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Nanodiscs: a versatile nanocarrier platform for cancer diagnosis and treatment

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Cancer therapy is a significant challenge due to insufficient drug delivery to the cancer cells and non-selective killing of healthy cells by most chemotherapy agents. Nano-formulations have shown great promise for targeted drug delivery with improved efficiency. The shape and size of nanocarriers significantly affect their transport inside the body and internalization into the cancer cells. Non-spherical nanoparticles have shown prolonged blood circulation half-lives and higher cellular internalization frequency than spherical ones. Nanodiscs are desirable nano-formulations that demonstrate enhanced anisotropic character and versatile functionalization potential. Here, we review the recent development of theranostic nanodiscs for cancer mitigation ranging from traditional lipid nanodiscs encased by membrane scaffold proteins to newer nanodiscs where either the membrane scaffold proteins or the lipid bilayers themselves are replaced with their synthetic analogues. We first discuss early cancer detection enabled by nanodiscs. We then explain different strategies that have been explored to carry a wide range of payloads for chemotherapy, cancer gene therapy, and cancer vaccines. Finally, we discuss recent progress on organic–inorganic hybrid nanodiscs and polymer nanodiscs that have the potential to overcome the inherent instability problem of lipid nanodiscs.

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1. Introduction

Circulating chemotherapeutics are significantly affected by their physicochemical properties (solubility, stability, pH,

etc.), pharmaceutical properties (release profile, target specificity, *etc.*), and biological barriers (dense desmoplasia, high interstitial pressure, blood–brain barrier, *etc.*). The tumor microenvironment (TME) presents an unprecedented complex barrier for chemotherapeutics to reach deep-seated cancer cells in solid tumors. The complex TME consists of the cancer cells as well as the endothelial, mesenchymal, and immune cells that are recruited to the tumor bed to help develop tumor

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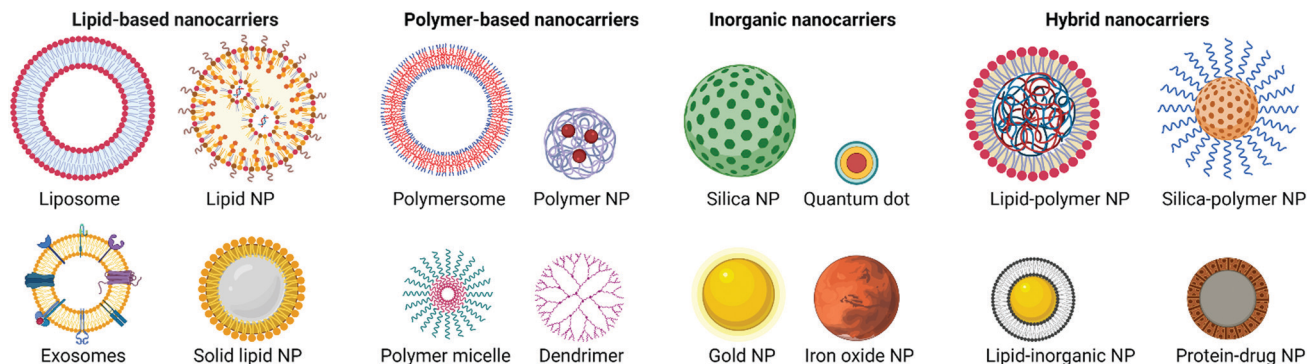


Fig. 1 Different classes of nanocarriers. Each class features multiple subclasses, with some of the most common subclasses highlighted here.

stroma. Together with the extracellular matrix, they form a strong barrier for chemotherapeutics,¹ resulting in suboptimal therapeutic effects and toxicities such as cardiotoxicity, nephrotoxicity, myelosuppression, and other side effects.² The suboptimal chemotherapeutic concentration likely helps the development of drug resistance.³ Incessant advancement in the molecular biology of TME and new anticancer agents including chemotherapy molecules, antibodies, siRNAs, miRNAs, plasmid DNA, peptides, and engineered immune cells continue to offer new and effective treatment options. However, their effectiveness is often not translated into clinical therapeutic breakthroughs due to the lack of efficient delivery systems.

Nanomedicines have great potential for cancer mitigation. They alter the pharmacokinetics of anticancer drugs, improve stability, provide specific targeting, exhibit a high surface-to-volume ratio, control drug release, and re-model immunosuppressive TME.⁴ Compared to conventional formulations, nano-formulations (*i.e.*, nanoparticle-based drug delivery carriers) rely on functional nanomaterials to realize on-demand drug release in a precise manner in response to internal stimuli (such as redox or oxidative environments, pH stimuli, tumor-specific

enzymes) or external triggers (such as UV or infra-red light, temperature and ultrasound, electric and magnetic fields).^{1,5,6} Nanocarriers have been shown to significantly improve the delivery of hydrophobic chemotherapy agents by enhancing their bioavailability and protecting them from various enzymes and other destabilizing factors.⁷ They have also been widely used for the delivery of hydrophilic molecules like small organic molecule as well as large biomolecules like siRNA, mRNA and miRNA.^{8–11}

The nanocarriers for anticancer drug delivery are broadly classified in four main categories, *i.e.*, lipid-based, polymer-based, inorganic, and hybrid nanocarriers. Each class of nanocarriers consists of multiple subclasses, among them some of the most common subclasses are presented in Fig. 1. Each class has its own advantages and disadvantages regarding cargo carrying efficiency, stability, and patient response. For more detailed discussions about these nanocarriers, we direct the readers to some excellent reviews in the literatures.^{12,13}

The development of nanocarriers may be subdivided into two major generations. The first-generation mainly aims to improve the water solubility of hydrophobic drugs and reduce their toxicities/adverse events, *e.g.*, encapsulation of drugs in lipids or



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albumin protein nanostructures, including simple liposomes (LIP) and other lipid vesicles shielded with polyethylene glycol (PEG) to prevent immune response and/or prolong circulation time. Those nanocarriers generally have controlled nanoparticle (NP) sizes that solely depend on the passive enhanced permeability and retention (EPR) effect for cellular distribution, such as the formulations of doxorubicin (DOX) (Doxil,[®] pegylated LIP) and paclitaxel (PTX) (Abraxane,[®] albumin-bound NP) approved by the Food and Drug Administration (FDA) for clinical use.² The second-generation nanocarriers not only provide the benefits of first-generation formulations but also actively search for tumors *in vivo*. Importantly, they allow tumor imaging, *in vivo* real-time tracking, and monitoring of the therapeutic efficiency of drugs.^{14,15}

Additionally, they use tissue-specific ligand coatings to target tumors with on-demand payload release (such as ThermoDox[®]) to provide increased drug accumulation at the tumor sites.¹⁵

The last two decades have witnessed extensive developments in both generations of nanocarriers that either physically encapsulate or chemically conjugate anti-cancer drugs, such as LIPs, polymeric nanocarriers, polymer-drug conjugates, lipid-drug conjugates, lipid-polymer conjugates, and inorganic nanocarriers including noble metals, silicon, silica, or iron oxide.^{1,16–18} Many of these nanocarriers are currently under clinical trials, with some being approved by the FDA for clinical use.^{15,19–21} A partial list of clinically approved nanocarriers for cancer chemotherapy or diagnosis is provided in Table 1.

Table 1 A partial list of clinically approved nanomedicines for cancer chemotherapy or diagnosis

No.	Name	Composition	Drug tested	Target cancer	Clinical trial phase	Year of approval	Ref.
Natural/synthetic polymer-based nanoparticles							
1	Eligard [®] (Tolmar)	(PLGH (poly(D,L-lactide-co-glycolide)))	Leuprolide acetate	Prostate cancer	—	2002	19
2	Oncaspar	Polymer protein conjugate	L-asparaginase	Leukemia	—	2006	21
3	Genexol-PM	PEG-poly(D,L-lactide) based micelle	Paclitaxel	Breast cancer	—	2007	15
4	Apealea	Micellar	Paclitaxel	Ovarian cancer, peritoneal cancer, and fallopian tube cancer	—	2018	22
Drug-lipid conjugates							
1	DHP107	Lipid nanoparticle	Paclitaxel	Gastric cancer	—	2016	15
2	PICN	Nanosuspension	Paclitaxel	Breast cancer	—	2014	15
Liposome formulations combined with drugs or biologics							
1	DaunoXome [®] (Galen)	Liposomal	Daunorubicin	Kaposi's Sarcoma	—	1996	19
2	DepoCyt [®] (Sigma-Tau)	Liposomal	Cytarabine	Lymphomatous meningitis	—	1999	19
3	Marqibo [®] (Onco TCS)	Liposomal	Vincristine	Acute lymphoblastic leukemia	—	2012	19
4	Onivyde [®] (Merrimack)	Pegylated liposomal	Irinotecan	Pancreatic cancer	—	2015	19
5	Doxil [®] /Caelyx [™] (Janssen)	Pegylated liposomal	Doxorubicin	Kaposi's sarcoma	—	1995	19
				Ovarian cancer	—	2005	
				Multiple myeloma	—	2008	
6	ThermoDox	Heat-sensitive liposome	Doxorubicin	Hepatocellular carcinoma	Phase III	—	21
7	Myocet	Liposomal	Doxorubicin	Breast cancer	—	2000	15
8	Lipusu	Liposomal	Paclitaxel	Breast and non-small-cell lung cancer	—	2007	15
9	Mepact	Liposomal	Mifamurtide	Osteogenic sarcoma	—	2009	15
10	Vyxos	Liposomal	Daunorubicin and cytarabine	Leukemia	—	2017	15
11	Mifamurtide (Mepact)	Liposome	Muramyl tripeptide phosphatidyl-ethanolamine	Nonmetastatic, resectable osteosarcoma	—	2009	21
Protein nanoparticles combined with drugs or biologics							
1	Abraxane [®] /ABI-007 (Celgene)	Albumin-bound nanoparticles	Paclitaxel	Breast cancer	—	2005	19
				NSCLC	—	2012	
				Pancreatic cancer	—	2013	
2	Ontak [®] (Eisai Inc)	Engineered protein	IL-2 and diphtheria toxin	Cutaneous T-cell lymphoma	—	2008	19
3	Nab-rapamycin (ABI-009)	Albumin NP	Rapamycin	Advanced malignant PEComa and advanced cancer with mTOR mutations	Phase II	—	21
Inorganic and metallic nanoparticles							
1	Nanotherm [®] (MagForce)	Iron oxide	—	Glioblastoma	—	2018	15,19
2	GastroMARK [™] ; Lumirem [®] (AMAG pharmaceuticals)	SPIO coated with silicone	—	Imaging agent	—	2001	19

The majority of nanocarriers under development for anti-cancer drug delivery are spherical in shape. In recent years, non-spherical nanocarriers such as nanodiscs (NDs) have attracted much attention. The idea of ND constitution is inspired from the biologically-derived solutions for the transportation of lipids – which are poorly water-soluble – in blood streams. Among various lipid nanocarriers in human blood, High-Density Lipoproteins (HDLs) plays a crucial role in transportation and metabolism of lipids, particularly cholesterol and triglycerides.²³ HDLs were first isolated in 1929 from equine serum and 1950s from human serum, subsequently, in 1966 it was established that HDLs deficiency might lead to ischaemic heart diseases (IHDs) and later on in mid-1970s, it was confirmed that low plasma HDL levels accelerate the development of atherosclerosis and IHDs due to reduced clearance of cholesterol from the blood vessels.²⁴ HDLs are known to transport signaling lipids, proteins, and microRNAs throughout the body and play multiple functions in complex inter-cellular communications.²⁵ These features, particularly the fact that nascent HDLs are essentially NDs encased by amphipathic apolipoproteins, have attracted much attention to develop HDLs and NDs as nanocarriers for various therapeutic agents.

In a broad view, NDs represent a patch of any nanoscale membrane encased by amphipathic molecules such as proteins, synthetic polymers, or short-chain lipids. Depending on the composition of the NDs, they may be classified into four categories (Fig. 2):

(1) Lipid nanodiscs (LNDs). LNDs consist of a disc-shaped lipid bilayer (typical diameter ~ 10 – 20 nm) surrounded and stabilized by amphiphilic biomacromolecules such as Apolipoprotein A1 (apoA-1) or membrane scaffold proteins (MSPs; Fig. 2A),^{28,29} saposin,³⁰ peptides,³¹ or nucleic acids;³²

(2) Styrene-maleic acid (SMA) lipid nanoparticles (SMALPs), which differ from LNDs in that the amphiphilic membrane scaffold biomacromolecules in LNDs are replaced with synthetic SMA or SMA-like copolymers (Fig. 2B);^{33–37}

(3) Polymer nanodiscs (PNDs). PNDs differs from LNDs in that the lipid bilayer of LNDs is replaced by a synthetic membrane patch consisting of amphiphilic block copolymers,

whereas the same choices of membrane scaffold polymers or biomacromolecules such as MSPs are used (Fig. 2C);³⁶

(4) Hybrid nanodiscs (HNDs) or hybrid bicelles. These disc-shaped nanostructures are not stabilized by amphiphilic macromolecules. Instead, they are made of long-chain cerasome-forming lipids (CFL) and short-chain phospholipids.²⁷ (Fig. 2D).

