility and stability in biological media, as well as its general bioavailability. The envisioned way of how the cargo will interact on a molecular level to cause a therapeutic effect, for example, binding to receptors or hindering cell division, typically determines the required delivery rate and dosage at the target location for minimal side-effects while maximizing therapeutic outcome.

2.3 | Polymeric nanoparticle design

The combination of the aforementioned demands and constraints allows to identify the specific challenges the DDS should address. Typical challenges that polymeric micelles can help overcome are increasing the water solubility of hydrophobic drugs, such as anticancer agents[67] like taxanes and platinates, reducing the cytotoxicity toward healthy tissue,[68] prolonging the circulation time of therapeutics in the bloodstream,[69] and facilitating cellular uptake via, for example, receptor-mediated endocytosis.[70]

Such requirements serve as a starting point for the design of polymeric NPs: Optimizing the size and shape, as well as mechanical properties such as stiffness,[71] will drastically affect the particle transport in vivo, tissue penetration, cellular uptake, and the clearance route (renal vs. hepatic/splenic macrophages) in case of systemic administration.[72–76] Typically, NPs with a size of about 40 nm to 200 nm with a narrow distribution are targeted. Smaller particles (hydrodynamic radius (R_H) of 4–9 nm, or molecular weight below 45 kDa) are rapidly removed from the body via the renal system. Larger particles (> several hundred nanometer) are actively trapped by the phagocytic cells of the reticuloendothelial system (RES; an immune system mainly located in the liver and spleen).[77]

Since micelles and polymersomes are in principle dynamic systems, (colloidal and) structural integrity needs to be evaluated upon dilution, in complex biological media, and under shear.[78,79] If necessary, additional non-covalent stabilizing interactions or covalent cross-linking can be introduced in the carrier system.[80]

The NP surface is the main interface between the DDS and the biological environment and therefore controls their colloidal stability and in vivo pharmacokinetic properties. The surface chemistry and its charge, but also parameters such as the grafting density[81] and hydration level of steric (polymeric) stabilizers should be adjusted according to the respective goal[77]: For example, employing a NP shell that promotes stealth properties can help to evade rapid clearance by the phagocytic cells of the RES and therefore facilitates prolonged circulation times in the blood stream.[82] This is beneficial for passive targeting of solid tumors via the enhanced permeability and retention (EPR) effect.[68,83] Though nowadays considered controversial, the EPR effect is characterized by leaky blood vessels and impaired lymphatic drainage in tumor

tissues, facilitating passive accumulation of NPs. Most commonly, stealth properties are obtained by using poly- or oligo(ethylene glycol) (PEG or OEG) based NP shells. Due to increasing concern about the immunogenicity of PEG,[84] alternatives, such as poly(*N*-(2-hydroxypropyl) methacrylamide) (PHPMA) or zwitterionic polymers, known for their antifouling properties, are investigated.[85]

As opposed to passive targeting, active targeting relies on modification of the surface with antibodies, cell penetrating peptides or ligands.[23,86,87] Either way, the final surface properties of the NPs determine the potential for (protein) corona formation, that is, adsorption of mainly proteins, but also other biomolecules on the NP surface when in contact with biological media. The amount and types of proteins, as well as how strongly they are bound (soft vs. hard protein corona), may play a major role in opsonization and removal of nanocarriers from the body. It may, however, also be beneficial as a way of in situ formation of a targeting surface.[88]

The chemistry of the polymeric carrier system can be selected based on the compatibility with the cargo and the desired encapsulation mode, for example, chemical conjugation to the polymer vs. non-covalent physical encapsulation/entrapment and the location in or on the DDS.[86] Depending on the desired cargo release mechanism (burst release vs. sustained release), functional groups may be incorporated that respond to target location-specific stimuli: Changes in pH can cause (de)protonation of, for example, carboxylic groups and amines, or degradation of acid-labile bonds, such as hydrazones, imides, esters, etc., overall leading to destabilization of the polymer system or release of the cargo.[89] Alternatively, one can exploit alterations in redox potential by incorporating reduction- or oxidation-sensitive linkages, such as thioether and disulfide bonds,[90] or the local upregulation of enzymes to facilitate the cleavage of enzyme-specific target bonds.[91] Responsiveness to external triggers, such as magnetic field, temperature, light, and ultrasound may also be an option to activate carriers, typically situated closely underneath the body surface, with good temporal and spatial control.[91–93] Particles responsive to different stimuli or multifunctional systems, for example, to combine therapeutic and diagnostic capabilities, have been reported as well.[91,93]

Overall biocompatibility, and, ideally, (bio)degradability of the polymer should be ensured to limit potential adverse effects from the carrier system itself.[33]

2.4 | Assessment, regulations, and upscaling

Finally, suitable in vitro (release kinetics, 2D and 3D cell studies) and in vivo evaluation of the NP formulations is required to assess the particle behavior and therapeutic effect in biologically relevant environments. Retention of functionality of the therapeutic cargo during the particle preparation until the release at the target location needs to be ensured. Other regulatory guidelines[94] should already be kept in mind at early developmental stages to facilitate entry to clinical trials later on: Aspects to consider are simplicity of the overall NP system and the preparation process, stability during prolonged storage, reproducibility and consistency between batches, tolerable levels of impurities (such as organic solvents), and potential for (economically favorable) upscaling.[64,95] Implementation of green chemistry principles[96] is desirable.

3 | (THERAPEUTIC) CARGO ENCAPSULATION IN PISA-NPS

The preparation of polymeric NPs, such as micelles and polymersomes, loaded with therapeutic cargo via PISA offers several advantages, but also certain challenges when compared to conventional self-assembly methods.[43] In situ cargo encapsulation, that is, concurrent polymer synthesis, self-assembly, and cargo encapsulation (Figure 4A, left), following a seemingly straightforward one-pot procedure is one of the key advantages. The reduction of necessary experimental steps compared to conventional SA approaches may significantly simplify and speed up the production of loaded NPs. Depending on the physical–chemical characteristics and encapsulation demands of the therapeutic cargo, it can be incorporated into NPs either via non-covalent (e.g., hydrophobic) interactions (physical encapsulation) or via covalent linkages. Non-covalent interactions allow for cargo retention within the particle, that is, the hydrophobic core (Figure 4, top). For hydrophilic cargo, entrapment in the aqueous lumen of polymersomes is also feasible.[97–99] With covalent linkages, the cargo can in principle be incorporated at any desired location on the BCP (Figure 4, middle and bottom). Most commonly this is realized with polymerizable prodrugs, which are functionalized, often inactive derivatives of a drug. Next to in situ cargo encapsulation, which is uniquely facilitated by the PISA processes, post-PISA cargo loading or functionalization is also possible (Figure 4B, right).

Regardless of the encapsulation strategy, high cargo loadings, that is, amount of cargo per NP are typically targeted to maximize the delivery efficiency of the NPs and to ensure cargo delivery at therapeutically relevant concentrations.[102] Consequently, high encapsulation efficiencies, that is, ratio of the amount of loaded cargo compared to the amount of cargo added during the encapsulation process, are desirable. Finally, biological

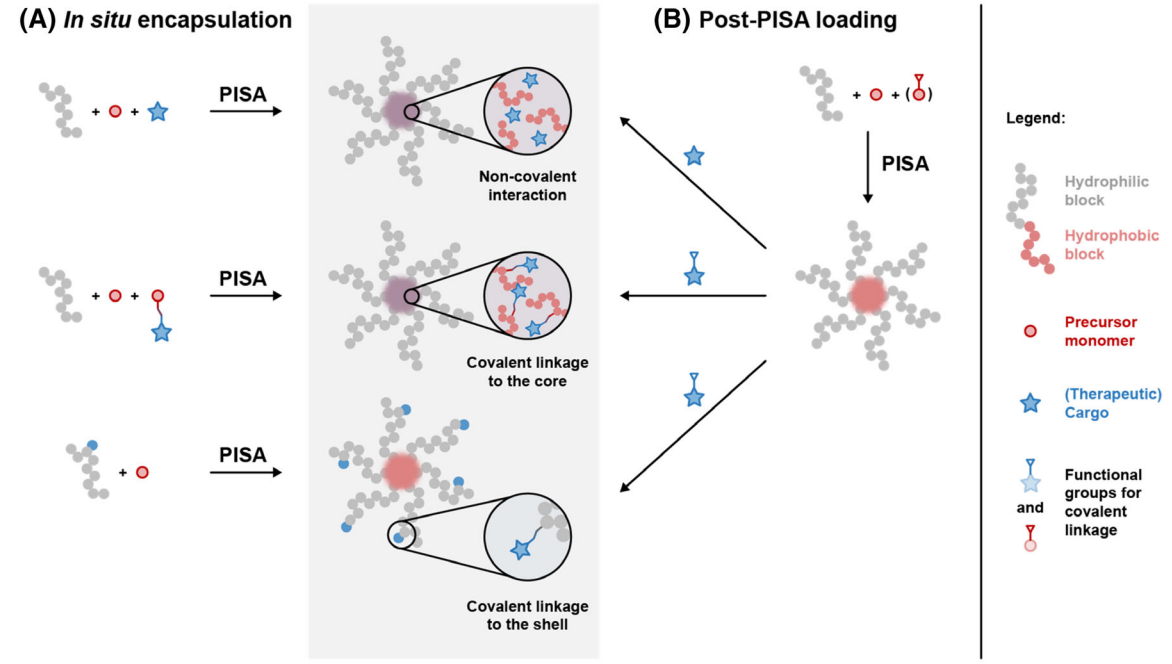

FIGURE 4 Preparation of loaded PISA-derived nanoparticles (NPs) via (A) in situ encapsulation and (B) post-PISA loading. (Therapeutic) cargo retention is possible via non-covalent interactions (top) or covalent linkage to the amphiphilic block copolymer in the core (middle) or the shell (bottom). Combinations of these general strategies are possible and have been reported.[100,101]

assessment of (loaded) NPs in vitro and in vivo generally requires fluorescent labelling of the BCPs. This is achieved via the same principles as in situ cargo loading via covalent linkages or by modifying the particles post-PISA.

