Recent applications of cell-penetrating peptide guidance of nanosystems in breast and prostate cancer (Review)

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Abstract. Cell-penetrating peptides (CPPs) are small peptides from natural sources or designed from other protein sequences that can penetrate cell membranes. This property has been used in biomedicine to add them to biomolecules to improve their capacity for cell internalization and as a guidance tool for specific cell types. CPPs have been shown to enhance cellular uptake *in vitro* and *in vivo*, improving the efficacy of anticancer drugs such as doxorubicin and paclitaxel, while also limiting their cytotoxic effects on healthy cells and tissues. The current study reviews the internalization and major therapeutic results achieved from the functionalization of nanosystems with CPPs for guidance into breast and prostate cancer cells *in vitro* and *in vivo*. In addition, the practical results obtained are specifically discussed for use as a starting point for scientists looking to begin research in this field.

Contents

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1. Introduction

Cancer is a common cause of death worldwide; its two essential characteristics are the loss of cell growth control, which leads to tumor formation, and the invasion of other tissues through metastasis. In addition, dissemination through blood or lymphatic vessels leads to secondary tumors. It has been estimated that ~18.1 million new cases and 9.6 million deaths were related to this disease in 2018. In women, the second most common type of cancer is breast cancer (BC), with 11.6% of cases, and in men, prostate cancer (PC) is the second most common type of cancer, with 7.1% of cases (1).

Hanahan and Weinberg described six major hallmarks of cancer progression. These are sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death (2). In recent years, most of the knowledge attained has been in elucidating cancer biology. Generally speaking, a single gene mutation seldom causes cancer. However, cancer can occur when a mutation falls in a key gene. These key genes can be categorized into three main groups: i) Proto-oncogenes; ii) tumor suppressor genes; and iii) DNA repair genes (3). Advances in cancer biology have identified possible molecular targets in BC and PC.

BC is a highly heterogeneous disease with complex classification subtypes such as Luminal A, Luminal B, HER2-positive and triple-negative. Most BC cases are invasive ductal carcinoma; however, inflammatory BC is also of concern due to its aggressiveness and occurrence in different patient populations (4). Similarly, PC is classified into three categories regarding its biology: i) Endocrine-driven; ii) microenvironment-dependent; and iii) tumor cell-autonomous (5). Several PC studies have demonstrated the role of the androgen receptor (AR) in its development and progression (6-10). The AR is located in the cytoplasm and, when bound with its ligand, translocates into the cell nucleus recognizing hormone response elements in regulatory genomic regions (11).

2. Standard cancer therapies vs. alternative treatments

The objective of cancer therapy is to keep the patient free of disease or in remission with a partial or complete decrease of

symptoms. The most common cancer therapies are surgery, radiotherapy and chemotherapy. In addition to being local treatments, they possess several disadvantages and limitations. For example, surgery requires diagnosis at an early stage of the disease, when metastasis development is less frequent; however, overdiagnosis has increased the number of mastectomies and prostatectomies performed each year (12).

Radiotherapy is applied to 30-40% of solid tumors, alone or combined with other treatments. However, it causes genetic damage to tumor cells and surrounding healthy tissues, with severe side effects. Radiation exposure also causes inflammation and, consequently, oxidative damage. High doses cause chronic damage that interferes with the healing ability of the tissue (12,13). Chemotherapy generally lacks specificity, leading to cytotoxic damage throughout the body and the development of resistance (14,15). Chemotherapy and radiotherapy have been shown to cause DNA double-strand breaks, increasing the production of reactive oxygen species (ROS) and a general stress response. This damage leads to several possible outcomes besides apoptosis, such as cell cycle arrest, senescence, mitotic catastrophe, inflammatory response and fibrosis at the tissue level (16).

Targeted therapies are directed at a biochemical pathway or target molecule required for tumor survival (15). Given the limitations of conventional treatments, the term precision medicine has emerged, in which therapy is directed according to the characteristics of the cancer to be treated. This therapy includes antibodies, drugs, nucleic acids and other therapeutic molecules. However, targeted therapies could be improved using nanosystems (NSs) as carriers whose surface can be modified to include the therapeutic principle and other components to protect the NS and facilitate target cell recognition and entry.