Although there are extensive reviews available on nanomedicines for cancer therapy that described the concepts, challenges, and opportunities,^{1,2,7,38,39} optimization strategies,⁴⁰ nano-formulation for cancer immunotherapy,^{4,41,42} and nucleic acid delivery,⁴³ along with specific reviews on LIPs^{44,45} or micelle-based nano-formulations,^{46,47} and reviews that focused extensively on the biological background, isolation, and characterization of LNDs as delivery vehicles for small molecules and siRNA,^{23,48–57} very few reviews discussed the development of NDs for cancer diagnosis and treatment. This review intends to provide an overview of recent advances that explore NDs for cancer therapy and present an outlook on future development.

2. NDs vs. traditional nanocarriers

The shape and size of NPs has a significant effect on their blood circulation time and cellular internalization.^{58–63} Before transvascular transport, a pivotal step is margination (radial drift) of NPs towards the blood vessel walls. The meaningful margination is an essential requirement for the transportation of NPs across the blood vessel. In contrast to spherical NPs, oblate shaped NPs experience torques in the blood flow, resulting in tumbling, rotation, and increased margination towards the blood vessel walls^{64–67} (Fig. 3A).

After margination, the transvascular transport is governed by many factors like fluid flow rate, filtration along a capillary and hydrostatic pressure gradient, particularly pressure difference between the vascular pressure and interstitial flow pressure.⁶⁸ Once these factors are overcome by the NPs, they are transported in the cancer cells through macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-caveolae or dynamin-independent endocytosis

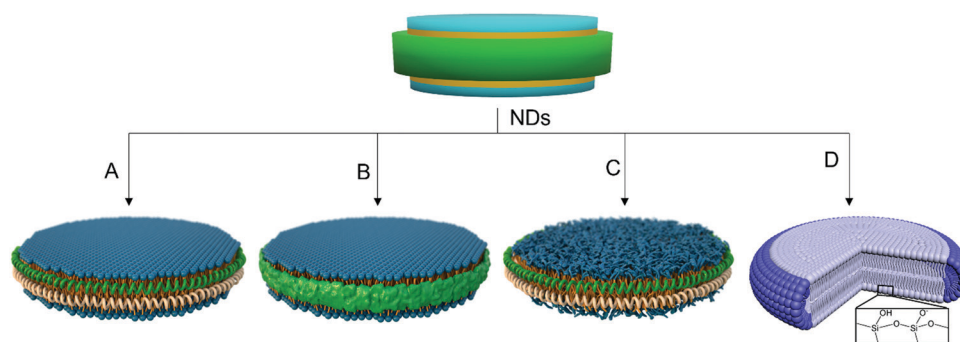


Fig. 2 Various ND platforms for cancer imaging and mitigation. Examples include (A) LNDs encased by MSPs,²⁶ (B) SMALPs stabilized by amphiphilic SMA-like random copolymers,²⁶ (C) PNDs encased by MSPs, which differs from LNDs in that the lipid bilayer patch in LNDs is replaced with an amphiphilic block copolymer membrane,²⁶ and (D) HNDs made from CFL and short-chain phospholipids.²⁷ Schematic representations of LNDs, SMALPs, PNDs, and HNDs are reproduced with permissions from ref. 26 Copyright 2020, Frontiers Media S. A. and ref. 27 Copyright 2017, John Wiley and Sons.

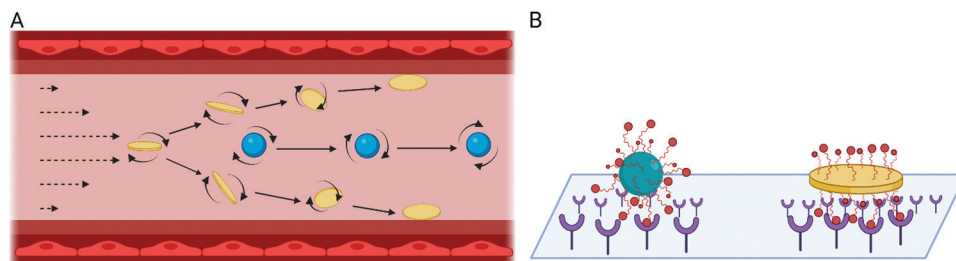


Fig. 3 Effects of particles shape on their margination in the blood flow and binding strength to the endothelium (illustrations adapted from ref. ⁶⁷). (A) Margination. NDs are subjected to torque forces within blood flow, experience drift and tend to tumble out of the general circulation toward vessel walls, whereas spherical NPs tend to follow the streamlines. (B) Binding avidity. Compared to spherical NPs, NDs have increased particle surface areas in contact with the endothelium, allowing a greater number of targeting ligand interactions to enhance the binding strength.

processes.⁶⁹ Overall, oblate shaped NPs (including disc or worm-like NPs) showed improved cellular internalization and favor efficient delivery of therapeutics owing to their large surface areas, multiple attachment points on cells, and reduced clearance by macrophages of the reticuloendothelial organs resulting in prolonged blood circulation half-lives.^{63,70–73} Generally, NPs with larger aspect ratios (*i.e.*, non-spherical NPs) are taken up in higher frequency and at faster rates.^{27,63} When decorated with targeting ligands, the oblate shape particles form a greater number of multivalent interactions leading to improved cellular uptake even at a high fluid flow rate⁷⁴ (Fig. 3B).

In this context, NDs offers many advantages over other nano-formulations due to their uniform ultra-small size, discoidal shape, and site-specific functionalization for cancer signature receptors. Among the other nanocarriers, LIPs offer advantages such as biocompatibility and biodegradability, low toxicity, facile surface functionalization, and possibility of controlled drug release, but they can trigger hypersensitivity reactions and have stability problems. Other organic nanocarriers such as micelles offer similar advantages but are limited to the encapsulation of hydrophobic drugs with relatively low efficiency and suffer from difficulty in scaling up and often unfavorable premature drug release profiles, aggregation, and toxicities.^{12,75–77} Inorganic NPs such as mesoporous silica NPs, superparamagnetic iron oxide NPs, carbon nanotubes, gold NPs, metal–organic frameworks, and quantum dots offer some distinct advantages such as large specific surface areas, uniformity in sizes, high stability, facile functionalization, and unique optical, electrochemical, magnetic properties suitable for theranostic applications.⁷⁵ However, limitations such as high toxicity, non-biodegradability, accumulation in vital organs, high cost of large-scale production, and particle aggregation restrict many inorganic NPs from translating to clinical applications.^{78,79}

Although LNDs encased by MSPs or SMALPs have gained increasing popularity in the study of membrane proteins shortly after their discovery in 2000s,^{28,33} their uniform, nano-scale and tunable sizes (*i.e.*, ~ 10 – 20 nm), large specific surface area, rapid cellular internalization, and high biocompatibility present them as a desirable drug-delivery platform.⁸⁰ For example, the size of LNDs can be precisely controlled by using suitable MSP constructs, typically ranging from ~ 10 to

~ 20 nm.^{54,81,82} Compared to spherical NPs, the disc-like structure of conventional LNDs and more recently developed PNDs or HNDs offers many advantages, such as improved circulation half-lives, cellular uptake, biodistribution, and microvascular adhesion.^{66,71,83,84} A brief comparison of the pros and cons for each type of ND platform (with respect to cancer diagnosis and mitigation) is provided in Fig. 4. The anisotropy effect can be further enhanced with ligand modification at specific locations on the NDs, including either planes or edge modifications.²⁷ These properties of NDs are highly merited for the development of novel cancer diagnosis and treatment options that exploit the NDs as carriers for a broad range of imaging probes, chemotherapeutics, vaccines, and anti-cancer genes.⁵³

Besides their well-defined nanoscale sizes and anisotropic shapes, NDs in general have excellent biocompatibility and biodegradability, a highly desirable feature for drug-delivery applications. Since the major component of NDs is either biodegradable lipids (for LNDs and SMALPs) or biodegradable and biocompatible polymers (PNDs), they can be metabolized by enzymes like protease, esterase, metalloproteases *etc.* Cross linked silicates in the HNDs may produce metabolic resistance and accumulation in the body, but silica is listed amongst the “generally regarded as safe” substances by the Food and Drug Administration (ID Code: 14808-60-7). The buffer incompatibility of conventional SMALPs is a concern, but this problem is solved by the recent development of SMA-like copolymers with unlimited buffer compatibility.^{35,85,86}

2.1 General methods for the assembly of NDs

The methods for preparing different types of NDs are varied. For example, the LNDs are assembled by mixing an optimal ratio of lipids of choice with suitable MSPs in the presence of an appropriate detergent, followed by removal of detergent below its critical micelle concentration (CMC) by different methods such as dialysis, dilution or using Bio-beads. Finally, the self-assembled LNDs are obtained by suitable affinity column purification;³⁶ For SMALPs assembly, the liposomes and SMA or SMA-like copolymers are incubated in a suitable buffer for varying length of times depending on the nature of the lipids and the copolymers. Once the liposomes are cut by the copolymers into SMALPs, the resultant products are

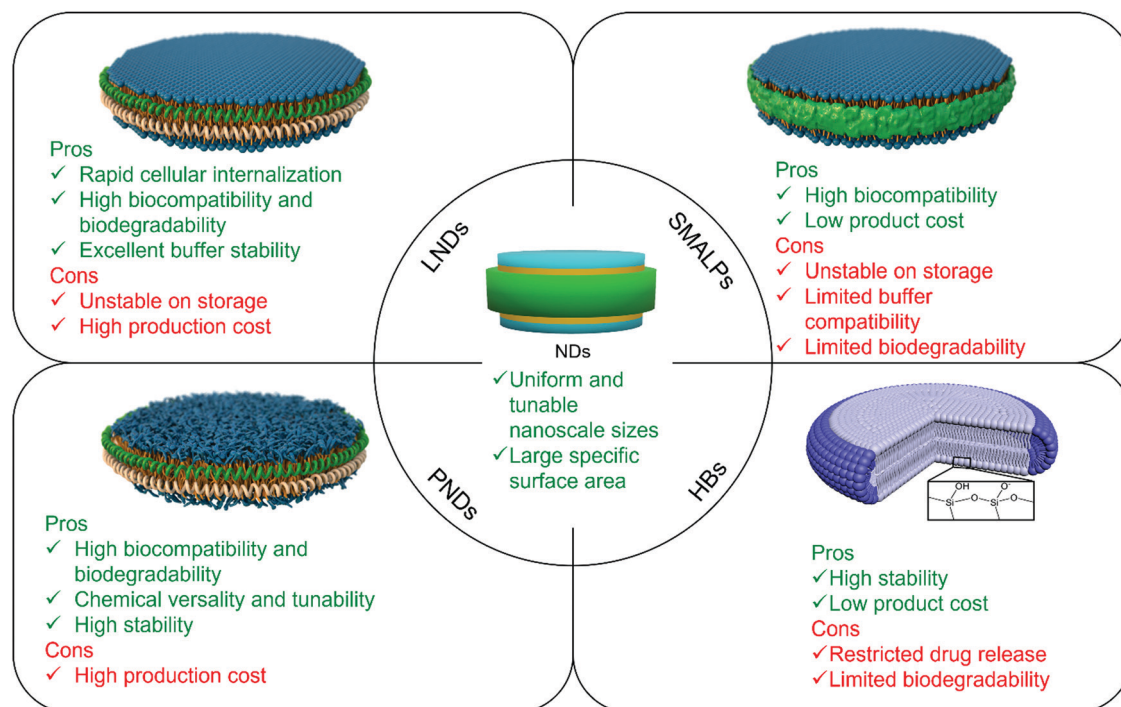


Fig. 4 The merits and limitations of different classes of anticancer NDs. NDs structure reproduced with permissions from ref. 26 Copyright 2020, Frontiers Media S. A. and ref. 27 Copyright 2017, John Wiley and Sons.

generally purified by size-exclusion chromatography; For PNDs assembly, an optimal ratio of polymersomes and MSPs are solubilized in a suitable detergent followed by removal of detergents similarly to the preparation of LNDs. The self-assembled PNDs are collected after purification by the affinity columns;³⁶ For HBs or HNDs, they are generally prepared by film hydration method. Typically, CFL and short-chain phospholipids at an optimal ratio were dissolved in organic solvent (e.g., CHCl_3), the solution was subsequently dried to obtain a lipid film. The lipid film was then hydrated with ultrapure water followed by continued hydration overnight.²⁷ For detailed protocols for the assembly of different NDs, we direct the readers to the research articles and protocols on LNDs,^{87,88} SMALPs,^{35,89} PNDs,³⁶ and HNDs,^{27,90} respectively.