This section highlights recently investigated strategies and systems for cargo encapsulation with PISA-derived NPs (Table 2). Examples of physical in situ loading (entries **1–15**), covalent in situ loading (entries **16–22**), and post-PISA loading (entries **23–27**) are presented. The majority of the reported systems focus on the encapsulation of small (hydrophobic) anticancer agents, for example, doxorubicin (DOX), camptothecin (CPT), cisplatin, and 4-(*N*-(S-penicillaminylacetyl)amino) phenylarsonous acid (PENAO), shown in Figure 5 (Structures **I**, **V**, **VI**, **IX**). Small hydrophobic dyes, such as Nile Red (Figure 5, Structure **III**), are frequently used as non-toxic analogues to these anticancer agents to assess cargo loading and release capabilities of new PISA-NPs via facile spectroscopy-based assays. Therefore, such systems are also included in the following sections.

3.1 | Physical in situ encapsulation

For physical in situ encapsulation, the cargo is initially solubilized in the reaction mixture. During PISA, the hydrophobic cargo partitions into the new, increasingly hydrophobic cores of the forming micelles and is retained there due to hydrophobic interactions. Hydrophilic cargo is encapsulated by chance in the aqueous lumen of forming polymersomes. In principle, the strategy is very simple and versatile when compared to encapsulation relying on covalent linkages (Section 3.2) since physical entrapment does not require the presence of polymerizable or reactive moieties on the cargo.

Karagoz[127] and the Armes group[128] pioneered in situ PISA encapsulation for different cargo types [hydrophobic dye Nile Red, silica NPs, and globular protein bovine serum albumin (BSA)] in polymeric micelles and polymersomes. Despite the diversity of suitable cargo types, the main focus of the PISA/drug delivery field remains, however, on the encapsulation of small hydrophobic molecules such as many anticancer agents.

While this concept seems very straightforward, the main challenge lies in balancing the polarity of all components to allow for solubilization of (hydrophobic) cargo, but still maintain sufficient driving force for the SA and cargo accumulation in the micellar cores. For aqueous systems, cargo solubilization may be facilitated by the monomer as it lowers the overall polarity of the reaction mixture in case of dispersion polymerization (Figure 1A). In case of emulsion polymerization, hydrophobic cargo can be solubilized in the monomer droplets.[103,104,109] Alternatively, the use of organic (co) solvents, such as methanol,[111] ethanol,[97,106,113] and

TABLE 2 Recent examples of (therapeutic) cargo encapsulation in situ or post-PISA, published 2019–2023.

	BCP[a]	PISA conditions[b]	NP morphology[c]	Cargo (encapsulation)	BCP modification and stimuli-responsiveness	In vitro/in vivo assessment	Ref.
1	PAA-*b*-PBA	TI RAFT	S	Doxorubicin[d]	Post-PISA functionalization with BODIPY-SH	Cytotoxicity, cellular uptake	103
2	PDMAPMA-*b*-PMMA	TI RAFT	S	Nile Red, Doxorubicin (NC, in situ)[d]	–	Cytotoxicity	104
3	PPEGMA-*b*-PMMA	(TI) RCMP	S, P	Rhodamine B (NC, in situ)	In situ crosslinking with divinyl monomer	–	99
4	P(PEGMA-*co*-PFBMA)-*b*-PHPMA	TI RAFT	S, W, P	Doxorubicin (NC, in situ); *N*-acetyl cysteine and doxorubicin (C, post-PISA)[d]	–	Cell viability, cellular uptake	105
5	PMPC-*b*-PMMA	TI RAFT	S, W, P	Curcumin (NC, in situ)	–	Cellular uptake, distribution in multicellular cancer spheroids (3D model)	106
6	PGMA-*b*-PHPMA	TI RAFT	S, W/R, P	Phenylacetic acid (NC, in situ)	–	–	107
7	POEGMA-*b*-PDEA	PI RAFT	S, R	Doxorubicin (NC, in situ)[d]	–	Cytotoxicity, cellular uptake	108
8	PHPMA-*b*-PDPA	MWI RAFT	S	Nile Red (NC, in situ)	pH-responsive	–	109
9	PEG-*b*-P(DIPEMA-*co*-BzMA)	TI RAFT	P	Rhodamine B (in lumen, NC, in situ)	pH-responsive	–	97
10	P(PEGMA-*co*-PEGMAOH)-*b*-PMTEAM	TI RAFT	S, (W), P	Nile Red (NC, in situ)	H_2O_2-responsive	Cytotoxicity, cellular uptake	110
11	PNAM-*b*-PNAT	TI RAFT	P	Calcein, Glucose oxidase (in lumen, NC, in situ)	H_2O_2-responsive	–	98
12	PPEGMA-*b*-(PGLMA/PBMOD)	(TI) RCMP	P	Fast Green FCF (in lumen, NC, in situ)	Reduction-responsive, in situ crosslinking	–	111
13	PEG-*b*-p(BMA-*co*-TPE-Azo-MA)	TI RAFT	S, P	Doxorubicin (NC, in situ)[d]	Tetraphenylethylene (TPE) as fluorescent probe; sensitive to hypoxic conditions	Cytotoxicity, cellular uptake	112
14	P(PEGMA)-ADP-Azo-P(BzMA)	TI RAFT	S, P	Doxorubicin (NC, in situ)[d]	Near-infrared fluorescent probe; sensitive to hypoxic conditions	Cytotoxicity, cellular uptake	113
15	PHPMAm-*b*-PMTEAM	TI RAFT	S, P	Nile Red (NC, in situ), sulfo-Cy5 NHS ester (in lumen, NC, in situ), Folic acid (C, in situ)	H_2O_2-responsive	Cytotoxicity	114
16	PHPMAm/PDEAEMA-*b*-PCPTM	TI RAFT	S	Camptothecin (C, in situ)	pH- and reduction-responsive	Cytotoxicity, cellular uptake	115
17	P(OEG)-*b*-P(CA NB DCI-*co*-quaternary amine NB DCI), P(OEG)-*b*-P(CA NB DCI-*co*-2-(diisopropylamino)ethyl NB DCI)[b]	ROMP	S	Cisplatin analogue (C, in situ)	pH-responsive	Cytotoxicity, cellular uptake	116
18	POEGMA-*b*-P(HPMA-*co*-RBMA)	PI RAFT	S	Rose Bengal (C, in situ)	Post-PISA modification with Sgc8 aptamer via SPAAC[e]	Cellular uptake	117

(Continues)

TABLE 2 (Continued)

	BCP[a]	PISA conditions[b]	NP morphology[c]	Cargo (encapsulation)	BCP modification and stimuli-responsiveness	In vitro/in vivo assessment	Ref.
19	POEGMA-*b*-PPBBMA	TI RAFT	S	Boronate ester (C, in situ)	Surface functionalization with phenylboronic acid	Cytotoxicity, cellular uptake	118
20	P(MPC-*co*-PENAO)-*b*-PMMA, P(PEGMA-*co*-PENAO)-*b*-PMMA	TI RAFT	S	PENAO (C, in situ)	Post-PISA functionalization with Cy5 or TPP for mitochondria targeting	Cytotoxicity, cellular uptake in vitro (2D, 3D), biodistribution in vivo	119, 120, 101
21	P(KLAAm-*co*-DMA)-*b*-P(DAAm-*co*-DMA)	PI RAFT	S	Proapoptotic KLA peptide (C, in situ)	–	Apoptosis efficiency	121
22	Sgc8-*b*-PHPMA, CpG-*b*-PHPMA	PI RAFT	S, W/R	Functional nucleic acids (FNAs): Sgc8 aptamer and CpG oligonucleotide (C, in situ)	–	Cytotoxicity, cellular uptake	122
23	PHEA-*b*-PNBA	TI RAFT	S	Nile Red (NC, post-PISA)	Polymer degradation upon UV exposure	–	123
24	PEG-*b*-PDAP-*b*-PHPMA	TI RAFT	S, W, P	Nile Red (NC, post-PISA)	Functionalization with thymine crosslinker	–	124
25	PEG-*b*-PCYS and PEG-*b*-P(CYS-*co*-BLG)	NCA-ROP	S	Indomethacin (NC, post-PISA)	Reduction-responsive; disulfide-based core-crosslinking	–	125
26	PGMA-P(HPMA-*stat*-GlyMARh)-*b*-PDPA	TI RAFT	Framboidal P	EGFP plasmid (in lumen, NC, post-PISA)	Phosphorylcholine-based targeting ligand; pH-responsive	Cellular uptake	100
27	P(PEGA)-*b*-PS	TI-RAFT	S	Recombinant His-tagged hemagglutinin (complexation[e], post-PISA)	–	Immune stimulatory efficiency in vivo	126