Nanomedicine development involves several types of nanoplatforms such as metallic and polymeric nanoparticles (NPs), nanoliposomes and nanomicelles, among others (17). Additionally, stimuli-responsive drug release systems increase cytotoxicity against cancer cells (18-20). Some of these nanoplatforms, along with the most common stimuli-responsive drug release systems, are shown in Fig. 1.

3. Strategies for site-directed cancer therapy using NSs

One highly advantageous characteristic of NSs is their ability to be functionalized in several ways for specific guidance to cancer cells and tumors. Several guidance tools are used, such as antibodies and aptamers. Some of these strategies can be seen in Fig. 2.

Other properties rely on the nature of the NP. Polymersomes, also called polymeric vesicles, have specific features varying by polymer composition. Generally, they have high stability, adaptable physicochemical properties, an easily-modified surface and a highly adjustable permeability membrane (21,22). Nanomicelles are commonly self-assembled; they can carry hydrophilic drugs, protecting them from unwanted interactions (23,24).

Similarly, nanoliposomes possess a specific characteristic of carrying both hydrophobic and hydrophilic drugs due to their double lipid membrane (25,26). Metallic NPs can serve as theranostics by carrying anticancer drugs and taking advantage of surface plasmon resonance for photothermal therapy and imaging (27,28). On another approach, nanoliposomes were used to develop a synthetic vaccine particle carryng rapamycin (SVP-rapamycin), with the objective of inducing a tolerogenic immune profile to NPs (29).

The present review discusses one of the most common guidance tools used, known as cell-penetrating peptides (CPPs). CPPs are small peptides, usually 5 to 30 amino acids in length, that are designed from other protein sequences, have a short life and can penetrate cell membranes in a non-selective way in most cases (30). However, as will be discussed further, some cell-specific CPPs have been studied and developed. Furthermore, the definition of CPPs has been changing throughout the years since their discovery >20 years ago. One definition that globally encompasses all descriptions known to date is 'any peptide that can, to a measurable degree, enter the interior of living cells in cell culture, or deliver a membrane-impermeant cargo' (31). This assumption will be considered for the present review.

The first reports of CPPs were from studies related to human immunodeficiency virus (HIV) and its cellular uptake (32,33). The TAT peptide, corresponding to the basic domain of HIV-1 TAT protein, was one of the first reported. Afterward, a homeodomain of the transcription factor antennapedia from *Drosophila melanogaster* also showed penetrating activity. This finding led to discovery of the CPP, penetratin, which corresponds to the third helix of the antennapedia homeodomain (34).

CPPs are added to molecules to improve their capacity for cell internalization (30). CPPs can deliver cargoes inside the cell through two primary mechanisms: Direct penetration and endocytosis, depending on several physicochemical properties (35). However, other reports mention some CPPs with a pore formation mechanism (31,36-39). Four major penetration models have been proposed through these mechanisms: Reverse vesicle endocytosis, direct translocation, adaptive translocation and pore formation (34). A general schematic of these models applied to NSs is presented in Fig. 3. The variety of mechanisms related to the heterogenicity of sequences of the CPPs impact their biochemical properties. Some of these properties, which must be considered when developing an NS, are shown in Table I, along with the basic classification used for most available CPPs (35). Most of the studies discussed in the present review involve cationic peptides, which are generally attributed to a direct penetration mechanism by being adsorbed onto cell surfaces due to anionic moieties (35,40). Each CPP sequence discussed in the present review is included when available.

The chemical reaction for CPP binding (also known as conjugation) to the NP depends most on the NP used. Lipid-based NPs rely on terminal amino and carboxyl groups from the peptide and lipids. The conjugation reaction is performed under constant stirring in a solution that contains both the lipids and the CPP (41,42). CPP conjugation to metallic NPs can be achieved by a thiol (SH)-ether bond between Cys from the CPP and a maleimide group in polyethylene glycol (PEG). Different sources of the required functional groups exist; one example is a functionalized SH-PEG and a maleimide-PEG (43).