2.2 Stability of NDs

Like self-assembled polymersomes, liposomes, or micelles, self-assembled NDs are subjected to disassembly-assembly equilibrium, and the energetics of individual equilibria depends on the structure and properties of the amphiphiles. Taking the self-assembly of micelles in aqueous solutions for example, when the concentration of amphiphiles increases above its CMC, they start to associate in order to minimize water contact with their hydrophobic moieties. This association favors their hydrophilic regions to make contact with surrounding aqueous solutions while shielding their hydrophobic regions away from water and towards the micelle center.⁹¹ By doing so, a hydrogen-bonded network of water molecules that would be otherwise needed to surround the hydrophobic region of individual amphiphiles are also freed and the overall free energy of

the system is minimized.⁹² Similarly, the self-assembly of NDs is also driven by minimizing the overall free energy of the system, where the amphipathic proteins, synthetic polymers, or short-chain lipids self-assemble to encase individual amphiphilic membrane patches and protect them from exposing their hydrophobic membrane edges to aqueous solutions. The stability of NDs depends on their chemical compositions. For example, just like polymersomes are in general more stable than liposomes, PNDs are more stable than LNDs.

In the following sections, we will discuss the application of various forms of NDs on selective delivery of imaging agents for early cancer detection and chemotherapeutics for cancer treatment. It is important to note that on a few occasions during our discussions, we use high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs) to represent the NDs following the nomenclature frequently used in the original publications, where HDLs/LDLs were used synonymously as NDs because many reported HDLs/LDLs were specified to be discoidal in shape.^{51,82,93} It should be pointed out that although HDLs and LDLs frequently assume the ND structures, they could acquire a spherical shape depending on the amount and type of lipids being encased by the apolipoproteins.²⁴

3. NDs for early cancer detection

Early detection of cancer is one of the paramount requirements for successful treatment and is directly associated with low cancer mortality.⁹⁴ Cancer imaging, including fluorescent imaging, computed tomography (CT), MRI, transabdominal

or endoscopic ultrasound imaging, and positron emission tomography (PET) imaging are often used for early-stage cancer detection.⁹⁵ Among these techniques, CT, MRI, and ultrasound imaging are anatomical imaging modalities. Fluorescent imaging and PET are molecular imaging techniques that complement anatomical imaging modalities by providing functional and molecular information.⁹⁶ However, the low number density of early-stage tumor cells often hidden deep in healthy organs and the lack of noticeable signs or symptoms possess major challenges in early cancer detection. In addition, molecular imaging for early-stage small tumors is often inadequate due to non-specific and insufficient accumulation of imaging agents in the tumor cells because of the poor delivery of those imaging agents.

Given that the increased metabolic demand of cancer cells as compared to the healthy cells is met by receptor-mediated access to large quantities of nutrients, cancer cells overexpress specific receptors known as “cancer signatures”, such as HER2/neu, scavenger receptor class B type-1 (SR-B1), epidermal growth factor receptor (EGFR), somatostatin, folic acid receptors (FAR), $\alpha_v\beta_3$ integrins, and low-density lipoprotein (LDL) receptors.⁹⁷ The overexpressed cancer signatures serve as an important tool to selectively target cancer cells for diagnostics and treatment (Fig. 5).

Because of their nanoscale size, discoidal shape, and ease of incorporation of a wide array of imaging agents, NDs have emerged as a powerful tool for the delivery of imaging agents for cancer detection. Generally, the payload can be loaded at three chemically distinct environments of NDs: (1) into the hydrophobic core; (2) onto the shell (or mantle), and (3) on the corona (or crust), depending on the physicochemical properties of the payload and ND design.^{51,53} Importantly, by chemically attaching specific tumor ligands either on the planes or edge of

the NDs, the tagged NDs can be routed to target tumor cells selectively.

Besides carrying chemotherapeutics directly with the NDs for their selective delivery to cancer cells, another often used strategies is to modify anticancer drugs into prodrugs and deliver the prodrugs instead.⁹⁸ There are excellent recent reviews describing the application of prodrugs for cancer-specific targeting,^{99–101} and linker-specific prodrugs such as nucleoside-based,¹⁰² disulfides-based,¹⁰³ pH-sensitive,¹⁰⁴ reactive oxygen species sensitive prodrugs,¹⁰⁵ and many others.¹ Prodrug strategy uses chemical functional groups such as esters (such as carboxyl, carbamate, carbonate, phosphate, or sulfate esters), amide, oxime, imine, disulfide, or thioethers groups between the drug and the promoity/nanocarrier system. The promoity attached with the drug plays a key role in overcoming various barriers, enhancing drug targeting, and improve drug-like properties. The conjugation of the drug and the prodrug moiety should be stable till it reaches the target site; however, once it reaches the target site, a fast drug release is expected to show the desired therapeutic effect. The release process is likely to take place in the TME either in response to a specific trigger such as elevated levels of cellular enzymes (*e.g.* esterases, phosphatases, sulphatases, matrix metalloproteases, thymidine phosphorylase, endopeptidase, cathepsin B, *etc.*),^{1,100} elevated levels of reactive oxygen species, low pH,¹⁰³ or specific ligand-receptor interaction or in response to external stimuli such as thermal,¹⁰⁶ ultrasound,¹⁰⁷ light¹⁰⁸ or magnetic.¹⁰⁹

3.1 Targeted delivery of fluorescent imaging agents

3.1.1 SR-B1 receptor mediated delivery of imaging agents.

The overexpression of SR-B1 receptors in some cancers (such as

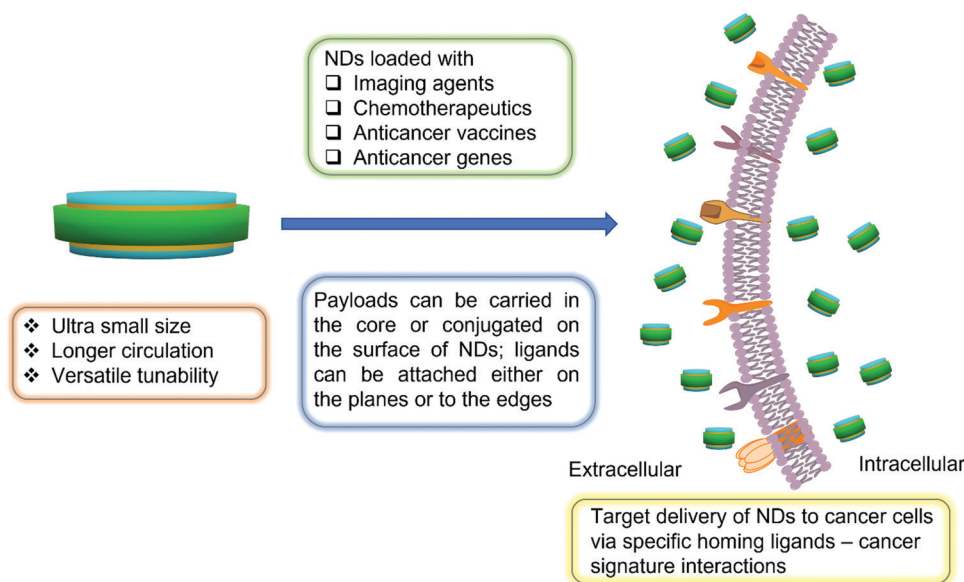


Fig. 5 Early detection and elimination of cancer cells enabled by ND platforms that target cancer signatures. NDs offer several advantages over other nanoparticles, such as well-defined sizes, long circulation time, high cellular uptake, and versatile surface functionalization options. A variety of diagnostic and/or therapeutic agents can be loaded either in the core or conjugated on the surface of NDs. Integration of homing ligands that target specific cancer signatures with NDs results in the highly efficient delivery of payloads into cancer cells.

prostate, breast, and ovarian cancers¹¹⁰) facilitates the selective transport of cholesterol esters from HDL to the cytosol through a hydrophobic channel in the cell membrane.¹¹¹ Using the SR-B1 receptors route, Zhang *et al.* reported that a HDL mimicking nanocarrier system can be assembled using apoA-1 mimetic peptide (FAEKFKAEVVDYFAKFWF) and loaded with a lipid-anchored near-infrared imaging dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide bisoleate (DiR-BOA). The DiR-BOA loaded nanocarriers are spherical, whereas empty nanocarriers have a discoidal shape with a diameter of ~ 16 nm. It was shown that Chinese hamster ovary cells having high expression of SR-B1 exhibited 55 times higher internalization and fluorescein signal from DiR-BOA loaded nanocarriers. In a mouse model, KB tumors (SR-B1⁺) showed a 3.8-fold higher fluorescence signal compared to HT1080 tumors (SR-B1⁻).¹¹¹ Cao *et al.* also reported that another fluorescent imaging agent, Bacteriochlorin e6 bisoleate (Bchl-BOA), loaded in HDL (HDL-Bchl-BOA) had an average diameter of ~ 12 nm. Each HDL-Bchl-BOA had an average of 2–3 molecules of apoA-1 and 6–9 molecules of Bchl-BOA. HDL-Bchl-BOA was preferentially taken up by Chinese hamster ovary cells (SR-B1⁺), resulting in a high fluorescence signal in KB cells in the athymic nude mice model.¹¹² Similarly, NDs of pyropheophorbide-conjugated with 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (pyro-lipid) were developed by Ng *et al.* The pyro-LNDs had a mean diameter of 10–30 nm with an elliptical structure. The pyro-LNDs were internalized and showed high fluorescence in Chinese hamster ovary cells stably transfected with SR-B1⁺.¹¹³

The effects of size, shape, and pegylation on tumor targeting were studied by Tang *et al.*¹¹⁴ They developed synthetic HDLs (sHDL) and their pegylated counterparts (PEG-sHDL), and compared the tumor-targeting efficiencies of those NDs with LIP and pegylated LIP (PEG-LIP). The particle sizes of spherical LIP and PEG-LIP were ~ 130 and ~ 100 nm, respectively, whereas the discoidal sHDL and PEG-sHDL showed average diameters of ~ 9.5 and ~ 12 nm, respectively. The efficient cellular uptake (in BHK-SR-B1 and HCT 116 colon carcinoma cells), tumor spheroids penetration, tumor accumulation, and

in vivo distribution of all the NPs was monitored by loading 3,3'-dioctadecyloxacarbocyanine perchlorate (DIO) or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) as a model drug and tracer. It was shown that sHDL, which targets the SR-B1 receptor, has significantly higher tumor targeting, penetration, and accumulation than LIP, PEG-LIP, and PEG-sHDL¹¹⁴ as shown in Fig. 6A.

3.1.2 FAR mediated delivery of imaging agents. FARs are extensively expressed on epithelial malignancies such as ovarian, breast, colorectal, cervical, and nasopharyngeal cancers.¹¹⁷ Covalently linked folic acid (FA) with macromolecules retains its high affinity for FARs and this approach to homing the tumor has been investigated extensively.¹¹⁸ The conjugation of FA to the NDs can be achieved either to apoA-1 protein (or similar proteins) or a long chain lipid anchor that may be inserted in both planes of the NDs. Using this strategy, Zhang *et al.* conjugated FA with highly basic lysine residues of apolipoprotein B (ApoB)-100, which required a minimum of $\sim 50\%$ lysine residue modification to abolish uptake of LDL by the low-density lipoprotein receptors and to direct them to the FRs. Compared with native LDL (~ 20 nm), the average particle diameter of FA modified LDL increased to ~ 26 nm when loaded with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) (DiI-LDL-FA) and ~ 24 nm when loaded with tetra-*t*-butyl-silicon phthalocyanine bisoleate. FA modified LDLs effectively deliver DiI, loaded on the surface or tetra-*t*-butyl-silicon phthalocyanine bisoleate core loaded dye. Both DiI-LDL-FA and reconstituted tetra-*t*-butyl-silicon phthalocyanine bisoleate folate-conjugated LDL (r-Pc-LDL-FA) were selectively taken up by FR⁺ KB and HepG2 (LDLR⁺) cells. This uptake was blocked by the addition of free FA.⁹⁷ Using a similar strategy, Corbin *et al.* developed engineered HDLs to load the fluorescent dye DiR-BOA. The engineered HDL had a mean diameter of ~ 9.0 nm and has approximately two molecules of DiR-BOA (core loaded) and 19 FA molecules attached to lysine residues of apoA-1. These particles accumulated selectively in FR⁺ KB cells *in vitro* and FR⁺ KB cell-derived tumors.¹¹⁹ In another strategy, Corbin *et al.* conjugated FA to apoA-1 after