Block copolymer (BCP) abbreviations: ADP, aza-BODIPY; Azo, azobenzene; BMA, butyl methacrylate; BMOD, bis(2-metharyloyl)oxyethyl disulfide; BzMA, benzyl methacrylate; CA, cisplatin analogue; DAAm, diacetone acrylamide; DIPEMA, 2-(diisopropylamino) ethyl methacrylate); DMA, *N*,*N*-dimethylacrylamide; GlyMA, glycidyl methacrylate; KLAAm, polymerizable KLA peptide; NB DCI, norbornene dicarboximide; P(OEG), poly(oligo(ethylene glycol); PAA, poly(acrylic acid); PBA, poly(butyl acrylate); PCPTM, polymerized camptothecin analogue; PCYS and P(CYS-*co*-BLG), (co)polymer obtained via NCA-ROP of L-cysteine *N*-carboxyanhydride (CYS-NCA) and γ-benzyl-L-glutamate *N*-carboxyanhydride (BLG-NCA); PDAP, poly(2,6-diacylaminopyridine methacrylate); PDEA, poly(*N*,*N*-diethylacrylamide); PDEAEMA, poly(2-(diethylamino)ethyl methacrylate); PDMAPMA, poly((*N*-[3-(dimethylamino)propyl] methacrylamide); PDPA, poly[2-(diisopropylamino)ethyl methacrylate]; PEG, poly(ethylene glycol); PEGA, poly(ethylene glycol) methyl ether acrylate; PEGMAOH, poly((ethylene glycol) methacrylate); PENAO, 4-(*N*-(S-penicillaminylacetyl)amino) phenylarsonous acid; PFBMA, poly(pentafluorophenyl methacrylate); PGLMA, poly(glycerol methacrylate); PGMA, poly(glycerol monomethacrylate); PHEA, poly(hydroxyethyl acrylate); PHPMA, poly([*N*-(2-hydroxypropyl)]-methacrylate); PHPMAm, poly(*N*-2-hydroxypropyl methacrylamide); PMMA, poly(methyl methacrylate); PMPC, poly(2-(methacryloyloxy)ethyl phosphorylcholine); PMTEAM, poly(*N*-(2-(methylthio) ethylacrylamide); PNAM, poly(*N*-acryloylmorpholine); PNAT, poly(*N*-acryloylthiomorpholine); PNBA, poly(*o*-nitrobenzyl acrylate); POEGMA, poly[oligo(ethylene glycol) methacrylate]; PPBBMA, 4-pinacolboronylbenzyl methacrylate; PPEGMA, poly(poly(ethylene glycol) methyl ether methacrylate); PS, polystyrene.; RBMA, Rose Bengal methacrylate; Rh, rhodamine; TPE, tetraphenylethylene; TPE-Azo-MA, TPE-azobenzene derived methacrylate. Other abbreviations: C, covalent linkage of the cargo to the BCP; NC, non-covalent interaction; RAFT, reversible addition-fragmentation chain-transfer (polymerization); RCMP, reversible complexation mediated polymerization; RO(M)P, ring-opening (metathesis) polymerization; SPAAC, strain-promoted azide-alkyne cycloaddition.

[a]Block copolymer (BCP) nomenclature: The hydrophilic block is mentioned first, then the hydrophobic block.

[b]TI, thermal initiation; PI, photoinitiation.

[c]S, spherical micelles; W/R, fused, worm- or rod-like micelles; P, polymersomes.

[d]The commercially available, hydrophilic salt form of doxorubicin (DOX·HCl) is usually converted with triethylamine to hydrophobic DOX prior to or during the encapsulation.

[e]Ni^{2+} with bis(nitrilotriacetic acid) (NTA)/histidine (His) interaction.

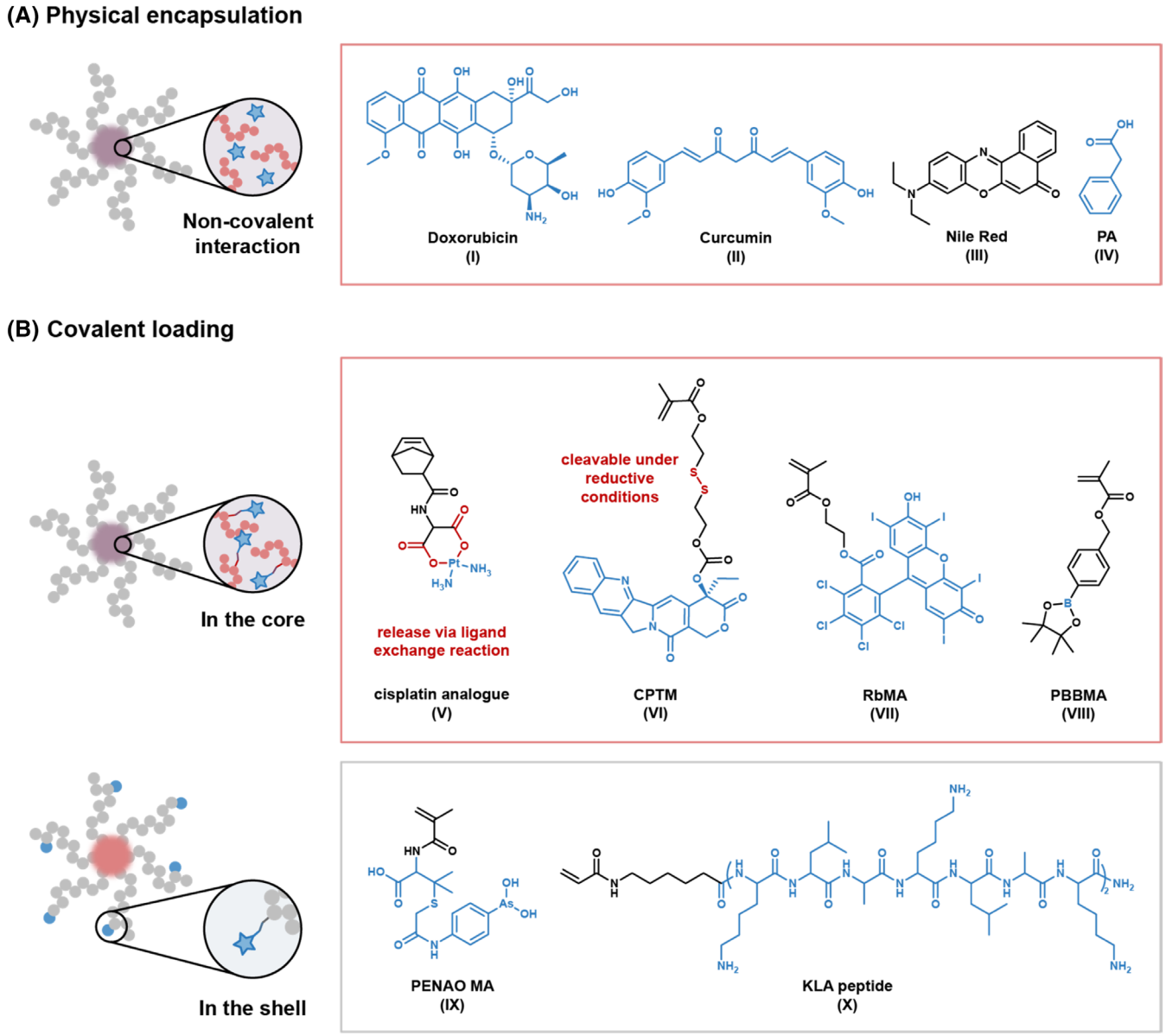

FIGURE 5 Recently reported examples of (therapeutic) cargo for in situ (A) physical encapsulation in the micellar core and (B) covalent loading in the core and the shell of PISA-derived nanoparticles (NPs). The bioactive parts and compounds are marked in blue. Cleavable groups for cargo release are marked in red.