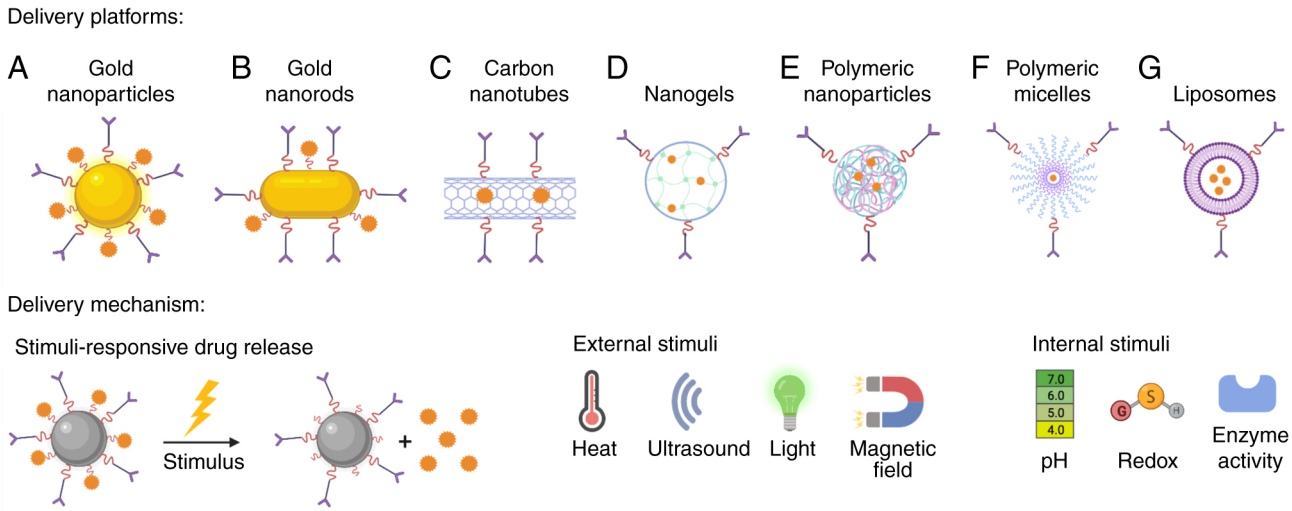

Figure 1. Common delivery nanoplatforms and stimuli-responsive drug release systems used in the treatment of several types of cancer. Stimuli-responsive drug release can be used for controlled delivery into a target tissue. However, internal stimuli have been mostly applied in metallic NPs, such as (A) gold nanoparticles and (B) gold nanorods, since their mechanism involves the activation and/or cleavage of an essential component of the nanosystem, releasing the drug. External stimuli generally have been applied in (C) carbon nanotubes, (D) nanogels, (E) polymeric NPs, (F) polymeric micelles and (G) liposomes, since their mechanism mostly involves the dissolution or disappearance of the NP. Examples of external stimuli are shown. Figure created with BioRender.com. NP, nanoparticle.