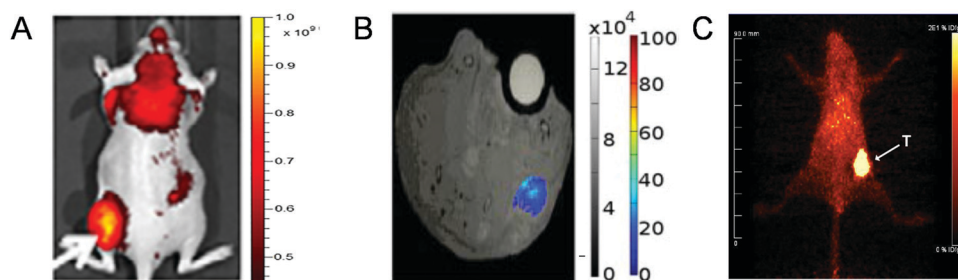


Fig. 6 Examples of ND platforms used for early cancer detection. (A) The *in vivo* fluorescence imaging of HCT 116 tumor-bearing nude mice 72 h after administration of DIO loaded sHDL.¹¹⁴ Tumors were located in the left flanks as indicated by the arrows, (B) representative *in vivo* T1-weighted MR images of Swiss nude mice bearing subcutaneous human EW7 Ewing's sarcoma tumors 24 h post-injection of reconstituted-rHDL (rHDL) loaded with amphiphilic Gd chelates.¹¹⁵ Enhanced pixels within the tumors were color-coded. Gray scale represents signal intensity; color scale represents the percentage of enhanced pixels above a threshold that is defined by the mean intensity of the whole tumor and noise of precontrast MR scanning, and (C) PET imaging of CEA positive tumors in CEA transgenic mice bearing CEA/E0771 cells in their right mammary fat pads with ⁶⁴Cu-DOTA-Antibody injected with 40 μ Ci of ⁶⁴Cu-DOTA-antibody and imaged after 46 h.¹¹⁶ Reproduced with the permission from ref. 114. Copyright 2017, Elsevier Ltd., ref. 115, Copyright 2010, John Wiley and Sons, and from ref. 116. Copyright 2020, American Chemical Society.

reconstitution of HDLs (rHDLs) preloaded with DiR-BOA. The rHDL(DiR-BOA) had a diameter of ~ 15 nm with four molecules of DiR-BOA and 44 molecules of FA on each particle. The FA-rHDL(DiR-BOA) was selectively internalized by FA-over-expressed IC5-MOSEC cell lines whereas, rHDL(DiR-BOA) was selectively taken up by SR-B1⁺ overexpressing (LdlA[mSR-B1]) cells and SR-B1⁺ tumor in mice model. The FA moieties on the surface of the rHDL(DiR-BOA) facilitates the uptake of the NPs into the IC5-MOSEC cells as confirmed by competitive uptake inhibition by adding excess of free FA.¹²⁰

Alternatively, FA can be conjugated to a lipid anchor and adsorbed in both planes of the NDs. In one such example, Tahmasbi Rad *et al.* used folate poly(ethylene glycol)-conjugated distearoyl phosphoethanolamine (DSPE-PEG₂₀₀₀ folate) to construct the NDs and loaded them with Nile Red or meta-tetra(hydroxyphenyl) chlorin for photodynamic therapy. A morphology comparison between LND and LIP (with identical chemical composition) was performed where the NDs had a hydrodynamic radius of 10–12 nm, whereas nanovesicles had 27–30 nm. In FR-overexpressed KB cells, a higher cellular uptake and high fluorescence intensity were observed in meta-tetra(hydroxyphenyl) chlorin-loaded NDs irrespective of conjugation of FA compared to similar vesicles. Similarly, KB tumor-bearing mice showed higher uptake and tumor penetration rate of FA-NDs compared to FA-vesicles.¹²¹

3.1.3 EGFR mediated delivery of imaging agents. EGFR are cell surface receptors and overexpressed in various solid tumors, including cancers of the brain, breast, colon, head, and neck, lung, ovary, and pancreas.¹²² Zhang *et al.* developed HDL-mimetic NPs using apoA-1 mimetic peptide (FAEK-KEAVKDYFAKFWD) and decorated with DSPE-PEG₂₀₀₀-EGF. The fluorescent NPs carry ~ 50 molecules of DiR-BOA with a diameter of ~ 15 nm. The functionalization of NPs with EGF ligand has minimal effect on particle size and showed almost two folds higher accumulation in EGFR-GFP-IIdA7 and KB cells (EGFR⁺). These NPs selectively accumulated in a xenograft KB cells tumor model (EGFR⁺) and showed 2.5-fold higher accumulation compared to EGFR[−] tumors.¹²³

3.2 Delivery of MRI contrast agents using NDs

The optical imaging for early cancer detection has problems such as limited penetration depth and lack of anatomical definition. Thus, other high-resolution techniques are required for imaging deep-seated tumors for molecular details. MRI offers high spatial resolution with anatomical details and excellent contrast. Gadolinium was shown as a highly efficient MRI agent co-delivered with a fluorescent dye (DiR) or 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) using the ND platform by Chen *et al.* for cancer imaging. To target angiogenic endothelial tumor cells, the Gadolinium and dye-loaded rHDL were decorated with $\alpha_v\beta_3$ -integrin-specific cyclic 5-*mer* RGD peptide. The rHDL-RGD NP loaded with DiR dye had a mean diameter of ~ 12 nm. Similar to the *in vitro* cellular uptake, the rHDL-RGD NPs had high tumor targeting efficiency in the human sarcoma xenograft model for deep-seated tumors and

provided high sensitivity and anatomical resolution as shown in Fig. 6B.¹¹⁵

3.3 Delivery of PET imaging agents using the NDs platform

PET uses small amounts of radioactive materials to evaluate organ and tissue functions at the molecular level. By identifying changes at the cellular level, PET can detect the early onset of disease. Among some popular radioactive agents, ⁶⁴Cu is an attractive radionuclide due to its relatively long half-life (12.7 hours) and low maximum positron energy (0.66 MeV). Delivery of radioactive materials required special functional modality on the nanocarriers systems to make a complex with radioactive material such as DOTA. One of the strategies applied by Huda *et al.* was to functionalize the lysine groups of α -helices of the scaffold protein MSP1E3D1 with DOTA to reconstitute ⁶⁴Cu labeled NDs. Conjugation of DOTA to the MSP had minimal effect on its amphipathic folding and ability to form NDs. Each NDs has an average of 5 DOTA per MSP with an average diameter of ND ~ 13 nm with discoidal shape. In the human lung carcinoid tumor model, the ⁶⁴Cu bearing NDs showed a steady increase of NDs concentration in tumors as visualized by PET and computed tomography (CT) images.¹²⁴

Recently, Wong *et al.* used a different approach for the delivery of ⁶⁴Cu using NDs where a lipid anchor was conjugated with DOTA for insertion on both planes of the NDs. Also, the tumor-targeting efficiency was enhanced by attaching carcinoembryonic antibody (CEA) to the surface of NDs. The anti-CEA antibody conjugated NDs had a diameter of 13–14 nm. In CEA-positive tumors in CEA transgenic mice, the antibody fragment fails to direct the NDs to the tumor, whereas attachment of intact anti-CEA antibody to the NDs provided the high tumor uptake of 40% ID/g (Fig. 6C and 7C).¹¹⁶

In another study, Pérez-Medina *et al.* used ⁸⁹Zr as PET agent incorporated in reconstituted HDLs (rHDLs) to image tumor-associated macrophages in the breast cancer mice model. The long-lived positron-emitting nuclide ⁸⁹Zr was incorporated in the NDs either by conjugating through phospholipid or apoA-1 to generate ⁸⁹Zr-PL-HDL or ⁸⁹Zr-AI-HDL. Both NDs were discoidal in shape with an average diameter of ~ 8.0 nm. Both types of NDs resulted in high tumor accumulation of radioactive material and good colocalization in tumor-associated macrophage-rich areas in tumor sections.¹²⁸

3.4 Delivery of inorganic NPs as imaging agents by NDs

Inorganic NPs such as gold or iron oxide NPs have distinct advantages for *in vivo* imaging. Gold NPs have a high X-ray attenuation in CT,¹²⁹ iron oxide NPs offer good contrast for MRI,¹³⁰ whereas quantum dots offer narrow and well-defined fluorescence emission peaks without photobleaching effects.¹³¹ These inorganic NPs can be attached to hydrophobic nanostructures that can be loaded in the core of NDs. The HDLs loaded with modified inorganic nanocrystals in the core are spherical with a diameter of ~ 10 nm while retaining all biological features of HDL. Such chemically modified iron oxide,¹³² gold,^{132,133} and quantum dot-loaded HDL^{132,134} were used for MRI, CT, and optical imaging, respectively for detection of

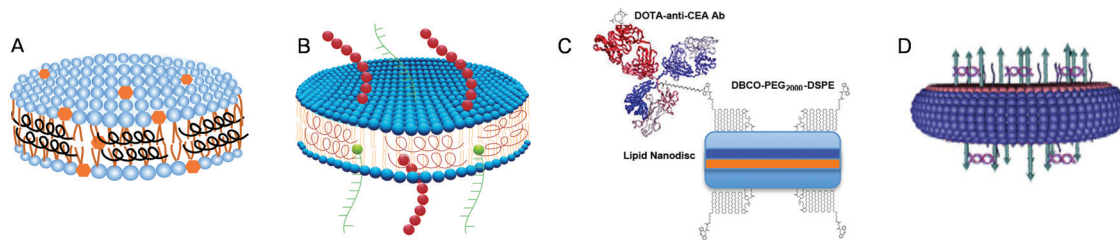


Fig. 7 Examples of different strategies that exploit NDs as carriers for cancer mitigation. (A) Hydrophobic WGA-TA (orange hexagonals) loading in the lipid core (blue dots with orange tails) of the NDs reconstituted by peptide 22A (black coils),¹²⁵ (B) NDs for delivery of cysteine-modified antigen (Ag) peptides (maroon filled circles as chains) and cholesterol-modified immunostimulatory molecules (Cho-CpG, green chains) inserted in LNDs,¹²⁶ (C) delivery of full-length anti-CEA antibody using NDs modified with doping of DSPE-PEG₂₀₀₀-DBCO,¹¹⁶ and (D) delivery of cancer therapeutic genes (siRNA) using NDs technology, where siSTAT3 (purple helix) was complexed with DOTAP (purple lines) and loaded on the NDs doped with DSPE-PEG₂₀₀₀-cRGD (green arrows). The HNDs were prepared from CFL (maroon filled circles) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (blue filled circles); both plane and edge loaded siSTAT3 NDs were synthesized.¹²⁷ Reproduced with the permission from ref. 125. Copyright 2017, Dove Press, ref. 126. Copyright 2017, Nature Publishing Group, ref. 116. Copyright 2020, American Chemical Society, and ref. 127. Copyright 2020, Elsevier Ltd.

atherosclerosis. These nanostructures are desirable platforms for cancer imaging; however, their efficiency for cancer imaging has not been explored yet.

4. NDs for anticancer drug delivery

Cancer is a pathophysiologically heterogeneous disease that needs varied strategies for effective control. The most popular strategies include surgery, radiation therapy, chemotherapy, and immunotherapy.¹³⁵ With an advanced understanding of molecular mechanisms in TME, novel anticancer drugs have been continuously discovered, which include small molecules, oligonucleotides, plasmid DNAs, siRNAs or miRNAs, antibodies, and engineered immune cells capable to control the specific protein or signaling pathways that are aberrantly expressed in the TME.^{1,136}

NDs have been extensively explored for drug delivery to cancer cells by utilizing the benefits of their small size and anisotropic geometry for enhanced cellular internalization. Different strategies such as physisorption, conjugation, or prodrug approaches have been explored to carry a variety of payloads by NDs. In the following sections, we will discuss the use of NDs for the delivery of chemotherapeutics, photosensitizers, chemoimmunotherapeutics, cancer vaccines, and anti-cancer genes for cancer mitigation.

4.1 Delivery of chemotherapeutics

Despite the significant advancements in cancer therapy, cancer remains the second leading cause of death globally.¹³⁷ Chemotherapy for cancer mitigation started after the use of nitrogen mustard during World War II. After this discovery, hundreds of cancer chemotherapeutic drugs have been developed.^{138,139} However, chemotherapy frequently fails in cancer treatments due to poor pharmacokinetics and wide distribution of drugs *in vivo*, insufficient delivery, and multiple drug resistance.¹⁴⁰ Various strategies have been explored to load hydrophobic drugs and hydrophilic peptides in the NDs and successfully deliver these payloads to cancer cells.