1,4-dioxane,[112] has been reported. However, removal of the organic solvent via dialysis prior to any biological experiments is typically necessary to prevent toxicity. An alternative approach to ensure sufficient driving force for encapsulation is adjusting the polarity of the cargo (Table 2, Footnote d).[104]

Considering the challenge of solubilizing hydrophobic cargo in the reaction mixture, PISA systems that aim for physical in situ cargo encapsulation generally yield low cargo loadings. In the exceptional cases where high cargo loadings are achieved[106] and/or amphiphilic drugs such as phenylacetic acid (PA; Figure 5, Structure **IV**)[107] are used, the cargo location within the nanoparticle should be considered as it may influence the NP behavior in a biological environment. The location of the cargo may not be limited to the hydrophobic core, but sequestration into the hydrophilic shell can occur.[129] A consequence of such dehydration of the hydrophilic shell can be reduced colloidal stability and/or cellular uptake.[106] This also indicates that aiming for the highest possible cargo loading might not necessarily lead to the DDS with the best performance in vitro or in vivo.[102]

Early examples of PISA studies[127,128] neglected the impact of cargo on the polymerization kinetics and the morphological outcome. However, recent studies refuted cargo inertness[106,108] and revealed significant

impact on the PISA process as a function of the physical–chemical properties of the cargo[106,107] and its concentration in the reaction mixture.[97,98] In a pioneering example, Cao et al. systematically studied the influence of drug loading on the morphology of PISA-NPs.[106] Conducting PISA in the presence of curcumin (Figure 5, Structure **II**) as a model drug revealed that its presence alters the morphological evolution during the PISA process (Figure 6). Li et al.[107] found similarly facilitated morphological transitions upon increasing the targeted loading with the drug PA.

The cargo may not just impact the SA process, but also the polymerization kinetics. Cargo-mediated radical chain transfer and/or scavenging may limit control over the polymerization kinetics and resulting polymer architecture, especially at high cargo concentrations.[98] For photo-initiated polymerizations, additional interferences may occur due to optical properties of the cargo, for example, if the cargo's absorption spectrum overlaps with the wavelength of the initiating light source. Zhang et al.[108] showed that DOX was able to act as a cocatalyst during photo-PISA, leading to increased polymerization rates. The rate increase was attributed to a higher radical concentration, leading to more dead chains and higher dispersities for the BCPs.

Loading the cargo is only half of the story. Next to relying on passive diffusion-based cargo release from the NPs, stimuli-triggered disassembly of PISA systems for on-demand release[53] has been developed. Inspiration for these designs was drawn from BCP-based DDS prepared via conventional SA methods.[91] In the development of such systems, a dye is typically encapsulated in situ to demonstrate and study the triggered release.[97,98,109–111,114] Often, the mechanism is based on a hydrophobic-to-hydrophilic transition of the core-forming block to disassemble the micelles and release the cargo (Figure 7, Strategy **i**). The transition can be either induced by a pH change, leading to the protonation of tertiary amino groups under slightly acidic conditions,[97,109] or by a change in the oxidation potential of the environment. Recent examples, such as

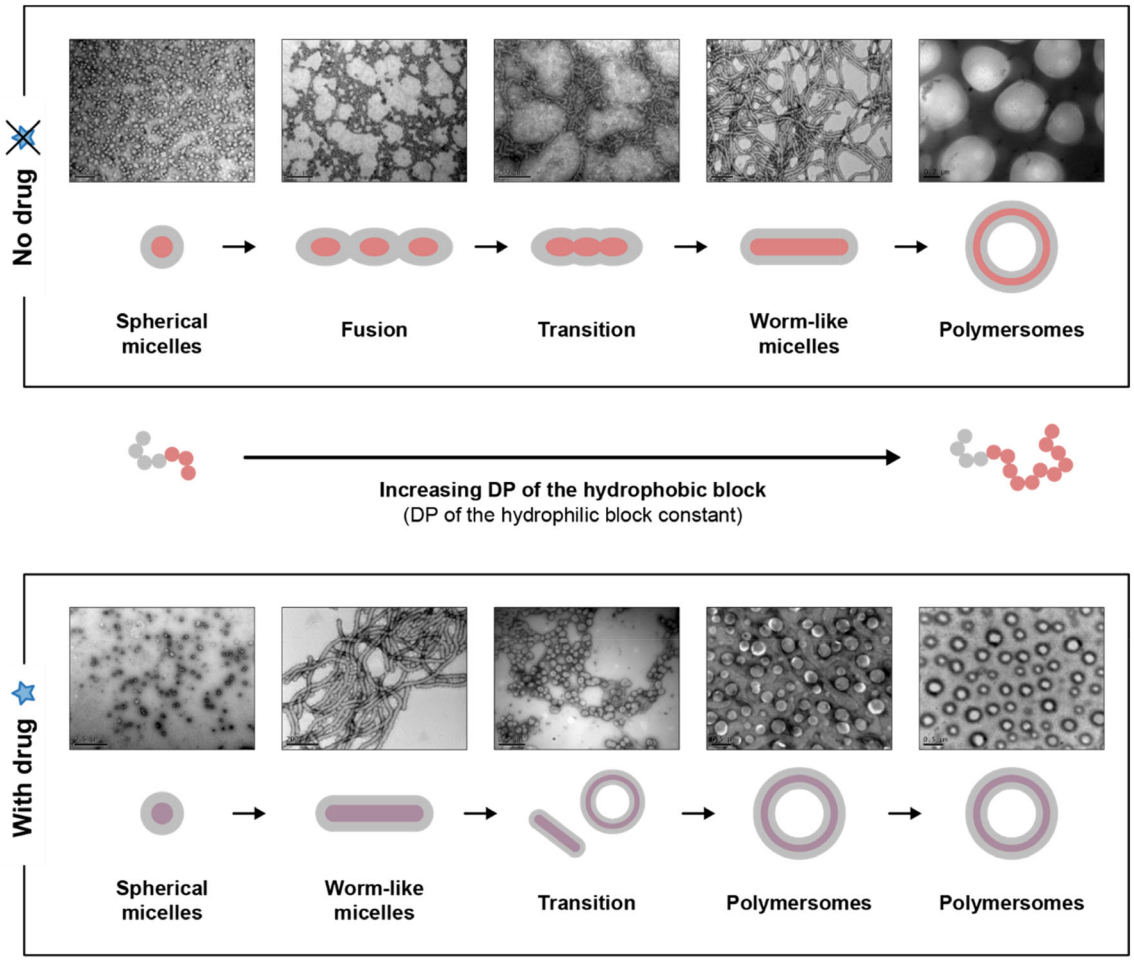

FIGURE 6 Schematic representation of the morphology evolution upon chain-extension of the hydrophobic block without drug (top) and in the presence of a drug (bottom). DP, degree of polymerization. Adapted with permission.[106] Copyright 2020, American Chemical Society.

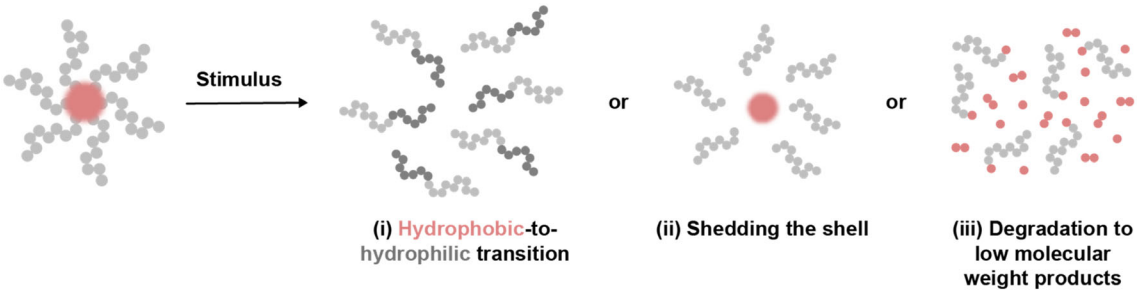

FIGURE 7 Common strategies in (stimuli-triggered) disassembly or degradation of the polymeric carrier system for on-demand drug release: (i) Hydrophobic-to-hydrophilic transition, (ii) shedding the shell, and (iii) degradation to low-molecular-weight products.

those of Sobotta et al.[98] and Phan et al.,[110,114] rely on the oxidation of the hydrophobic thioether groups on the repeat units in the core by radical oxygen species (ROS), such as H_2O_2, to hydrophilic sulfoxides.

The disassembly and release mechanism can also be controlled by the reductive cleavage of chemical bonds. Sarkar et al.[111] prepared crosslinked PISA-derived polymersomes featuring reduction-sensitive disulfide groups. Upon exposure to a reducing agent like glutathione (GSH), the crosslinkers are cleaved, the polymersomes fully disassemble, and an encapsulated hydrophilic dye is released from the aqueous lumen. The group of Zhu exploited azobenzene linkers that can be cleaved by reductants such as azoreductase in two different DOX-loaded systems. In the first system,[112] enzymatic hydrolysis of the azo bond releases a fluorescent probe (tetraphenylethylene, TPE) from the repeat units of the core-forming block. This not only triggers the disassembly of the nanoparticles and DOX release, but also activates aggregation-induced emission fluorescence (AIE) of TPE due to the elimination of the azobenzene-based fluorescence resonance energy transfer (FRET) process. In the second system,[113] the same concept was applied, but the azobenzene linkages were incorporated into the BCP backbone as a connector between the two blocks. Hence, (enzymatic) bond cleavage equals shedding of the stabilizing shell[130,131] (Figure 7, Strategy **ii**), which leads to DOX release and the formation of large irregular aggregates, accompanied by a near-infrared fluorescence switch-on.