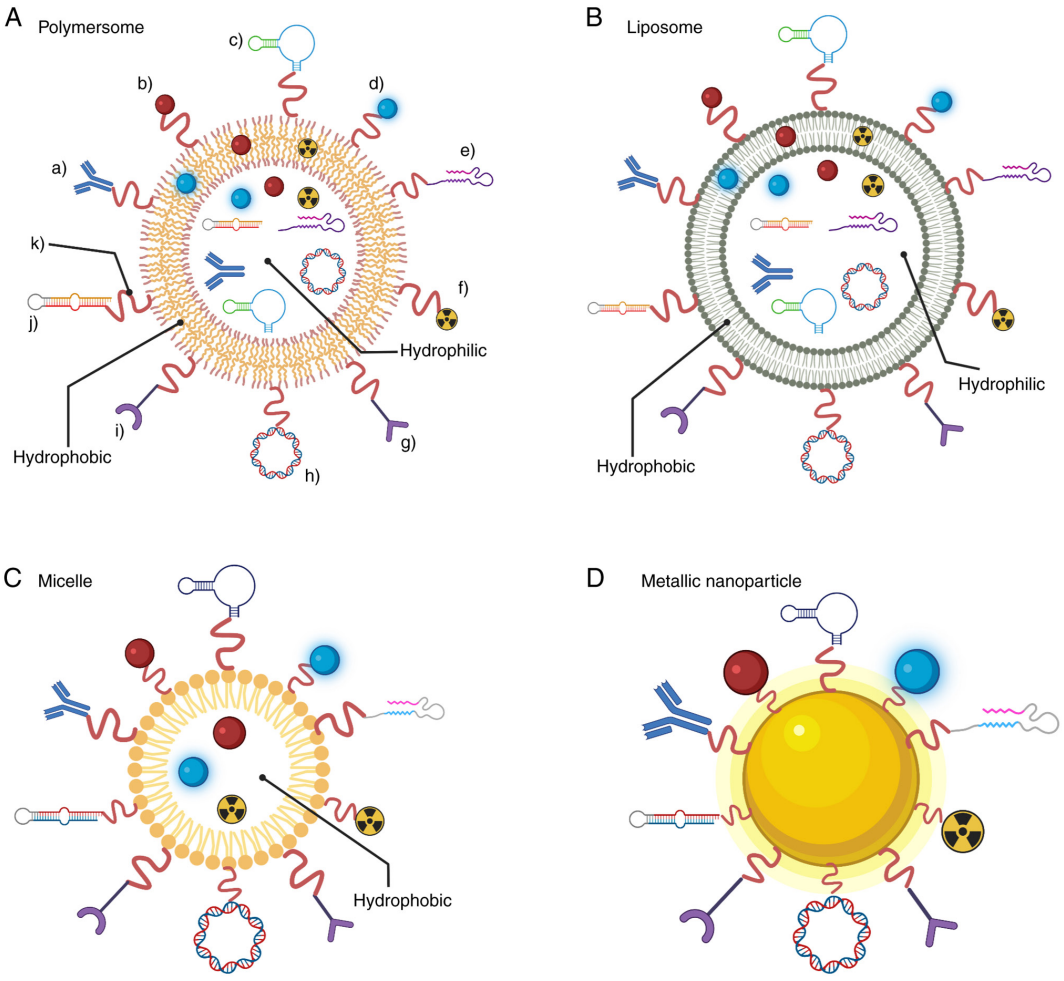

Figure 2. Functionalization strategies for specific site-direction of NPs. Antibodies, targeting ligand and aptamer guidance have been used for highly specific delivery into a particular cell type. Fluorescent dyes and radioligands have been used for diagnostic purposes. Drug, DNA vector and pre-miRNA coupling have been studied for therapeutic purposes. CPPs and enzymes have been shown to increase cell uptake. A linker chain is required for coupling to the NP surface. The nature of (A) polymersomes, (B) liposomes and (C) micelles allows them to be used as a vehicle for delivering hydrophobic and/or hydrophilic anticancer agents, among others. The nature of (D) metallic nanoparticles allows them to be used as theranostics for both drug delivery and imaging applications. Figure created with BioRender.com. Key: a), antibodies; b), drug; c), targeting ligand; d), fluorescent dye; e), CPP; f), radioligand; g), aptamer; h), DNA vector; i), enzyme; j), pre-miRNA; k), linker chain. NP, nanoparticle; CPP, cell-penetrating peptide; miRNA, microRNA.