Loading a hydrophobic drug into the lipid core of NDs using hydrophobic and electrostatic interactions is a common approach where degradation of the apoA-1 or apoA-1 mimetic peptide by protease results in disassembly of the NDs and subsequent drug release. For example, McConathy *et al.* have developed rHDL particles loaded with PTX that were assembled using apoA-1 with an incorporation efficiency of ~50% of the initial drug load. The PTX loaded rHDL showed improved cytotoxicity against several cancer cell lines (5 to 20 times) when compared to the free drug. More importantly, the PTX loaded rHDL were well tolerated by mice and showed significantly low toxicity compared to free PTX. The higher cellular uptake mechanism of PTX loaded rHDL showed SR-B1 mediated internalization in SR-B1-transfected ldl A7 and in PC3 cells.¹⁴¹ These rHDLs, when functionalized with FA *via* apoA-1 modification, were rerouted to the FR overexpressed cells (OVCAR-3 cells).¹⁴² Similar to the natural HDL development process, the engineered discoidal HDLs become spherical by the action of the lecithin-cholesterol acyltransferase. Such maturation can affect the loaded drugs in the NDs. The effect of lecithin-cholesterol acyltransferase on discoidal PTX loaded HDL, PTX (P-d-rHDLs) was studied by Jia *et al.* and showed that re-modeling of the NDs to a spherical shape, increasing their diameter from ~68 nm to ~83 nm, enhances drug leakage, reduces cellular uptake *in vitro*, and reduces the cytotoxicity of P-d-rHDLs by ~3 times compared to the non-lecithin-cholesterol acyltransferase treated group in MCF-7 cells.¹⁴³

TAT peptide (YGRKKRRQRRR) is capable of traversing the plasma membrane and induces apoptosis in cancer cells.¹⁴⁴ A genetically fused TAT peptide to a truncated apoA-1 protein was developed by Murakami *et al.* resulting in a mutant apoA-1 (Δ apoA-1) that overt its recognition from SR-B1. The mutant Δ apoA-1 and Δ apoA-1-TAT used to form the corresponding DOX-r Δ HDL and DOX-r Δ HDL-TAT by loading DOX into the lipid core. The empty NDs had a mean diameter of ~18 nm for r Δ HDL-TAT and r Δ HDL, whereas DOX loaded DOX-r Δ HDL-TAT had a mean diameter of ~24 nm and DOX-r Δ HDL had a diameter of ~155 nm with a DOX loading of ~10% for both NDs. The DOX delivery efficiency of DOX-r Δ HDL-TAT was

approximately two times higher than that of DOX-rΔHDL in NCI-H460 cells and A549 cells. Also, the DOX-rΔHDL-TAT showed higher anti-tumor activity in the mice tumor model.¹⁴⁵ In a different approach, PEG-stabilized bilayer NDs loaded with DOX (DOX-Disks) were developed by Zhang *et al.* These NDs had a mean diameter of ~80 nm, and high encapsulation efficiency of 96% for DOX, with a pH-sensitive release. The DOX-disks showed long-circulating times in rat blood compared to DOX in solution and showed ~10-fold higher tumor accumulation with lower heart toxicity. The DOX-disks were likely to be internalized in the cancer cells *via* energy-dependent endocytosis processes, like clathrin-mediated, macropinocytosis-mediated, and non-clathrin- and non-caveolae-mediated endocytosis pathways.¹⁴⁶

The apoA-1 mutant, apoA-1_{Milano} (apoA-1_M) was used by Zhang *et al.* to construct 10-hydroxycamptothecin (HCPT) loaded reconstituted HDL (rHDL_M-HCPT) with a drug loading capacity of ~4% (w/w) and a diameter of ~22 nm. HCPT was slowly released from rHDL_M-HCPT and had 70 times improved cytotoxicity compared to the free drug, whereas conventional LIP and rHDL_{wt}-HCPT displayed 27- and 58-times enhanced cytotoxicity, respectively, at an equal dose in SKOV-3 cells. This improved cytotoxicity is likely due to the improved receptor-specific binding of apoA-1_M to the SR-B1 receptors. The improved receptor-specific binding was also observed in organ-specific delivery of HCPT as a significant increase in drug concentration was observed in almost all tissues except the heart and brain when compared to free HCPT treatment.¹⁴⁷

Similarly, HCPT loaded lipid HDL were constructed by Yuan *et al.* using apoA-1 mimetic amphipathic helix peptide 5A (DWLKAFYDKVAEKLKEAF-P-DWAKAAYDKAAEKAKEAA) for drug delivery to SR-B1 overexpressed colon carcinoma. The HDLs had a discoidal shape with a diameter of ~10 nm. The incorporation of HCPT in HDL provided metabolic stability to HCPT and improved 3-fold cytotoxicity in colon HT29 carcinoma cells. The HCPT loaded HDL showed higher serum concentration–time curve (AUC_{0–t}) and C_{max} for HCPT-HDL relative to the free HCPT after intravenous administration in rats.¹⁴⁸

Ghosh *et al.* formulated curcumin loaded NDs with an average diameter of ~50 nm and solubilization efficiency of 70%. The curcumin-loaded NDs were significantly more effective in inducing apoptosis than the free curcumin.¹⁴⁹ Further, a detailed mechanistic study of curcumin loaded NDs showed that the apoptosis induction is a result of enhanced generation of reactive oxygen species along with decreased expression of proteins that include cyclin D1, pAkt, pIkBα, and Bcl₂ and enhanced FoxO3a and p27 expression as well as caspase-9,-3, and poly(ADP-ribose) polymerase cleavage. All these effects led to enhanced G1 arrest in MCL cells.¹⁵⁰

Naturally occurring 4,19,27-triacetyl withalongolide A (WGA-TA) isolated from Solanaceae family of plants is a potent anti-tumor compound. Its low solubility in plasma and short circulation half-life (~1 h) was improved by formulating WGA-TA in sHDL. WGA-TA loaded sHDL (Fig. 7A) were composed the apoA-1 mimetic peptide 22A and had a diameter of 10–12 nm. The WGA-TA loaded-sHDL selectively accumulated

in SR-B1 positive neuroblastoma (NB),¹⁵¹ adrenocortical carcinoma,¹²⁵ and triple-negative breast cancer mice models and produced tumor regression.¹⁵²

Melittin is a large peptide isolated from European bee venom with high potential as an anticancer agent.¹⁵³ Its severe hemolytic effects were addressed by developing melittin-loaded NDs. The flat circular lipid bilayer has a diameter of ~50 nm in diameter, surrounded and stabilized by PEG-lipids on the rim, and functionalized with c(RGDyK) to target overexpressed α_vβ₃ and α_vβ₅ integrins. The NDs protected melittin against trypsin digestion and prevent hemolysis when injected in mice. The NDs provided higher cellular internalization, improved cytotoxicity, and enhanced tumor regression in integrin overexpressed U87 tumor cells.¹⁵⁴

The effect of drug loading on the stability of very-low-density lipoprotein (VLDL), LDL, and HDL was studied by Kader *et al.* by loading 5-fluorouracil (5-FU), 5-iododeoxyuridine (IudR), DOX, and vindesine. The broad molecular weight and large hydrophobic variation among these drugs have a significant effect on drug loading. The relative loading efficiency was vindesine > IudR > DOX > 5-FU among all three classes of lipoproteins. The drug loading did not significantly affect size, morphology, thermal transition temperature *T_m*, or transition enthalpy Δ*H* of the lipoprotein core compared to the native particles. However, the Δ*H* for LDL-DOX and LDL-vindesine complexes were lower compared to the native particles due to drug immiscibility in the LDL core lipids. The drugs loaded in LDL and HDL were more cytotoxic than the free drug for MCF-7 cells, whereas VLDL-drug complexes had the same cytotoxicity as free drugs.¹⁵⁵

In another study, Subramanian *et al.* evaluated the combination of cytotoxic drugs loaded in HDLs to synergize the effect of cholesterol-free HDLs. The cholesterol-free sHDL was formulated using peptide 22A and loading the standard regimen of cisplatin, etoposide, DOX or mitotane used for adrenocortical carcinoma chemotherapy. The cisplatin, etoposide, and mitotane had a synergistic effect with cholesterol-free sHDL, whereas DOX acted as an antagonist in NCI-H295R and SW13 cells. This synergistic effect was also observed in improved clonogenic inhibition, increased adrenocortical carcinoma cells apoptosis, and decreased mitochondria membrane potential compared with monotherapy.¹⁵⁶

4.2 Delivery of photodynamic therapeutics

Photodynamic therapy (PDT) is one of the safest and novel non-invasive treatments for various forms of cancer, including cutaneous T cell lymphoma, colorectal cancer, melanoma, and breast cancer.^{157,158} PDT uses a nontoxic drug that is activated by irradiation, which causes the generation of cytotoxic reactive oxygen species, particularly singlet oxygen (¹O₂) that kills the cancer cells.¹⁵⁹ For high therapeutic benefits and minimal toxic effects, a high accumulation of PDT drug in the cancer cell is required. PDT agents were successfully delivered using the ND platform. For example, Ge *et al.* have developed LNDs loaded with the photodynamic therapy agent hypocrellin B that were constructed using MSP expressed and purified from

E. coli. The hypocrellin B-ND was discoidal in shape with a diameter of ~ 11 nm and loading efficiency of $\sim 40\%$. The lipid environment of the NDs had no detrimental effect on the light absorption and its potential to generate reactive oxygen species. The hypocrellin B-ND displayed enhanced internalized in MCF-7 cells and proved more cytotoxic upon exposure to light compared to cells treated in the dark.¹⁶⁰

4.3 Delivery of chemoimmunotherapeutics

Cancer immunotherapy proved successful in achieving long-term survival in 10–30% of cancer patients; however, immune therapy utilizing immune checkpoint blockers is ineffective in most cases¹⁶¹ because the therapeutic efficacy largely depends on pre-existing anti-tumor T-cells. Thus, most tumors where a low population of tumor-specific T-cells are available did not provide the desired therapeutic output.¹⁶² To improve the abundance of anti-tumor T-cells, immunotherapy is combined with therapeutic vaccines,¹⁶³ radiation therapy,¹⁶⁴ and chemotherapy¹⁶⁵ for strong anti-tumor immunity. Among chemotherapeutics, DOX and PTX can activate anti-tumor T-cell responses through a special form of tumor-cell killing known as immunogenic cell death.¹⁶⁶ Tumor cells undergoing immunogenic cell death up-regulate the expression of calreticulin and high-mobility group box 1, which are “eat me” and “danger” signals, respectively, and produce triggered antigen-specific T-cell responses.¹⁶⁷ Delivery of DOX to stimulate the immune system using a synthetic HDL has proved beneficial (Fig. 8).

The sHDL developed by Kuai *et al.* were composed of the apoA-1 mimetic peptide 37A and DOX was incorporated in the sHDLs by conjugation with 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol through *N*- β -maleimidopropionic acid hydrazide for pH-sensitive release in the TME. The DOX-loaded sHDL (sHDL-DOX) has a loading efficiency of $\sim 2\%$ with a diameter of ~ 10 nm. The sHDL-DOX showed enhanced tumor accumulation and triggered robust expression of danger signals associated with immunogenic cell death within tumors and generated potent anti-tumor T cell responses. The sHDL-DOX treatment broadens the T-cell mediated epitope recognition for tumor-associated antigens (CT26 gp70 (AH1) (H-2L^d-restricted SPSYVYHQF)), neoantigens (Adpgk protein), and the intact whole tumor cells (CT26 tumor cells). The co-treatment of sHDL-DOX with α PD-1 (IgG antibody) induced complete regression of established colon carcinoma in 80–88% mice and provide 100% protection among all survivor mice when re-challenged with tumor cells.¹⁶⁸

In another example, Kadiyala *et al.* loaded DTX in LNDs to treat glioblastoma by the chemoimmunotherapy approach. To enhance the immune response, NDs were loaded with the Toll-like receptor-9 agonist CpG oligodeoxynucleotide through its conjugation with cholesterol. The DTX loaded sHDL NDs (DTX-sHDL) were assembled using 22A peptide and had a diameter of ~ 10 nm and a discoidal shape. The DTX-sHDL-CpG treatment resulted in tumor cell death with concomitant release of the damage-associated molecular pattern molecules calreticulin and high-mobility group box 1 into the TME in glioblastoma bearing mice. Release of CpG from the NDs activates macrophages and dendritic cells resulting in simultaneous