Next to strategies based on internal stimuli, such as pH and redox potential, also external stimuli such as light are of interest as they may provide additional options for micelle degradation and drug release. For example, Chaabouni et al. reported a novel class of PISA-NPs with a UV-inducible self-immolation mechanism in the core-forming part of the polymer backbone, leading to full degradation into low-molecular-weight products (Figure 7, Strategy **iii**) and release of cargo and free radicals.[123] For this specific system, the cargo, a fluorescent dye, was, however, loaded post-PISA (Section 3.3).

3.2 | In situ covalent loading of cargo

For many drug delivery applications, NP systems with physically encapsulated cargo that is solely retained by hydrophobic interactions are unsuitable due to limited cargo retention in complex biological media.[132] To reduce premature leakage, cargo can be covalently linked to the hydrophobic core as well as the hydrophilic shell. Either way, functionalization of the cargo is required: For small molecules and peptides, the cargo is commonly equipped with a polymerizable group, enabling its use as a (co) monomer during PISA or during the formation of the hydrophilic block (Figure 5B). If the cargo needs to be cleaved off from the polymer backbone to fulfill its function at the target location, (stimuli-responsive) cleavable groups may be used to tether the cargo to the BCPs. A well-reported prodrug example is a camptothecin-based monomer (CPTM; Figure 5, Structure **VI**) that has been successfully incorporated in the core of PISA-derived micelles – either as the sole building block[115] or as a comonomer.[133,134] Under reducing conditions, for instance after cellular uptake in the cytosol due to sufficiently high concentrations of GSH, the drug CPT is released from the polymer backbone via degradation of the disulfide linker. Aiming for a different release principle, Wright et al.[116] incorporated a cisplatin prodrug monomer (Figure 5B, Structure **V**) via ROMPISA in the core of poly(oligo ethylene glycol) (P(OEG)) stabilized micelles. For the release of the therapeutically active platinum species, nucleophiles, such as chloride or water, are expected to diffuse into the core to promote ligand exchange reactions that lead to the replacement of the labile carboxylate ligands.[135]

In other cases, the cargo remains bioactive even when attached to the polymer backbone. Incorporating the cargo in the hydrophilic shell can increase cargo stability and prolong circulation times, while still providing accessibility to the biological target. For instance, Noy et al.[119] reported the incorporation of PENAO (Figure 5 Structure **IX**) via copolymerization with zwitterionic

2-methacryloyloxyethyl phosphorylcholine (MPC) into the hydrophilic block of spherical micelles. PENAO is a hydrophilic organo-arsenical drug that exhibits its anticancer efficiency by binding to cysteine in the mitochondria,[136] also when incorporated into a polymeric matrix.[137] In follow-up work,[120] the potential of this system was extensively investigated in vitro as well as in vivo with a biodistribution study in mice where the NPs exhibited rapid accumulation in the liver. With the goal of enhancing the therapeutic performance, the NP surface was modified post-PISA with triphenylphosphonium (TPP) as a mitochondria-targeting ligand.[101] However, there was no significant improvement of mitochondrial accumulation noticeable in vitro, as TPP was likely not sufficiently exposed on the surface due to the hydrophobic character of the ligand.

Accessibility of the cargo combined with the benefit of being incorporated into a nanoparticle was also exploited by Sun et al.[121] for the amino acid sequence KLAKLAKKLAKLAK ("KLA" peptide; Figure 5, Structure **X**). This peptide is a potential anticancer therapeutic that promotes programmed cell death. However, it suffers from low proteolytic stability and poor cellular uptake efficiency on its own. The functionalized peptide KLAAm was therefore incorporated into the hydrophilic block via copolymerization with *N*,*N*-dimethylacrylamide (DMA). This peptide brush was then chain-extended with a combination of diacetone acrylamide (DAAm) and DMA via aqueous photo-PISA at room temperature to form nano-sized particles. Incorporation into a NP structure indeed increased the proteolytic resistance compared to the peptide alone, and significantly enhanced cellular uptake in HeLa cells. The enhanced stability was reasoned to be based on steric hindrance imposed by the polymer brush, limiting access of the peptides into the enzymatically active centers of proteases.

Yang et al.[122] went a step further by employing functional nucleic acids (FNAs) as both the hydrophilic stabilizing block and therapeutically active cargo. FNAs possess remarkable modularity, offering diverse bioactivities, including targeting, catalytic and immune-stimulation functions. Nonetheless, similar to the KLA peptide, molecularly-dissolved FNAs face challenges, such as susceptibility to nuclease-mediated degradation and limited cellular uptake. To overcome these issues, the authors used mild photo-PISA to chain-extend two different FNAs as examples (Sgc8 aptamer for tumor targeting and CpG oligonucleotide for immune therapy) with HPMA. The resulting three-dimensional (3D) FNA nanostructures exhibited higher resistance to nuclease degradation in serum, improved cellular uptake efficiency, and, consequently, enhanced bioactivity.

If accessibility of the cargo to the local environment is not required, for example, in case of photodynamic therapy (PDT) or boron neutron capture therapy (BNCT), the cargo may also remain incorporated in the core. Korpusik et al.[117] developed PEG-based NPs via photo-PISA featuring Rose Bengal methacrylate (RBMA; Figure 5, Structure **VII**) with HPMA in the core. In this system, RBMA serves multiple functions: The monomer acts as a photocatalyst for self-catalyzed photo-electron transfer RAFT (PET-RAFT) PISA upon irradiation with low energy yellow light, as a singlet oxygen ($^1O_2$) generator for PDT, and as fluorophore for imaging in vitro. To improve selectivity for tumor tissue, the NP surface was functionalized post-PISA with a targeting ligand (Sgc8 aptamer) via strain-promoted azide-alkyne cycloaddition (SPAAC).

BNCT is an anticancer therapy based on (i) the delivery of non-toxic boron drugs to tumor cells and (ii) irradiation with a thermal neutron beam that triggers nuclear fission of boron-10. The subsequently produced high-energy alpha particles then eliminate tumor cells. To work toward solving the hurdle of specific accumulation of boron in high concentrations in tumor cells, Huang et al.[118] developed the monomer 4-pinacolboronylbenzyl methacrylate (PBBMA; Figure 5, Structure **VIII**) and successfully used it as a building block for the core-forming block in PHPMA-stabilized PISA-NPs.

3.3 | Post-PISA loading and functionalization

In situations where in situ loading poses significant challenges, such as cargo degradation, limited monomer conversion, or hindering BCP assembly into stable micelles or polymersomes, post-PISA loading may be a viable solution. Some of the hallmarks of PISA, such as high solid content during NP preparation and fast polymerization kinetics, remain hereby intact. Moreover, post-PISA functionalization proves beneficial in creating loaded PISA-NPs with dual functionality, like combining active targeting and cargo delivery.[101]

Post-PISA loading methods are in essence analogous to those explored for conventionally assembled NPs: Physical encapsulation of hydrophobic cargo can be achieved by dissolving small amounts of cargo in the dispersion medium[124] or via latex swelling with a suitable organic solvent.[18,123] Crosslinking is recommended for improved retention of the NP morphology during the swelling/loading procedure.[124,125,138] For vesicles, hydrophilic cargo can be encapsulated in the aqueous lumen through a different approach: For instance, Mable et al.[100] took inspiration from cell transfection procedures to incorporate EGFP plasmid

DNA in PISA-derived, pH-responsive framboidal polymersomes via electroporation.

Loading and retaining cargo via covalent linkages in the core or the shell can be achieved through chemical coupling reactions with functional groups incorporated in the BCP via (co)monomers or the polymer end groups. Common moieties that can react with (functionalized) cargo under mild conditions include hydroxy groups,[101] aldehydes,[55,139] epoxides,[100] carboxylic acids,[103,140] thiols,[140] and *para*-fluorines.[105] Alternatively, cargo can be immobilized on the PISA-NP surface via complexation, such as Ni^{2+}-NTA/His interaction.[126]

4 | ADVANCES OF PISA FOR POTENTIAL FUTURE DDS

While significant progress has been made in (therapeutic) cargo encapsulation via PISA, several challenges remain that limit in vivo applicability: Incompatibility of sensitive (biological) cargo with most PISA reaction conditions,[39,141] often poor control over NP morphologies with new monomers and cargo,[34] premature NP disintegration upon exposure to biologically relevant conditions,[142] risk of adverse side-effects from the polymeric carrier,[33] accommodating a wider range of (therapeutic) cargo,[143–146] and small scale NP production with batch-to-batch variability.[147] Although the solutions to these challenges are not all clear-cut and straightforward, we can draw inspiration from our experience with conventional polymeric micelles as DDS[148] and the wider PISA toolbox, that is, PISA literature that concerns systems without (therapeutic) cargo encapsulation. Recent developments in (i) exploiting milder reaction conditions and other polymerization techniques, (ii) achieving control over NP size and shape, (iii) enhancing structural integrity, (iv) incorporating (bio)degradability, (v) expanding the PISA concept to other driving forces, and (vi) upscaling PISA are discussed in the light of developing the next-generation PISA-derived DDS that can be applied effectively and safely in vivo.