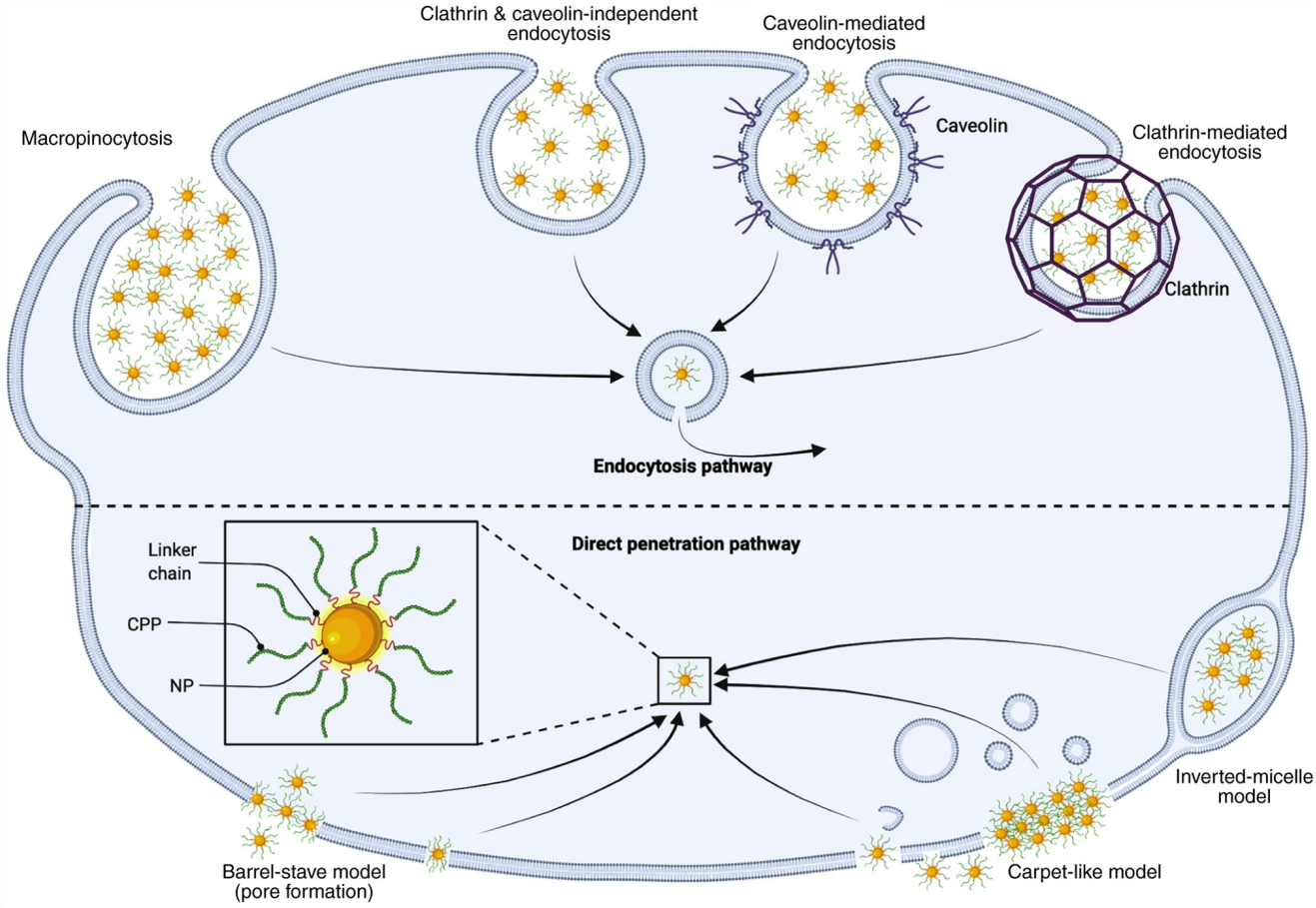

Figure 3. Previously proposed models for CPP internalization mechanisms when coupled to NPs. These mechanisms can be divided into two major pathways: Endocytosis and direct penetration. Endocytosis is an energy-dependent process that may be further subclassified as macropinocytosis, clathrin- and caveolin-independent endocytosis, caveolin-mediated endocytosis and clathrin-mediated endocytosis. Endocytosis is usually related to cell membrane receptor internalization. The direct penetration pathway is an energy independent process that involves the interaction of positively charged CPPs with the negatively charged cell membrane. This interaction can lead to pore formation or membrane destabilization (carpet-like model and inverted-micelle model), which will lead to CPP-NS penetration. Figure created with BioRender.com. NP, nanoparticle; CPP, cell-penetrating peptide; NS, nanosystem.

4. Improving NS properties

One of the challenges in the construction of NSs is to improve stability, biocompatibility and cell penetration. For instance, one strategy for inducing apoptosis and inhibiting tumor cell proliferation involves using small interfering RNAs (siRNAs) (44-46). The main limitation of this technology is that when administered in the systemic circulation, the siRNA faces two main problems. The first is its limited cell-penetrating properties, and the second, its rapid degradation by circulating ribonucleases. Incorporating polymers such as PEG and hyaluronic acid (HA) contributes stealthiness against the immune system of the host, and improves biocompatibility and biodegradability (47-50). In addition, cationic polymers such as chitosan are implemented when the therapeutic agent is RNA, protecting it against degradation and further cell internalization (51).