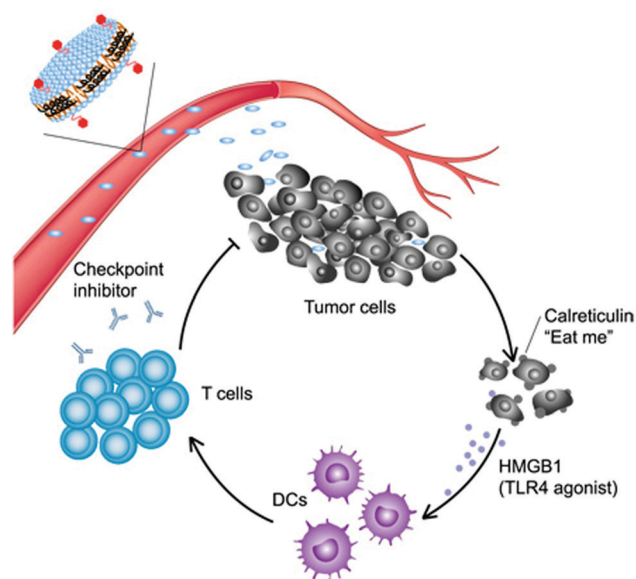


Fig. 8 NDs as nanocarriers for the delivery of chemoimmunotherapeutics. The sHDL enables intratumoral delivery of DOX followed by internalization and pH-responsive release of DOX in the endosomes/lysosomes. Released DOX kills tumor cells and triggers ICD, promoting up-regulation of CRT (the “eat me” signal) and release of danger signals such as HMGB1. DCs recruited to the immunogenically dying tumor cells phagocytose them, process tumor antigens, and cross-prime tumor antigen-specific T cells. Antitumor immunity primed with sHDL-DOX synergizes with immune checkpoint blockade, leading to efficient elimination of established tumors and prevention of tumor relapse.¹⁶⁸ Reproduced with the permission from ref. 168. Copyright 2018, American Association for the Advancement of Science.

uptake and processing of tumor antigens. The activated dendritic cells migrate to the draining lymph nodes, presenting tumor antigens to CD8⁺ T cells resulting in anti-tumor CD8⁺ T-cell-mediated immunity. The therapeutic efficiency of DTX-sHDL-CpG was further enhanced by combination with radiation therapy, leading to tumor elimination from 80% of the glioblastoma bearing animals. More importantly, the immunotherapy developed a long-term immunological memory, providing 100% mice survival after tumor rechallenging without any further treatment.¹⁶⁹

4.4 Delivery of cancer vaccines

Peptide-based cancer vaccines are rapidly gaining popularity owing to their excellent safety profile, ease of manufacturing, and quality control. However, peptide-based vaccines have frustrating weak immunogenicity and generally require co-delivery of immunological adjuvants for potent immune response.¹⁷⁰ For example, delivering peptide-based vaccines to the draining lymph nodes is challenging and subsequently leads to immunological tolerance and cytotoxic T lymphocytes fratricide.¹⁷¹ Recently, Kuai *et al.* developed sHDL to deliver peptide-based cancer antigen mixed with adjuvants and tumor-specific mutant neopeptides. ApoA-1-mimetic peptide 22A was used for lipid solubilization and intracellular release of Ag from the sHDL was controlled by a reduction-sensitive conjugation

(disulfide linkage) between the Ag and sHDL. The sHDL NDs were loaded with Ag peptides, OVA₂₅₇₋₂₆₄ (a model CD8 α^+ T-cell epitope Ag from ovalbumin), and Adpgk (neoantigen in MC-38). The final NDs co-loaded with Ag and CpG (Fig. 7B) had ~ 6.5 Ag peptides and ~ 1 CpG molecule per NDs, with discoidal morphology and diameter of ~ 10 nm. The sHDL markedly promoted the delivery of Ag/CpG to the Delphian lymph nodes and induced CD8 α^+ T-cell responses. Notably, the sHDL-Ag/CpG induced a peak frequency of $\sim 21\%$ Ag-specific CD8 α^+ T cells and Ag-specific cytotoxic T lymphocytes responses after the third vaccination, whereas the mixture of free Ag peptides (SIINFEKL or CSSIINFEKL) and CpG induced 1–3% Ag-specific cytotoxic T lymphocytes. When mice immunized with sHDL-Ag/CpG were challenged with B16OVA cells there was no detectable tumor for up to 28 days and there was no toxicity. In contrast, mice vaccinated with free Ag peptides + CpG or Ag + CpG + the immunoadjuvant Montanide succumbed to tumors with marginal survival benefits. A combination of sHDL-Adpgk/CpG with anti-PD-1 treatment generated robust neoantigen-specific cytotoxic T lymphocytes responses with complete tumor regression in most mice and 100% survival of mice after rechallenging with cancer cells. The strong anti-tumor T-cell responses produced by the NDs in combination with immune checkpoint inhibitors showed the remarkable potential to eliminate tumors in $> 85\%$ of animals¹²⁶ (Fig. 9).

Recombinant proteins and peptide antigen-based vaccines have low immunogenicity and necessitate the administration of

immune-stimulating adjuvants such as toll-like receptor agonists (TLR agonists) to promote the immune response. Recently, Kuai *et al.* developed sHDL loaded with monophosphoryl lipid A (MPLA, a TLR4 agonist), CpG-rich oligonucleotide (CpG, a TLR9 agonist), and the antigen protein ovalbumin, or the E7 antigen peptide that produces strong humoral immune responses in animal models. The adjuvant-loaded NDs had an average diameter of ~ 10 nm and an encapsulation efficiency $> 80\%$ for MPLA and $> 95\%$ for cholesterol-CpG. The NDs co-loaded with dual adjuvants (ND-MPLA/CpG) effectively activated dendritic cells when compared with free dual adjuvants or even NDs containing either MPLA or CpG. The ND-MPLA/CpG admixed with ovalbumin significantly improved antigen-specific CD8 $^+$ T cell responses in B16F10-OVA tumor-bearing mice, inducing regression of established melanoma tumors. Similarly, when TC-1 tumors in mice were treated with ND-MPLA/CpG admixed with E7 antigen peptide, $\sim 20\%$ E7-specific antigen-specific CD8 $^+$ T cells were produced, leading to potent anti-tumor efficacy against established TC-1 tumors.¹⁷²

In general NP-based vaccines are administered *via* the subcutaneous (SC) route, whereas the conventional vaccines by the intramuscular route, presumably due to the “depot” effect for prolonged vaccine delivery. Recently, sHDL based vaccines loaded with CpG and neoantigen Adpgk (sHDL-Adpgk/CpG) along with the immune checkpoint blockers anti-PD-1 and anti-CTLA-4 IgG antibodies, significantly enhanced NP delivery by the SC route to draining lymph nodes. The SC route improved NDs uptake by antigen-presenting cells and generated a 7-fold

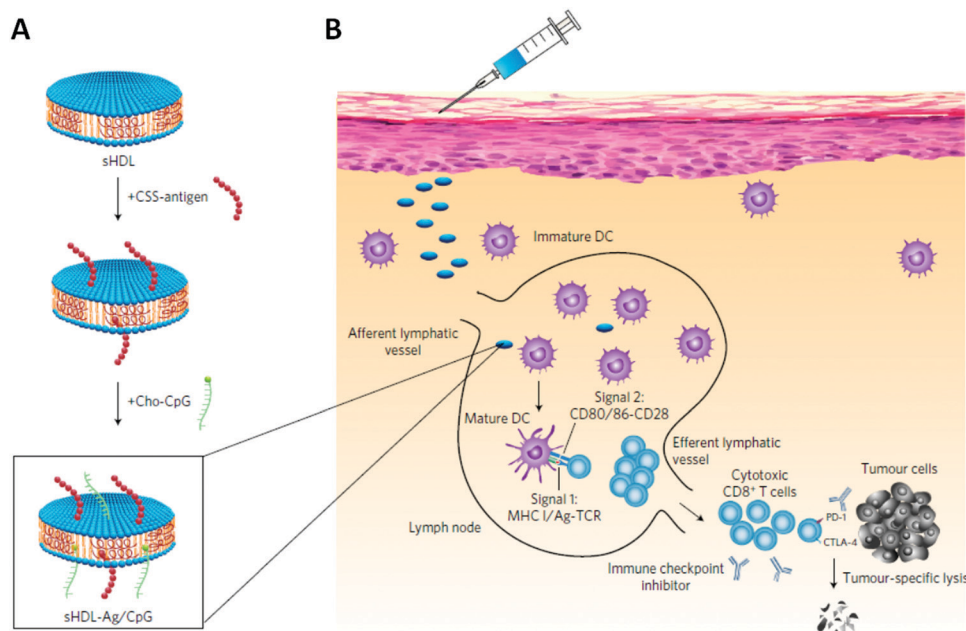


Fig. 9 NDs platform for personalized cancer vaccine delivery. (A) the NDs are composed of phospholipids and ApoA-1 mimetic peptides (22A) and post assembly loaded with cysteine-modified Ag peptides, including tumor-specific mutated neoantigens, and cholesterol-modified immunostimulatory molecules (Cho-CpG) (sHDL-Ag/CpG). (B) Following administration, NDs efficiently co-deliver Ag and CpG to draining lymph nodes, promote strong and durable Ag presentation by dendritic cells (DCs) (Signal 1), and induce DC maturation (Signal 2), eliciting a robust Ag-specific CD8C cytotoxic T-lymphocyte (CTL) responses. Activated CTLs recognize and kill their target cancer cells in peripheral tissues and exert strong anti-tumor efficacy. Combination immunotherapy with immune checkpoint blockade further amplifies the potency of ND vaccination, leading to elimination of established tumors.¹²⁶ Reproduced with the permission from ref. 126. Copyright 2017, Nature Publishing Group.

higher frequency of neoantigen-specific T cells compared with the intramuscular route, confirming the SC route as a more specific way to deliver a peptide vaccine to Delphian lymph nodes for immunotherapy against advanced cancers.¹⁷³

Cancer stem cells (CSCs) exist primarily in an inactive cell cycle and may escape from standard chemotherapy resulting in chemoresistance, tumor relapse, and metastasis.¹⁷⁴ Aldehyde dehydrogenase (ALDH) is a functional biomarker for CSCs and ALDH isoforms A1 and A3 are identified in human CSCs of melanoma and breast cancer patients.¹⁷⁵ Recently, Hassani Najafabadi *et al.* have identified antigenic sequences from ALDH1-A1 and ALDH1-A3 and used them to develop two antigenic peptides, LLYKLADLI from ALDH1-A1, and LLHQLADLV from ALDH1-A3. These antigenic peptides were loaded in LNDs constructed from apoA-1 mimetic peptides to activate APCs for T cell responses against ALDH^{high} CSCs. The LNDs loaded with cholesterol-CpG and antigen peptides form ALDH-A1-CpG-ND and ALDH-A3-CpG-ND and have particle diameters of 9–13 nm. The SC injection of NDs at the tail base of mice increased antigen trafficking to lymph nodes and generated robust ALDH-specific T cell responses in D5 melanoma and 4T1 cell mammary carcinoma mouse models. When NDs were combined with anti-PD-L1 (IgG), prolonged survival in both animal models indicates amplified immune response from the antigenic peptides and reduced the frequency of ALDH^{high} CSCs in tumor tissues, leading to strong anti-tumor effects against both tumors.¹⁷⁶

In another example, Kuai *et al.* reported that NDs prepared with the apoA-1 mimetic peptide 22A loaded with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[3-(2-pyridyldithio)] propionate modified antigen E7 peptide (GQAEPDRAHYNIVTFCKCD) and cholesterol-CpG induced a high E7 specific CD8⁺ T cell response (~32%). In TC-1 models of HPV-associated lung metastasis of head/neck and cervical cancer, the SC delivery of these NDs vaccines generated superior T cell responses resulting in the elimination of established TC-1 tumors. Another peptide, Gp33 (CSSKAVYNFATM), when loaded in NDs had a comparable T cell response and tumor regression rate with the Listeria-based live vector vaccine.³¹

Recently, Scheetz *et al.* developed three neoantigen peptides, namely AALLNKYLA (NeoAg1, H2-D^b-restricted), MSLQFMTL (NeoAg2, H2-K^b-restricted), and GAIFNGFTL (NeoAg3, H2-D^b-restricted) for immunotherapy and tested them in mice glioma models. All three neoantigen peptides and cholesterol-CpG can be loaded onto apoA-1 mimetic peptide-based sHDL and had a diameter of ~12 nm. The cocktail NeoAgs-CpG-NDs vaccination-induced robust expansion of IFN γ leading to expression of neoantigen-specific CD8 α ⁺ T cells. When the peptide ND treatment was combined with anti-PD-L1 (IgG) a robust induction in the maturation of intratumoral dendritic cells was observed, followed by intratumoral infiltration of CD8 α ⁺ T cells and CD107 α effector phenotype into the TME, leading to improved survival and protective immunity against tumor relapse. Importantly, the animals that survived from a combination treatment of NDs and the anti-PD-L1 group remained tumor-free without any treatment when rechallenged with GL261 cells. The sHDL

loaded with peptide neoantigen epitopes (mIDH1_{123–132} and mIDH1_{126–141}) in the genetically engineered murine mIDH1 glioma model significantly extended animal survival and provided long-term immunity against mIDH1 tumors.¹⁷⁷

4.5 Delivery of anticancer genes

Gene therapy is one of the effective therapeutic approaches for cancer treatment.¹⁷⁸ Cancer development initiates alteration of the oncogene, tumor suppressor gene, and other genes. Gene silencing reduces the expression of a specific gene in organisms being the promoter of tumor growth. RNA interference (RNAi) is the most commonly used technique for gene silencing.¹⁷⁹ The RNAi (*e.g.*, small interfering RNA [siRNA]) effectively silent genes that are difficult to target with conventional approaches such as antibodies or small molecule inhibitors.¹⁸⁰ However, naked DNA or RNA are easily cleared by the phagocytes or nucleases and their cellular uptake is limited, and thus requires specific delivery vectors to reach cancer cells.¹⁸¹ A variety of vectors such as non-viral lipids or protein carriers, including cholesterol, LIP, antibody protomer fusions, cyclodextrin NP, fusogenic peptides, aptamers, biodegradable polylactide copolymers, and polymers^{182,183} had partial success with limitations such as toxicity, instability, and non-targeted delivery. Cationic NDs provide a suitable platform for gene delivery and can be used to load the genes on the corona. The electrostatic interaction of cationic NDs and anionic genes neutralizes the charge from the NDs and allows intracellular transportation. Another potential approach is to chemically modify the RNA with a lipophilic anchor to make it suitable for loading on the NDs.