4.1 | Exploring milder reaction conditions and other polymerization techniques

In recent years, PISA protocols have been extended to work for a large range of polymerization techniques, monomer types, solvents, and initiation mechanisms. However, many of them are poorly compatible with sensitive (biological) cargo or NP building blocks, such as proteins, (oligo)peptides, nucleic acids, and carbohydrates, due to the presence of organic solvents or high reaction temperatures.[33,141,149] Hence, specific emphasis has been on moving toward mild reaction conditions, that is, synthesis in water and at ambient (or below) temperatures. RAFT-PISA has been explored by far the most due to its excellent compatibility with aqueous conditions and a broad range of monomers.[39,40] The reduction of the polymerization temperature from 40 °C to 90 °C, which is typically required for thermal initiation of radical polymerization, is primarily facilitated by the development of photo-mediated PISA: Light-triggered decomposition of a photoinitiator as radical source allows PISA to proceed at ambient conditions, providing temporal (by turning irradiation on/off), and spatial control. Next to using a photoinitiator as an exogeneous radical source, the chain transfer agent (CTA) can be activated by light due to thiocarboylthio photoactivity[150]: Either directly (photoiniferter) or via photo-redox catalysis (PET-RAFT).[151] PET-RAFT is particularly intriguing as biocompatible examples of water-soluble photocatalysts[152] such as Eosin Y (derivatives) are available, and it can offer oxygen tolerance during the polymerization.[153] For general aqueous RAFT-PISA, oxygen tolerance has only been realized so far via the addition of glucose oxidase (GOx) and glucose to the reaction mixture.[122,154,155] Oxygen-tolerant PISA allows circumventing the need to purge with inert gas or tedious freeze-pump-thaw cycles, which drastically improves handling of reaction mixture volumes down to the μL scale. Such small-scale experiments facilitate the use of expensive biological components[122] and high-throughput screenings in, for instance, 96-well microtiter plates.[156]

Next to photoinitiation, other exogeneous initiation mechanisms have been explored for aqueous RAFT-PISA, such as redox-initiation, enzymatic initiation, and sono-RAFT (initiation via ultrasound) in which radicals are formed in situ via homolysis of water.[39]

Aside from advancing RAFT protocols, PISA has been extended to other polymerization techniques,[41–43] most notably radical ring-opening polymerization (rROP) with cyclic ketene acetals and (aqueous) ROP of *N*-carboxyanhydrides (NCA-ROP).[157] These techniques allow for incorporating biodegradable groups into the polymer backbone; something not possible with the commonly applied RAFT polymerizations for PISA (Section 4.4).[33]

4.2 | Achieving control over NP size and shape

PISA offers excellent control over particle properties, such as size and shape, for many BCP systems.[34,158] This control enables optimization of, for example, particle transport in the body and cellular uptake.[34,45,55,106,140,159,160]

However, the morphological evolution during PISA depends not only on the micelle packing parameter p (Figure 2), but also on the polymer chemistry, reaction and rearrangement kinetics, and the (chemical) environment during self-assembly.[161] Carrying out PISA in an (oil-in-water) emulsion instead of a dispersion polymerization adds further complexity due to differences in nucleation and BCP reorganization during and after chain growth.[162] Consequently, the development of new PISA systems, featuring novel (prodrug) monomers, may demand tedious screening of reaction conditions before sufficient control over size and shape is achieved. To understand the mechanistic details of PISA better, in-depth studies have been conducted on, for example, nucleation[163] and parameters influencing the morphological evolution.[164–168] As a result, a more rational design approach to steering final NP morphologies in new PISA protocols is now feasible, also supported by computational methods.[169–174] This subsection provides insight into the parameters that may be leveraged to direct the morphological evolution during PISA, and how intra-/intermolecular interactions can be used to improve accessibility of anisotropic NPs.

4.2.1 | Kinetically-arrested states versus (facilitated) reorganization

In principle, continuous growth of the solvophobic segment during PISA corresponds to a time-dependent volumetric aspect ratio that provides access to different NP morphologies within a single PISA protocol (first spherical micelles, then worm-like micelles, and finally polymersomes).[166,167] The NP size can be controlled by adjusting the degree of polymerization (DP) of the BCPs.[34]

However, in practice, the morphological evolution during PISA often does not proceed in such a straightforward manner: Hindered rearrangement may prevent reaching thermodynamic equilibrium states, leading to kinetically trapped morphologies, typically spheres. The underlying reason is related to limited chain mobility of the core-forming polymer (determined by the (solvated) glass transition temperature T_g).[20,175] Even if there is sufficient chain reorganization for morphological evolution, particle isolation during transitions can result in mixed phases or morphologies, termed jellyfish, donuts and others.[166,176,177] Such particles are challenging to characterize and lack batch-to-batch reproducibility, making them impractical as drug delivery vehicles.

While kinetically-trapped spheres may be suitable for certain applications, eventual relaxation during long-term storage may lead to changes in the particle characteristics. Therefore, targeting (higher-order) morphologies closer to equilibrium is more desired. Overall, two main strategies are followed to promote morphological transitions: (i) Improving chain mobility and (ii) increasing the available relaxation time. Chain mobility can be tuned on a polymer level through, for instance, copolymerization,[178] parallel synthesis of BCP and homopolymer (polymerization-induced cooperative assembly, PICA),[179] or using a combination of (two) different macro-CTAs in case of RAFT polymerization. Alternatively, modifying the reaction conditions, that is, solvent composition,[180] reaction temperature,[177,181] or additives (e.g., CO_2,[106,182] electrolytes,[183] and cargo[106,119]) can have a significant impact, particularly with pH- or temperature-responsive monomers/polymers.[184] The polymerization kinetics, and hence the available relaxation time, are also influenced by the reaction conditions, such as the reaction temperature or the initiation mechanism, and, in case of RAFT polymerization, the CTA.[185] Spatiotemporal control, such as photoinitiation with on/off cycles, may provide additional time for BCP reorganization during the PISA process.[108] In addition to in situ strategies, post-PISA annealing[20,186] or rearrangement driven by exposure to so-called "transformers", that is, small molecules acting as plasticizers, can aid in obtaining higher-order morphologies.[187]

Next to NPs based on linear, amphiphilic BCPs, more complex NP architectures, like onion-like or framboidal polymersomes, can be achieved by incorporating additional polymer blocks (facilitated by the living polymerization) in the polymer backbone.[100,173,188,189] This additional compartmentalization opens up possibilities for co-delivery of two different cargo types. Moreover, branched polymer structures can be included in the NP design.[190] Although more challenging to reproduce and characterize, branching offers an additional handle of control, for example, by promoting morphological transitions at lower DPs compared to linear BCPs.[191,192]

4.2.2 | Additional intra-/intermolecular interactions to imprint directionality

While PISA allows for the general accessibility of higher-order morphologies, the preparation of anisotropic particles, such as rod- or worm-like micelles or nanotubes (Figure 8A), remains challenging due to the typically narrow experimental window in which these structures are accessible.[192] Obtaining pure phases of such particles is often practically not feasible or requires tedious screening of reaction conditions. Given the potential of anisotropic particles for biomedical applications,[72,73,75,193] there is significant

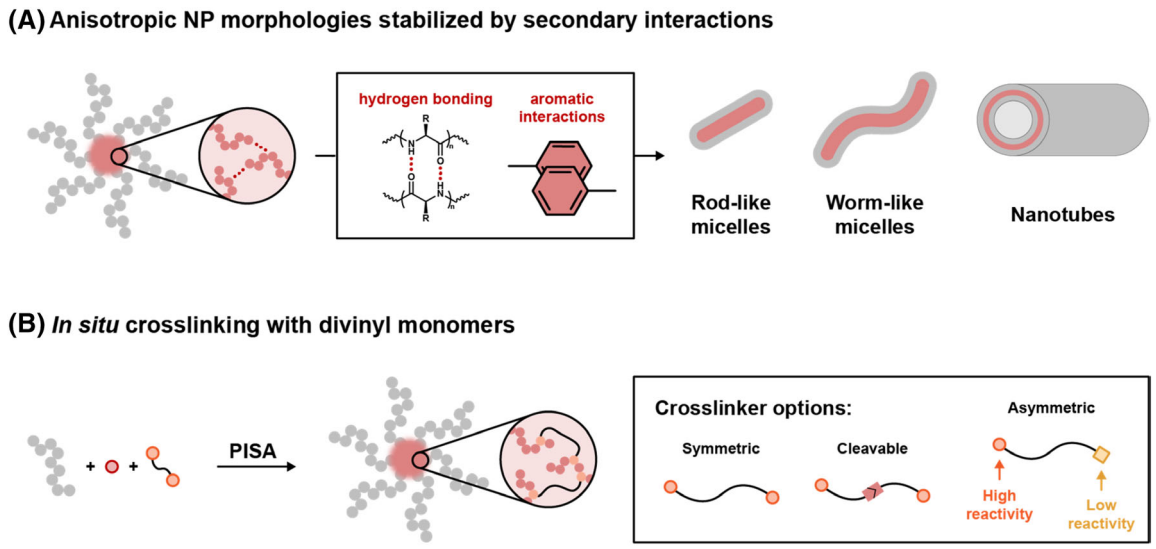

FIGURE 8 (A) Chemical motifs to imprint directionality in PISA-derived nanoparticles (NPs) via secondary interactions, that is, hydrogen bonding[157,194–198] and aromatic interactions (π-π stacking),[199–201] leading to stabilization of anisotropic nanostructures, such as rod-like micelles, worm-like micelles, and nanotubes. (B) In situ PISA core-crosslinking with divinyl monomers.

interest in developing strategies that make their synthesis via PISA more robust.