5. Guided delivery of NSs by CPPs

The most common uses of CPPs include improving cancer treatment with drugs and gene therapies, among others. *In vitro* and *in vivo* experiments have been conducted to improve cell penetration, stability, viability and drug cytotoxicity, and to reduce tumor size. However, a valid concern raised by several researchers is the lack of specificity of some CPPs, which could lead to drug delivery to cells in healthy tissues. Some modifications, such as antibody or antibody fragments and aptamers, could be incorporated into the NS for specific targeting. Other researchers have developed chimeric CPPs that specifically target cancer cells (52-54). Next, the present review discusses some of the NSs coupled to CPP for BC and PC.

6. CPPs coupled to NSs for BC

NSs coupled to CPP to guide drug release in BC

Doxorubicin (DOX). DOX is commonly used to treat various types of cancer, including BC. However, despite wide use, it has well known undesirable side effects, such as cardiotoxicity (55). Various types of CPP have been coupled to a variety of NSs, including liposomes, gold, dendrigraft, iron oxides and polymeric structures. These NSs carrying DOX and/or other anticancer drugs have been tested *in vivo* and *in vitro* to improve delivery and reduce side effects. Below, the present review briefly describes some of the NSs that have been coupled to CPPs.

Table I. Examples of CPPs, their classification and other biochemical characteristics.

Classification	CPP name	Sequence length, aa	Molecular weight, kDa	Isoelectric point
Amphipathic	p28	28	2.91	3.49
	VT5	26	2.60	6.17
	Pep-1	21	2.84	10.48
	BPrPr	28	3.19	10.65
	Transportan	27	2.84	10.75
	MAP	18	1.87	11.27
	MPG	27	2.80	11.74
	ARF	22	2.65	12.49
	pVEC	18	2.21	12.59
	Bac7	24	2.93	13.00
Hydrophobic	Pep-7	15	1.80	3.28
	PFVYLI	6	0.75	5.54
	C105Y	17	1.99	8.05
Cationic	DPV1047	19	2.31	12.29
	Penetratin, pAntp	16	2.24	12.44
	HIV-1 TAT protein	13	1.80	3.28
	HIV-1 TAT protein 9 aa	9	1.34	12.80
	Polyarginines (R_7-R_9)	Variable, 7-9	Variable, 1.10-1.40	Variable, 12.78-12.90

Table created using Geneious Prime 2021.2.2. (https://www.geneious.com). CPP, cell-penetrating peptide; HIV, human immunodeficiency virus.

CPP PVF (PFVYLI). Hydrophobic CPP PVF coupled to a 100-nm liposome was evaluated in MCF-7, MDA-MB-435S, MCF-7/Adr (Adriamycin-resistant human mammary adenocarcinoma) and 4T1 murine BC carcinoma cell lines. Even when the cell survival rate was higher in cells treated with the NS *in vivo*, the NS decreased the tumor weight up to 0.4 g compared with the control (0.9 g) and free DOX (0.7 g), while keeping the mice at a healthier weight compared with the free DOX-treated mice (41).

HIV-derived TAT CPP (YGRKKRRQRRRTAT). With the ability to permeate the blood-brain barrier, the HIV-derived TAT CPP was evaluated in MDA-MB-231 cell lines and metastatic *in vivo* tumors using gold NPs coupled with PEG as the carrying vehicle for DOX. *In vivo* cell uptake increased 4.8-fold compared with that of the control, while DOX half maximal inhibitory concentration (IC_{50}) decreased 80% compared with that of free DOX. Furthermore, no significant adverse effects were found when the DOX dose of the NS ranged from 1 to 5 μg. However, at 10 and 15 μg DOX, the mice exhibited significant weight loss, which a common side effect of this drug. Also, the development of ascites and peripheral edema was found in the mice, which a relatively common adverse effect of DOX metabolism. These results suggest further targeting with this NS should be achieved before moving into clinical trials (43).