One commonly activated gene in many tumors is the signal transducer and activator of transcription 3 (STAT3) that mediates key processes involved in malignant transformation and progression.¹⁸⁴ STAT3 silencing using small molecule inhibitors or non-specific delivery methods results in severe adverse effects.¹⁸⁵ The siRNA for STAT3 and focal adhesion kinase were loaded on rHDL by Shahzad *et al.* to treat ovarian and colorectal cancer, respectively. The rHDL is composed of apoA-1 and efficiently incorporates (>90%) of siRNA onto rHDL, which was pretreated with oligolysine peptides (~40 lysine residues) to neutralize the anionic charge for stabilization. The siRNA-loaded NDs had a diameter of ~10 nm. The rHDL had a robust payload carrying capacity (up to 4 mg of siRNA/ml) with high stability and no siRNA leakage for up to 2 weeks. The STAT3 and focal adhesion kinase siRNA-loaded rHDLs produce a significant gene silencing and had a synergistic effect with DTX or oxaliplatin to reduce the tumor burden in both orthotopic mouse models. Combination treatment with STAT3 siRNA/rHDL and DTX had a 30-fold increase in tumor cell apoptosis in TME when compared with the control group, suggesting highly efficient delivery of siRNA to the target tissue.¹⁸⁰

In another study, Chen *et al.* developed STAT3 siRNA loaded on two different HNDs bearing cyclic RGD peptide (cRGD) to target $\alpha_v\beta_3$ integrin receptors. The cRGD was attached to the HNDs either at the edge or to both planes to produce E-cRGD-NDs or P-cRGD-NDs, respectively (Fig. 4D). These HNDs had

high stability and rigid structure due to *in situ* polymerization of organosiloxane to form a sol-gel coating on the surface of the NDs. The empty HND has a particle diameter of ~ 50 nm, whereas the siRNA loaded HNDs have a slightly larger size. The ligand anisotropy endowed the HNDs show diversified cellular interactions resulting in different efficacies for E-cRGD-NDs and P-cRGD-NDs. The edge modification of cRGD efficiently separated the targeting domain and siRNA loading field, thus establishing the functional anisotropy of NDs. This segregation resulted in the collaborative superiority in siRNA loading, cellular uptake, gene silencing efficiency, and protein expression when compared to P-cRGD-NDs and Lipofectamine 3000. This superiority of E-cRGD-NDs was further enhanced by co-administration of PTX, which showed the most significant tumor inhibition and resulted in almost complete tumor suppression, suggesting the potential benefits of anisotropic E-cRGD-NDs as a delivery platform for combined delivery of gene and chemotherapeutic agent.¹²⁷

In another example, Ghosh *et al.* reconstituted NDs using apoA-1 (particle diameter 20–50 nm) loaded with antisense siRNA for GAPDH after complexing dsOligo with the cationic lipid 1,2-dimyristoyl-3-trimethylammoniumpropane at a 1 to 1 charge ratio. The dsOligo complexed NDs induced $\sim 60\%$ knockdown of the GAPDH gene in HepG2 cells, a value comparable to that of Lipofectamine.¹⁸⁶

Besides these examples, some spherical HDL were also utilized as a vehicle for siRNA transport using surface-modified gold NP¹⁸⁷ or calcium phosphate¹⁸⁸ for siRNA loading in the HDL. In addition, conjugation of siRNA with cholesterol, bile acids, or long-chain fatty acids provides lipophilic siRNA that can be loaded in the rHDLs for high stability and higher cellular uptake.¹⁸⁹ The cholesterol-conjugated siRNA loaded in rHDLs were successfully utilized for Pokemon gene silencing in hepatocellular carcinoma.¹⁹⁰

5. HNDs for anticancer drug delivery

HNDs or hybrid bicelles (Fig. 2D) are a relatively new class of NDs. In contrast to conventional LNDs, HNDs are structurally stable and expect to retain their discoidal structure in long term *in vivo*, at elevated temperatures, or in the presence of other amphiphiles. The organic-inorganic hybrid bicelles are assembled using long-chain alkoxysilane lipids and short-chain phospholipids. Their high stability is achieved by the formation of a crosslinked siloxane network on the surface of HNDs *via* a sol-gel reaction of the organoalkoxysilane lipids. The resultant HNDs consist of a silicate surface encompassing a lipid bilayer, with typical diameters in the 20–50 nm range. HNDs are morphologically stable even after drying in the air or in the presence of an excessive nonionic surfactant.⁹⁰ The successful sol-gel reaction is often confirmed by Fourier-transformed infrared spectroscopy, which shows a peak at 1100 cm^{-1} corresponding to the asymmetric stretching vibration of the siloxane bond (Si–O–Si), whereas morphology and size is confirmed by transmission cry-electron microscopy, small-angle X-ray scattering, dynamic light scattering and/or atomic force microscopy.

The HNDs can be easily modified with suitable ligands to target specific cancer receptors. Target specificity may be introduced by modifying either short alkyl chains that form the edges of HNDs (*i.e.*, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC)) or long alkyl chains that form both planes of HNDs (*i.e.*, CFL). For example, the octa arginine sequence of cell-penetrating peptides can be introduced to modify HNDs and enhance their penetration into cancer cells.²⁷ The lipid core of the HNDs is suitable for loading hydrophobic drugs such as DOX for cancer drug delivery.^{84,191} The partial silica coating on the HNDs improves their stability to a great extent while compromising the bilayer fluidity, which may retard drug release. However, drug release may be improved by doping lipid-modified PEG such as DSPE-PEG₂₀₀₀. Such modification improves drug release and enhances the therapeutic efficacy of the encapsulated drug.⁸⁴ Like targeting ligands, drugs may also be suitably modified to load into the lipid core or conjugate with short or long-chain lipid components.

In general, HNDs maintain their discoidal morphology above the phase transition temperature of long-chain lipids.¹⁹² The siloxane bonds can be only degraded at very high temperatures¹⁹³ or at extreme chemical conditions (pH 2–4 and 9–12),¹⁹⁴ which present a challenge regarding their biodegradability. However, siloxane polymers are known for their low toxicity, good blood compatibility, and physiological inertness.¹⁹⁵ The uses of HNDs for the delivery of chemotherapeutics for cancer treatment have been explored in recent years.

Lin *et al.* fabricated HNDs from CFL and DHPC and loaded them with DOX, resulting in nanostructures with a diameter of ~ 60 nm and a thickness of ~ 6 nm. These HNDs had a DOX loading efficiency of $\sim 2\%$ without affecting particle size and showed extended stability on long-term storage or in the presence of nonionic detergent. The HNDs displayed high cellular uptake *via* endocytosis related to clathrin and micropinocytosis and showed pH-dependent DOX release. The pH sensitivity is most likely due to the protonation of the DHPC at low pH and subsequent disruption of the nanostructure.¹⁹¹

The silica coating on the lipid NDs may retard the encapsulated drug release, whereas incorporating a permeability enhancer in the lipid bilayer may increase membrane fluidity and enhance drug release. To study such an effect, Lin *et al.* assembled HNDs with different concentrations of PEG doped in the lipid bilayer and monitored DOX release. Among various combinations, HNDs prepared by 5% PEG doping proved best and had high DOX loading of $\sim 2.4\%$ (DOX@HNDs) with a particle diameter of ~ 50 nm and discoidal morphology. The DOX in HNDs exhibited higher cellular uptake and therapeutic efficacies than free DOX in mice model.⁸⁴

Along with the shape of NPs, target biological cells have a crucial impact on cellular behavior for the bio-nano interactions. The effect of shape anisotropy, functionalization anisotropy, and phagocytic/endocytic nature of cells was screened by Wang *et al.*, who compared hybrid nanospheres, HNDs, as well as HNDs with edge modification and plane modification. The HNDs prepared from CFL and a DHPC were decorated with the octaarginine sequence of cell-penetrating peptides after

conjugating with short alkyl chain (C8-R8) and long alkyl chain (C18-R8), respectively, for edge or plane modification. The HNDs had a diameter of ~ 50 nm and a thickness of ~ 5 nm. The shape anisotropy significantly influenced the cellular internalization and followed a regular rule: strong phagocytic cells were more sensitive to the change in ligand location but relatively insensitive to the alteration in shape, whereas the weak-phagocytic cells were the opposite. The shape anisotropy effect is most likely because plane modified HNDs might firmly adhere to the cell surface *via* its larger contact area and up to 50% of active R8, which impeded the biomembrane motion, resulting in decreased membrane fluidity. In contrast, edge-modified HNDs might contact the cell membrane on its R8 modified edge, and such a small contact area might lead to less restriction to the cell membrane fluidity.²⁷

6. PNDs as a versatile new ND platform for cancer therapy

The most recently discovered PNDs are potentially another powerful ND platform for cancer diagnostic and treatment (Fig. 2C). PNDs support membrane proteins as LNDs but with much improved stability. Unlike LNDs that tend to aggregate in 1–2 days even when stored at 4 °C and in a few hours at elevated temperatures, PNDs are largely stable at 4 °C, room temperature, or 37 °C for at least one week that was tested.³⁶ PNDs differ from LNDs in that the lipid bilayer of LNDs is replaced by

a patch of amphiphilic block copolymer membrane, which in turn is encased and stabilized by the same choices of membrane scaffold macromolecules as used in LNDs.³⁶ Those amphiphilic block copolymers by themselves self-assemble in aqueous solution into polymersomes, the synthetic analogues of lipid vesicles.^{196,197} The amphiphilic block copolymers (di-block, AB; or triblock, ABA or ABC type polymers) contain adjacent blocks with different compositions, solubility, and sequence distributions. Depending on the hydrophobic block sizes, polymersomes can be several folds thicker compared to LIP, enabling mechanical and chemical stability and decreasing the premature release of encapsulated payloads.¹⁹⁸ Polymersomes offer benefits due to the customizable and flexible design of copolymers, enabling improved control over properties such as size, surface charge, functionalization, and architecture, along with increased complexity in design, such as stimuli responsiveness.^{199,200}

The polymersome-forming characteristic of amphiphilic block copolymers is the prerequisite for their self-assembly with membrane scaffold macromolecules into PNDs. The morphology of self-assembled amphiphilic block polymers depends on the packing of copolymer chains, which can be determined based on the 'packing parameter' p .²⁰¹ In practice though, it is difficult to calculate p based on the structure of the amphiphilic block polymers. Alternatively, block copolymers can be characterized by a synthetically accessible hydrophilic block fraction ($f_{\text{hydrophilic}}$). As a simple rule of thumb, a $f_{\text{hydrophilic}}$ of approximately $35 \pm 10\%$ of an amphiphilic diblock copolymer yields

Table 2 Examples of polymersome-forming amphiphilic block polymers used for cancer drug delivery