Incorporating functional groups in the core-forming block that imprint directionality during the SA process via secondary intermolecular interactions, such as hydrogen bonding,[157,194–198] aromatic interactions such as π-π stacking,[199–201] and electrostatic interactions,[202] has been shown to widen the experimental window. Such interactions effectively overrule the packing parameter *p* as the main criterion defining NP morphology. The overall concept shows resemblance to crystallization-driven self-assembly (CDSA), which readily allows the formation of non-spherical NPs and more complex, hierarchical assemblies during conventional solvent exchange self-assembly.[32,33]

Imprinting directionality via hydrogen-bonding can be effectively realized by introducing a supramolecular moiety, such as a bis-urea sticker at the (CTA) chain end. Mellot et al.[194] demonstrated that this modification leads to fiber-like morphologies over a wide range of block lengths for the core-forming block. In contrast, using a comparable CTA without the bis-urea units resulted in spherical micelles for similar block ratios.

Another approach to leverage hydrogen-bonding involves exploiting the secondary structures of polypeptides. Aqueous NCA-ROPISA allowed the incorporation of γ-benzyl-L-glutamate as the core-forming repeat unit, leading to a significant amount of β-sheet and α-helix formation.[157] This secondary structure formation is believed to be linked to the well-defined needle-like NP morphologies that were obtained. Subsequently, this ROPISA methodology was extended to L-leucine NCA to gain a better understanding of how the secondary structure of the polypeptide influences the NP morphology and anisotropy.[203]

In addition to hydrogen bonding, aromatic interactions[199–201] have also been exploited: Zhu et al.[199] demonstrated the synthesis of polymeric nanotubes with remarkable length, reaching up to over 11 μm, by employing an aromatic monomer with an anthracene residue for the core-forming block. Weakening the aromatic interaction by increasing the reaction temperature or adjusting the monomer composition resulted in spherical nano-objects. Moreover, controlling the polymer concentration allowed for tuning the aspect ratio of the nanotubes, a feat previously only achieved by altering the polymer composition.[200]

Other strategies toward anisotropic structures involve using host-guest complexation[204–206] or liquid crystalline block copolymers based on, for example, fluorinated repeat units.[207–209]

Next to exploiting secondary interactions to imprint directionality, they could also be leveraged for cargo retention in the core.[132,210,211]

4.3 | Enhancing structural integrity

Self-assembled polymeric NPs are typically stable in the polymerization medium (dissociation- and aggregation resistant). However, when transferred to biologically relevant conditions (e.g., aqueous medium, high dilution, the presence of proteins, surfactants, or salts, and shear forces), their stability may be compromised.[78] Assays, such as NP incubation in serum or cell growth medium,

are often a sufficient indicator of potential colloidal instabilities in in vitro studies. However, to preserve the NP morphology and to prevent premature disintegration in vivo, relying solely on hydrophobic interactions in the core seems to be rarely sufficient[142]; yet, most of the recently developed PISA systems do (Table 2).

Instead, to retain structural integrity in vivo, higher thermodynamic stability (extremely low CMC) or kinetic stability through a highly viscous core with low chain mobility is crucial. This may be accomplished via kinetic trapping, by introducing intermolecular stabilizing interactions in the core-forming polymer block (as described in Section 4.2.2), or by implementing (non-)covalent crosslinking in situ or post-PISA.

Covalent crosslinking can be achieved either during PISA by incorporating divinyl monomers in the core in situ[161,192,212–215] (see Figure 8B), or via (photo-)chemical post-PISA processing.[216] In situ crosslinking can reduce experimental steps, but is prone to inhibit morphological evolution due to progressively restricted chain mobility during PISA.[212,213] To overcome this limitation, incorporating the crosslinker can be delayed until the morphological transitions have been largely concluded: For example, by adding the crosslinker at a later stage during PISA[161] or by choosing a crosslinker with a lower reactivity than the monomer.[217–219]

An elegant alternative strategy, first reported by Qu et al.,[214] circumvents these delayed crosslinker additions. In their work, they use an asymmetric crosslinker, allyl acrylamide (ALAM), with two vinyl groups of different reactivity. As a result, one side of the crosslinker is incorporated during the polymerization, but crosslinking only occurs toward the end, enabling the morphological transition to polymersomes during PISA. The range of asymmetric crosslinkers has been extended since.[215]

Post-PISA crosslinking approaches are similar to the strategies used for conventionally assembled micelles.[142] Reactive functional groups, such as epoxides, aldehydes or ketones, are first introduced into the BCP via (co)monomers. Subsequently, crosslinking can be achieved by adding chemical crosslinkers, for example, hydrazine,[220] or based on dihydrazide,[221] diamines,[222–224] alkoxyamines,[225] or dithiols.[226] Alternatively, photo-mediated crosslinking[138] is possible and has the advantages of typically mild reaction conditions, no additives or byproducts, and an easily controllable extent of crosslinking by irradiation time and light intensity.[52] Reported examples of photo-crosslinkable monomers applied in PISA systems are based on UV-induced dimerization of coumarin units,[138,227] cycloaddition of cinnamate groups,[228] and disulfide exchange reactions.[229] Wavelength-orthogonality may be exploited for successive photo-PISA and crosslinking.[227,229]

Given the complexity of incorporating covalent crosslinking in PISA-NPs as well as its frequent need for biologically unfavorable chemicals, non-covalent crosslinking relying on hydrogen bonding[124] or electrostatic interactions[230] may be considered. For example, Abad et al.[124] incorporated repeat units bearing the nucleobase analogue 2,6-diacylaminopyridine (DAP) between the PEG-based shell and the hydrophobic PHPMA-based micellar core. The DAP units were employed for non-covalent crosslinking with a molecule containing four thymine units, driven by triple hydrogen bonding per unit.

4.4 | Incorporating (bio)degradability

The vast majority of PISA-NPs developed for biomedical application rely on the use of biocompatible polymers to limit toxicity from the carrier system. However, to further minimize the risk of adverse effects from polymer accumulation in the body, (bio)degradability of (at least part of) the polymer backbone is desirable.[33]

If triggered by location specific stimuli, such as pH or redox potential (and accompanied by cargo release), polymer degradation can go hand in hand with stimuli-responsiveness, which has already been well explored for PISA-NPs (Section 3.1).

However, achieving biodegradability, that is, degradability governed by biological activity, by incorporating hydrolysis-sensitive groups throughout the polymer backbone, specifically the core-forming block, is more difficult. Including biodegradable functional groups, such as esters from cyclic ketene acetals (CKAs),[231] thioesters from thionolactones,[232] disulfide groups from cyclic allylic sulfides,[233] and peptides and peptoides from *N*-carboxyanhydrides (NCAs),[157,234] requires ROP of cyclic monomers, which often exhibit high sensitivity to protic solvents or water. Given this technical difficulty, ROPISA is still very niche in the overall PISA field. Strategies to circumvent premature monomer degradation during ROPISA rely on adjusting the pH and reaction temperature,[157] or resort to NP transfer from polar aprotic solvents to water post-PISA.[231] Alternatively, reverse sequence PISA, a novel approach to PISA recently established by the Armes group,[235,236] could be an interesting work-around to incorporate hydrolysis-sensitive groups in the core.[237] Biodegradability may also be incorporated in the hydrophilic block, for example by chain-extending poly(sarcosine),[234] or in a terpolymer between the hydrophilic and the hydrophobic block, circumventing the need for ROPISA.[189]

4.5 | Expanding the PISA concept to other driving forces

Nowadays, the therapeutic arsenal is much wider than the traditional small-molecule, hydrophobic compounds. Sugars, proteins and nucleic acids (DNA and RNA) or complex combinations thereof are gaining popularity in the pharmaceutical field due to their high selectivity and low toxicity.[143–146] Typically, these biologics are not compatible with the stereotypical PISA as outlined in this Review. This is mainly caused by the fact that these (macro)molecules are either heavily charged or highly polar and therefore not miscible or dispersible in the hydrophobic cores of amphiphilic BCP micelles. Encapsulating biologics is in principle possible in the aqueous lumen of PISA-derived polymersomes.[97,98,100,111,114] However, these higher-order morphologies can be difficult to access experimentally and are generally rather polydisperse in size.

More robust systems can be obtained by substituting the hydrophobic attraction with other attractive forces to drive the self-assembly process. Gu et al. recently reviewed current endeavors in this direction and identified the use of electrostatic interactions, stereocomplexation (or chirality), hydrogen bonding and crystallization as alternative driving forces.[238] The favorable benefits of performing one-pot procedures operating at high concentrations and tunability of particle morphology remain valid. Extending these strategies to therapeutically relevant cargo presents a promising strategy to widen the cargo scope for PISA. Recent examples moving toward this direction include the encapsulation of small-interfering RNA (siRNA) and plasmid DNA,[202,239] dendrimers,[240] and synthetic polypeptides.[241]

4.6 | Upscaling PISA

PISA is considered to be economically more favorable for large-scale NP production compared to conventional SA methods due to the typically higher solid content and fewer experimental steps. Despite the higher solid content, upscaling from small laboratory scale remains necessary for eventual commercial application.