TAT peptide fragment (RKKRRRQRC). A gold nanostar coupled with mesoporous silica (MS), TAT fragment and the photosensitizer drug protoporphyrin IX was used as a theranostic system. Also, Raman detection for diagnostics, photodynamic therapy (PDT) and cytotoxicity were evaluated in the BT-549 cell line. Through Raman imaging, it was determined that TAT CPP activity allowed cell penetration while maintaining cell viability, although once PDT was applied, the cell viability was decreased (56). A similar study used an NS containing TAT and gold NPs along with anti-HER2 antibody for increased specificity and surface-enhanced Raman spectroscopy, which was assessed for DOX release rate into SK-BR-3 cells. After 24 h, *in vitro* cell viability was decreased by 39.48% compared with that of the control (57). Another study designed a synthetic peptide containing TAT, L-lysine residues, fusogenic GALA peptide and cell targeting peptide (DMPGTVLP) with stearic acid to improve condensation and stability to a dioleoylphosphatidylethanolamine (DOPE)-based nanoliposome. This NS was evaluated against MCF-7 cells delivering an siRNA targeting BCL2 mRNA. The chimeric peptide used in this NS increased cell uptake, and BCL2 silencing was decreased by ~80%. However, cell viability was decreased by only 20% (58).

Angiopep-2 CPP (TFFYGGSRGKRNNFKTEEY). The size of the NS directly impacts tumor accumulation and diffusion abilities. Keeping this in mind, researchers developed an NS that could be decreased in size by the action of metalloproteinase-2, while keeping high specificity for triple-negative BC (TNBC). Gelatin and dendrigraft polylysine were used as a carrying vehicle and stabilization agent, while DOX was used as the anticancer agent in 4T1 cells. As expected, *in vitro* cell uptake was increased from 15 to 40%, which translated into a higher inhibition rate. *In vivo*, there was an ~3-fold decrease in tumor volume and a 4-fold decrease in tumor weight compared

with that for free DOX treatment. Furthermore, no difference in mouse bodyweight was reported, suggesting the relative safety of this NS (59).

Arginine-rich amphiphile CPP (lauryl-PPPPRRRR). A nanoliposome-based system carrying the arginine-rich amphiphile CPP and DOX or paclitaxel (PTX) was tested in MCF-7 cells. After 3 h, cell uptake had increased 5-fold with rhodamine B (a hydrophilic dye) and 30% with Nile red (a hydrophobic dye) to show this dual amphiphile activity. The drug uptake and effectiveness of the liposome improved with the NS compared with the use of free drugs. *In vitro*, cancer cell viability was decreased by 50 and 25% in the CPP-DOX-NS and NS-PTX groups, respectively (60). These results show a significant difference between NSs with CPPs and those that do not have them coupled.

QLPVM CPP peptide. The QLPVM CPP was attached to a 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE)-PEG-based nanoliposome carrying tamoxifen (TAM) and DOX, evaluated in MCF-7 cell tumor-bearing mouse models. *In vitro* cell viability was dose-dependent for the NS. However, cell viability was lower than that in the NS-free TAM and NS-free DOX groups. *In vivo* tumor inhibition with the combined NS showed the best results. In a synergistic effect, tumor volume decreased 7-fold compared with that of the control, with similar results in tumor weight and no noticeable bodyweight loss or cardiac toxicity (61).

Chimeric R_8 CPP ($AVPIR_8$). A disadvantage of drug therapy is the existence of drug-resistant tumors. Thus, the chimeric R8 CPP was fused with an apoptotic peptide to create a co-delivery nanocomplex in MCF7-DOX resistant cells. The nanocomplex consisted of binding several wild-type p53 proteins, chimeric CPPs and p53 DNA. Assembly was enabled by preparing a mixture of these components and incubating them at 37˚C for 30 min to allow the formation of the nanocomplex. There was a 36-fold *in vitro* increase in apoptosis in the synergistic NS compared with that in free DOX, leading to a 97.9% decrease in cell viability. *In vivo*, there was a significant tumor volume and weight decrease when the mice were treated with the NS. Tumor volume decreased ~10-fold, while tumor weight was 9-fold lower at 21 days post-injection (62).