No.	Polymer	Hydrophilic block	Hydrophobic block	Molecular wt (kD)	$f_{\text{hydrophilic}}$	Ref.
Diblock polymers, AB						
1	PEO ₄₀ -PEE ₃₇	PEO	PEE	3.9	0.39	204
2	PEO ₂₆ -PBD ₄₆	PEO	PBD	3.6	0.28	204
3	PEG ₄₅ -PBOx ₄₈	PEG	PBOx	10	—	205
4	PIAT ₅₀ -PS ₄₀	PIAT	PS	—	—	206
5	PEO ₄₆ -PCL ₂₄	PEO	PCL	4.7	0.42	207
6	PEO ₄₃ -PLA ₄₄	PEO	PLA	6	0.33	207
7	PEO ₈₀ -PBD ₁₂₅	PEO	PBD	10.4	0.29	207
8	PEG ₄₅ -PCL ₂₉ ; DEX ₂₂ -PCL ₆₆	PEG; DEX	PCL	DEX-PCL = 17.8; PEG-PCL = 5.3	DEX-PCL = 0.32; PEG-PCL = 0.37	208
9	PEG ₁₁₄ -PLGA ₃₈	PEG	PLGA	10	—	209
10	PAA ₁₆ -ONB-PMCL ₇₆	PAA	PMCL	11.3	0.11	210
11	PTMC ₂₆ -b-PGA ₂₀	PGA	PTMC	5.2	—	211
12	PEG ₁₁₄ -P(TMC- <i>co</i> -DTC ₂₉)	PEG	P(TMC- <i>co</i> -DTC)	24.2	—	212
13	PEG ₁₇ -PPS ₃₀	PEG	PSS	2.7	0.28	213
14	PMPC ₂₅ -PDPA ₁₂₀	PMPC	PDPA	55.0	—	214
15	PEO ₄₃ -P(DEA ₉₄ -CMA ₅)	PEO	P(DEA-CMA)	17.7	—	215
16	PGMA ₅₈ -PHPMA ₂₅₀	PGMA	PHPMA	58.9	—	216
17	PEO ₄₅ -PTTAMA ₂₅	PEO	PTTAMA	13.6	—	217
18	PEG45-P(Asp)100; PEG45-P(Asp-AE)100	PEG	PAsp; P(Asp-AE)	—	—	218
Triblock polymers, ABA and ABC						
1	PMOXA ₂₅ -PDMS ₇₅ -PMOXA ₂₅	PMOXA	PDMS	9.8	—	219
2	PLA ₁₁₅ -F127-PLA ₁₁₅	F127	PLA	29	—	220
3	PEO ₄₅ -PLA ₈₅ -PAA ₁₁₀	PEO and PAA	PLA	19.5	—	221
4	PEG ₁₁₄ -PCL ₁₆₀ -PDEA ₂₄	PEG and PDEA	PCL	27.3	—	222
5	P(EO ₁₉₆ - <i>co</i> -AGE ₉)- <i>g</i> -PCL ₂₃₇	P(EO- <i>co</i> -AGE)	PCL	13.8	0.27	223
6	PEG ₁₁₄ -P(CL- <i>co</i> -LA) ₅₉ -PEG ₄₅	PEG	CL- <i>co</i> -LA	50.5	0.38	224
7	P(LA ₁₂₃ - <i>co</i> -DAC _{3.5})- <i>g</i> -PEG ₁₁₄	PEG	P(LA- <i>co</i> -DAC)	15	0.33	225
8	PEG ₁₁₃ -PAA ₂₀ -PNIPAM ₂₁₁	PEG and PAA	PNIPAM	26.44	—	226

polymersomes;^{202,203} Other nanostructure morphologies such as micelles and worm-like micelles are obtained at $f_{\text{hydrophilic}} > 0.50$, while inverse micelles or solid-like particles are observed at $f_{\text{hydrophilic}} < 0.20$.¹⁹⁸ This simple rule may not apply to amphiphilic triblock copolymers though. An incomplete list of reported amphiphilic block copolymers used to construct polymersomes for cancer drug delivery is presented in Table 2. All of those block copolymers are potential candidates for the assembly of PNDs.

Like LNDs and HNDs, PNDs can be modified for drug delivery to cancer cells by similar synthetic strategies. PNDs offer distinct advantages over LNDs and HNDs, such as improved stability, facile conjugation chemistry, and biodegradability. PNDs may be fine-tuned by carefully selecting polymer blocks with desired functionality for degradation in stimuli-responsive manners such as elevated pH, TME redox potential, or sensitivity to specific enzymes, as that explored in the design of polymersome-based drug delivery systems.^{227–230} Similarly, the thermal stability and biodegradability of the PNDs may also be tailor-made through permutation and combination from a large array of synthetic block polymers.^{231–234} PNDs may accommodate lipophilic drugs in the hydrophobic core or conjugated to the copolymer backbone through chemo-responsive linkages. The mechanism of chemo-responsiveness and drug release relies on the disassembly or swelling of nanocarriers in response to the actions of organic molecules or enzymes in the TME.^{235–237} This phenomenon can be applied to PNDs for controlled delivery of drugs triggered by a disease-related abnormal level of chemicals in the TME, such as acidic pH, hypoxic microenvironment, elevated levels of reactive oxygen species, and essential enzymes (*i.e.* MMP-9, MMP-2, cathepsin B, FAP- α , legumain, *etc.*).^{237,238}

7. Future directions and outlook

The lipid-based NDs including LNDs and SMALPs showed continuous aggregation during storage even at low temperature, which jeopardizes the reliability and efficacy of ND-based drug formulations. The clinical translation of MSP or apoA-1 mimetic peptides based NDs is also partially limited by the high production cost of large quantities of pure apoA-1 proteins either recombinantly or by plasma-purification.¹⁴⁸ In addition, the use of MSP and apoA-1 to encase NDs raises potential safety concerns due to their human protein origin,²³⁹ and post-translational modifications of apoA-1 that occur in the context of systemic inflammation (oxidative damage, glycation or carbamylation) may transform anti-inflammatory apoA-1 into a pro-inflammatory protein. Humoral autoimmunity to apoA-1 and HDL indicative of modulated inflammatory and immune responses was indeed observed in populations of high cardiovascular risk.²⁴⁰

Significant developments in the constitutional elements of NDs have taken place in recent years, expanding the horizon to exploit NDs for cancer mitigation. For example, to circumvent the often fluidic and labile nature of LNDs, HNDs were developed and showed remarkable stability even at elevated

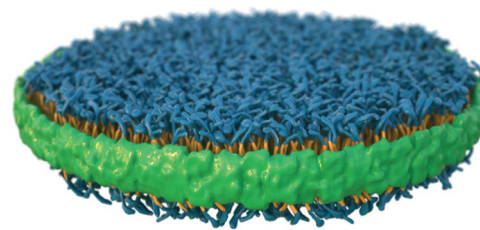


Fig. 10 Proposed structure of fully synthetic PNDs. A nanoscale membrane patch comprised of amphiphilic block copolymers (blue: hydrophilic block; gold: hydrophobic block) is encased and stabilized by amphipathic belt-like polymers (green).

temperatures and/or under drying conditions. On the downside, the larger particle sizes of HNDs, their limited choices of constitutional components, difficult body clearance, and potential organ accumulation may limit their clinical applications. Another very promising development for anticancer drug delivery is PNDs, *i.e.*, the lipid bilayer of the NDs is replaced with an amphiphilic block copolymer membrane in the formation of MSP-encased PNDs.³⁶ We expect that the MSPs derived from apoA-1, the essential constitutional element of the NDs, may be also replaced with fully synthetic small peptides^{111,123,126,148,168} or synthetic amphipathic random copolymers such as SMAs^{33–37} and many of the SMA-like alternatives.^{85,241} The enhanced buffer stability of zwitterionic SMAs and other SMA-like copolymers in the presence of divalent cations or under low pH environment expands their utility to support NDs for pharmaceutical applications.^{35,85,86,241,242} With the exciting potential of NDs for cancer therapy as well as some of their outstanding challenges in mind, we envision that the flexibility of the self-assembly process that produces NDs may open up a new avenue to realize fully synthetic PNDs (Fig. 10).

The fully synthetic PNDs will consist of suitable amphiphilic block copolymers that form the hydrophobic membrane patch, and amphiphilic SMA-like random copolymers that act as the membrane scaffold to encase and stabilize the NDs. By taking advantage of the expertise gained during the last two decades on the molecular engineering of polymer-based drug delivery systems,^{243–247} this new type of fully synthetic PNDs can be designed to have the long shelf life needed for industry-scale drug formulations, and the versatility to deliver a wide range of anti-cancer agents to tumor sites with high specificity, efficiency, serum stability, low toxicity, and excellent biodegradability.

8. Conclusion

The clinical translation of nanomedicines is challenging due to various limiting factors such as controlled and reproducible synthesis at the industrial scale, stability of drug formulations before and after drug administration, inconsistent toxicity, and differences in efficacy between benchtop tests and clinical trials, just to name a few. Many of the challenges come from poorly understood *in vivo* responses to nanomedicines. For example, NPs properties such as size, shape, and targeting ligands can be

significantly altered from the original designs once in the bloodstream.⁷ The interaction of nanomedicines with plasma proteins in the bloodstream forms a 'corona' around the NPs that redefines their pharmacokinetics and targeting efficiency. Although second-generation nanomedicines are actively pursued for cancer diagnostic and treatment, many of the conventional spherical NP-based formulations proved inefficient in clinical trials. For example, it has been found that the complex biological barriers result in suboptimal therapeutic benefits, as <1% (median) of the NPs generally reach the tumor sites.²⁴⁸

NDs, including LNDs, PNDs, and HNDs, have emerged as effective tools for delivering diagnostic and chemotherapeutic agents to cancer cells. We discussed many examples where NDs effectively delivered diagnostic agents, including agents for fluorescent imaging, MRI, CT, and PET, along with chemotherapeutic agents, peptide-based cancer vaccines, and therapeutic genes (siRNA). Notably, cellular internalization of NDs is not limited to the EPR effect since they are taken up following the binding to their natural receptors (SR-B1) or receptors of choice by adding specific receptor-ligand on the NDs. This is an important property of NDs, as it was shown recently that the EPR effect by itself is not sufficient to account for the observed number of NPs in a cancer cell.²⁴⁹

Looking forward, we believe that the field of adapting NDs for drug delivery in general and cancer mitigation in particular, has great potential to grow. Developing fully synthetic PNDs as nanocarriers for the diagnosis and treatment of cancers is a new frontier waiting to be explored.

Abbreviations

PEO-PEE	poly(ethyleneoxide)-poly(ethylethylene)
PEO-PBD	poly(ethyleneoxide)-poly(butadiene)
PBOx	poly(styreneboroxole)
PEG	poly(ethylene glycol)
PMOXA-PDMS-	
PMOXA	poly[(2-methyloxazoline)-poly(dimethylsiloxane)-poly(2-methyloxazoline)]
PS-PIAT	poly[styrene- <i>b</i> -poly(1-isocyanoalanine(2-thiophen-3-yl-ethyl) amide)]
PLA-F127-PLA	poly(lactic acid)- <i>b</i> -Pluronic F127- <i>b</i> -poly(lactic acid)
PEO- <i>b</i> -PCL- <i>b</i> -PAA	poly(ethylene oxide)- <i>b</i> -poly(caprolactone)- <i>b</i> -poly(acrylic acid)
PEO- <i>b</i> -PCL	poly(ethylene oxide)- <i>b</i> -poly(ϵ -caprolactone)
PEG-PCL-PDEA	poly(ethylene glycol)- <i>b</i> -poly(ϵ -caprolactone)- <i>b</i> -poly(2-(diethylamino) ethyl methacrylate)
DEX-PCL	dextran- <i>b</i> -poly(ϵ -caprolactone)
PEAG	poly(ethylene oxide- <i>co</i> -allyl glycidyl ether)
PEG- <i>b</i> -PLGA	poly(ethylene glycol)- <i>b</i> -poly(L-lactic- <i>co</i> -glycolic acid)
mPEG-P(CL- <i>co</i> -LA)-PEG	methyated poly(ethylene glycol)- <i>b</i> -poly(caprolactone- <i>co</i> -lactide)- <i>b</i> -poly(ethylene glycol)

P(LA- <i>co</i> -DAC)- <i>g</i> -PEG	poly(lactide- <i>co</i> -diazidomethyl trimethylene carbonate)- <i>g</i> -poly(ethylene glycol)
PAA-ONB-PMCL	poly(acrylic acid)-ONB-poly(methyl caprolactone)
PTMC- <i>b</i> -PGA	poly(trimethylene carbonate)- <i>b</i> -poly(L-glutamic acid)
PEG-P(TMC-DTC)	poly(ethylene glycol)- <i>b</i> -poly(trimethylene carbonate- <i>co</i> -dithiolane trimethylene carbonate)
PEG-PPS	poly(ethylene glycol)- <i>b</i> -poly(propylene sulfide)
PMPC-PDPA	poly(2-(methacryloyloxy)ethyl phosphorylcholine)- <i>b</i> -poly(2-(diisopropylamino)ethyl methacrylate)
PGMA-PHPMA	poly(glycerol monomethacrylate)- <i>b</i> -poly(2-hydroxypropyl methacrylate)
PEO- <i>b</i> -PTTAMA	poly(ethylene oxide)- <i>b</i> -poly(2-(((5-methyl-2-(2,4,6-trimethoxyphenyl)-1,3-dioxan-5-yl)-methoxy)carbonyl)amino)ethyl methacrylate)
PEG-P(Asp)	poly(ethylene glycol)- <i>b</i> -poly(aspartic acid)
PEG-P(Asp-AE)	poly(ethylene glycol)- <i>b</i> -poly([2-amino-ethyl]-aspartamide)
PEG-PAA-PNIPAM	poly(ethylene oxide)- <i>b</i> -poly(acrylic acid)- <i>b</i> -poly(<i>N</i> -isopropylacrylamide)

Author contributions

The manuscript is written through contributions from all authors. All authors approved the final manuscript.

Conflicts of interest

The authors declare no competing interests.

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