Given the strong influence of the reaction conditions on the NP morphology during PISA, the choice of reactor type and tunable parameters is crucial,[242] even for robust PISA systems. For example, in case of photo-initiated PISA, (semi-)continuous production in flow reactors may be preferred over batch reactors as short optical path lengths lead to more uniform irradiation, and hence better reproducibility.[243,244]

While upscaled PISA remains a relatively unexplored area, some promising works have been reported.[147,155,209,245,246] Photo-initiated RAFT-PISA in flow has been successfully realized under both aqueous[147,155,209] and non-aqueous conditions.[246] Different morphologies (spherical micelles, worm-like micelles, and polymersomes) have been prepared following the same general principles as in small-batch mode, that is, adjusting the solid content[209] or targeting specific DPs, for example, by varying the residence time in the reactor.[147] Finally, the potential of flow-PISA for drug delivery has been demonstrated by physical in situ encapsulation of DOX.[147]

As an alternative to flow-PISA, further simplification of the BCP synthesis with PISA via forced gradient copolymerization, that is, gradual injection of core-forming monomer, may be considered for large-scale NP manufacturing.[247]

5 | CHALLENGES TOWARD IN VIVO STUDIES AND CLINICAL TRANSLATION

In the academic world, new DDS are being reported at a staggering rate. (Block co)polymer-based systems are no exception. Every day, a handful of papers are published that introduce new particle designs, loading strategies, targeting concepts and so forth. While these systems contribute to our overall understanding of the extremely challenging processes involved in drug delivery, the impact on the clinic has been minimal.[248] Obvious reasons are the extremely lengthy clinical trials and enormous capital investments that are needed to start them. The trials themselves are even more demanding and there is no room for error, as a single failure can doom the system's translation. As outlined recently by Metselaar et al., the only way to increase the acceptance of DDS systems on the market is to be more aware of the end user's perspective directly from the early developmental phase.[249] Additionally, economic and clinical feasibility need to be considered. Only when the newly developed DDS is anticipated to add significant cost reduction or therapeutic benefit over existing treatments is the start of development justified.

With this in mind and realizing that PISA is still a relatively new kid on the block when it comes to DDS synthesis, it would be beneficial for the PISA field to start thinking beyond the academic lab rather sooner than later. Here we would like to provide some pointers for technical and scientific challenges specific for PISA-derived DDS that in our opinion should be addressed by the polymer science community. We would like to remind the reader that these challenges are not an extensive list and come on top of the hurdles associated with the clinical translation

of BCP-based systems in general, for example, low structural stability and cargo retention upon dilution, polymer-induced toxicity, unfavorable biodistributions/pharmacokinetics, and high production costs.[248,249]

1. **Structural integrity of the cargo.** One of the hallmarks of PISA is the simultaneous polymer formation, assembly and in situ cargo encapsulation. Considering that most PISA systems rely on radical-based polymerization techniques, it is not unreasonable to assume that these highly reactive radical species could alter the chemical nature of the cargo. Such changes could lead to loss of function, reduced bioactivity, and, in the worst case, toxic byproducts. Although some studies attempted to evaluate cargo degradation with spectroscopic assays,[103–105] higher analytical precision is required to, for example, exclude formation of non-spectroscopically active side products. Ultra-performance liquid chromatography (UPLC) coupled to mass spectrometry might be the analytical technique to isolate, identify and quantify the type and concentration of generated impurities.[121] Furthermore, the proneness to degradation will be highly dependent on the chemical details of the cargo and therefore needs to be evaluated on a case-to-case basis.
2. **Sample purification.** Samples suitable for drug delivery require a well-defined and consistent chemical composition.[94] Impurities, that is, anything besides the encapsulated cargo and targeted BCPs, could be toxic or reactive. Residual monomer, non-chain extended hydrophilic blocks, residual initiator fragments, non-micellized BCPs and non-encapsulated cargo are likely suspects. For each of these undesired components, the concentrations and associated risks once exposed to in vitro or in vivo environments should be quantified.[250] If toxicity risks are identified, efficient purification needs to be applied. A crucial consideration in selecting these methods is the non-compromising effect they should have on the NP morphology. Furthermore, common methods, such as dialysis, filtration, and (ultra)centrifugation, could be less efficient due to partitioning of the trace impurities in the hydrophobic pockets of the self-assembled structures.
3. **Shelf life & structural integrity in biological environments.** Considering that PISA is an inherently out-of-equilibrium process, the (kinetic) stability of the formed structures can be hard to predict and is only sparsely addressed in current literature.[98,126] Changes to particle size, shape, and cargo retention should be carefully monitored over extensive periods of time to ensure consistency in these structural parameters and evaluate applicability to real-life scenarios. Evidently, monitoring should be performed in biologically relevant buffers which are used for administrating. Equally important is assessing the structural integrity of the PISA particles once exposed to complex biological fluids. Advanced characterization tools, for example, asymmetric flow field-flow fractionation (aF4) might be instrumental in these studies.[78,251] Structural changes or premature disintegration might have severe consequences for the DDS performance. Having no knowledge of these processes greatly hampers the formulation of clear structure-performance relationships.
4. **Reproducibility.** Performing polymerization, assembly and encapsulation simultaneously makes PISA a sensitive process. Particularly true when targeting higher-order morphologies, changes or drift in, for example, temperature, concentration and reactant purity can severely impact the properties on the resulting particles. Evidently, this can propagate to undesired surprises when these PISA particles are evaluated in vivo. If ever to be applied on larger scales for pharmaceutical applications, robustness of the synthetic process should be guaranteed.
5. **Drug compatibility.** Compared to the total number of active pharmaceutical compounds currently on the market or in development, the variation in cargo molecules actually used in combination with PISA is low (Table 2 and Figure 5). Commonly applied drugs, such as DOX and curcumin, seem to be the first choice for the development of new encapsulation systems. Although justifiable from an academic and fundamental point of view, it would be highly beneficial to expand the cargo scope and evaluate if PISA can not only serve as an alternative, but also enabling technology for certain cargos. These larger scale screening studies on typically expensive cargos could be facilitated by recently reported oxygen-tolerant RAFT systems enabling polymerizations in μL volumes,[122,156] simulation studies to minimize the tedious optimization processes typically involved in multi-parametric PISA systems[169–172] and fully automated RAFT platforms.[252]
6. **Benchmarking against other DDS.** From a commercial point of view, it would be paramount to compare the PISA-derived systems directly to more commonly applied DDS, for example, conventional (polymeric) micelles, lipid nanoparticles, liposomes or against therapies relying on the same pharmaceutically active cargo. PISA will only be able to make an impact beyond the academic drug delivery field if it can produce significantly more effective, safer, or cheaper therapeutic formulations.

To summarize, PISA has a long way to go from its current academic status to implementation in clinically relevant experiments. We greatly encourage an intimate and early dialogue between polymer chemists, pharmacists and biologists to safeguard applicability and feasibility of the future PISA formulations in the early stages of their development.

6 | CONCLUSION

In a relatively short time, PISA has established itself as one of the go-to methods for the preparation of BCP micelles and related higher-order morphologies. This strategy, that combines polymerization under mild (aqueous) conditions, assembly, and cargo loading in one single step, sparked interest across the chemistry and material science fields. Drug delivery is most frequently suggested as the final application of choice. Indisputably, significant progress has been made in this context. Numerous examples showcased the synthetic capabilities of PISA in the encapsulation of small hydrophobic drugs and in some cases the resulting particles were evaluated in vitro or even in vivo. Having passed this initial wave of promising results, it is now up to the field to take the leap and try to push PISA beyond an academic exercise for DDS. Tailoring of the particle characteristics for specific DDS needs in terms of stability, integrity, biodegradability, and shape fidelity can be found within the broader PISA literature. The same holds true for expanding the PISA concept to other driving forces to accommodate the ever-growing class of biological pharmaceuticals, procedures to scale-up particle synthesis, and perform rapid screenings of future PISA formulations. We expect to see implementation of these concepts and ideas in the near future. The ultimate challenge is to simultaneously address the aforementioned aspects in a single PISA-derived platform without imposing unsurmountable complexity to the particles and their fabrication procedure. Only then, the highest chances of success in vivo can be expected.

In addition to these academic endeavors, the practicalities of pushing drug delivery vehicles to the clinic cannot be ignored. Polymer chemistry alone is not enough to realize an impact in the world of drug delivery. Systematic in vivo studies and clinical trials are extremely regulated processes. Collaborating with pharmacists, biologists, and even medical experts will be paramount to navigate through this complex experimental landscape. Only with the combined knowledge of these fields, we can start to explore the ultimate potential of PISA for drug delivery. We sincerely hope this Review provides a starting point for future research and inspires the community to engage in this highly multidisciplinary journey.

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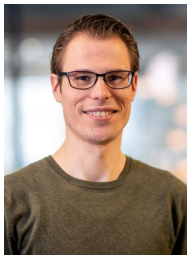

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