R_7 CPP (RRRRRRR). The R_7 CPP was coupled in a poly-lactic-co-glycolic acid-PEG polymer with folic acid and the cell-cycle anticancer agent, vincristine sulfate (VCR). Cell uptake mediated by R_7 was not significantly different from cell uptake mediated by folic acid and its receptors. However, cell viability was decreased to 30% when 5 nM VCR was added, compared with 60% cell viability without VCR. These results were further confirmed by determining an increase in MCF-7 cell percentage in the G2/M arrest phase from 12.28±1.74% to >33% (63).

R_9 CPP (RRRRRRRRR). A DSPE-PEG-based nanoliposome coupled with R_9 was developed to deliver cabazitaxel in the 4T1 cell line and murine models. Cell uptake was increased 1.78-fold after 8 h of exposure compared with the control. *In vitro* cytotoxicity was enhanced 223-fold to 0.03 μg/ml due to NS activity. *In vivo*, the NS localization in the lymph nodes was reported to increase 1.73-fold at 24 h post-injection. This finding demonstrates a penetrating activity with long retention times. Also, primary tumor growth decreased 75.3%, while tumor inhibition in the lymph nodes was reported at 89.1%, showing that this NS can inhibit metastasis. The size of this NS was 13 nm; NSs with sizes <30 nm have been reported to have better access to lymph nodes (64-66).

iRGD peptide (CRGDK/RGPD/EC). Vesicles extracted from red blood cell membranes and coupled to iRGD peptide were used as biological NSs carrying PTX. *In vitro* cell uptake in 4T1 cells was higher in the NS compared with that in the control. However, *in vitro* cell viability was similar compared with that of free PTX. *In vivo*, the NS decreased tumor growth to 8.5%, 5.6-fold less compared to the control (67). In a similar study, the iRGD CPP coupled to iron oxide nanoworms was used as an NS to inhibit or lower BC metastasis to the brain, targeting MDA-MB-231 and 4T1 xenografts in 231Br and 4T1-BR5 mouse models. Brain imaging showed that 30 days post-injection, the number of tumors was decreased by 60%, and their size had decreased 5-fold compared with the controls. However, researchers found a tight window of 12 days for this metastasis decrease (68).

Pentapeptide CALNN CPP (CALNN). The pentapeptide CALNN CPP coupled with gold NPs was used to deliver linalool (a monoterpenoid derived from plants) and evaluate its anticancer activity in MCF-7 cells. *In vitro* cytotoxicity was reported at >80% after 48 h of treatment, while clonogenicity was inhibited. *In vivo*, the NS conferred no statistical change in mouse health, suggesting the safety of the NS for further study. Unfortunately, the study did not include *in vivo* anticancer activity assays (69).

CF CPP (CREKA-CN2-CKDEPQRRSARLSAKPAPPKPEPK PKKAPAKK-NH2). CREKA, a linear pentapeptide designed to bind to fibrous protein in the tumor microenvironment, was fused to the CF peptide (a CPP for nucleus translocation) using a polylactic acid-based NS as the vehicle. This NS was designed for targeted delivery of the anticancer drug erlotinib into MDA-MB-231 cell lines to combat drug resistance in TNBC. Cell uptake was increased 2.3-fold when the pH of the assay was similar to that of the tumor microenvironment. *In vitro* cell viability decreased <20% in the pH simulating the tumor microenvironment, compared with that in the physiological pH, which maintained 50% viability. *In vivo*, a 2.8-fold tumor decrease was observed in mice treated with the NS, while their body weight was maintained, suggesting an effective method for TNBC tumor treatment that lacked adverse effects (70).

tLyP-1 CPP (CGNKRTRG). The tLyP-1 CPP, which specifically targets tumors, was used to develop an NS targeting MDA-MD-231 cells. The polymeric NPs consisted of HA and D-α tocopheryl succinate as a vehicle. The NS carried the anticancer drug docetaxel. Cell uptake compared with that of the control increased 2.36-fold, while *in vitro*, cell cytotoxicity was higher in the NS with tLyP-1. *In vivo* tumor weight decreased 3.5-fold compared to the control (71).

PEGA-pVEC CPP (CPGPEGAGC-LLIILRRRIRKQ AHAHSK). The PEGA-pVEC CPP that targets BC cells and tumors was coupled to an NS of colloidal MS as a vehicle for the anticancer agent epigallocatechin-3-gallate (EGCG) in MCF-7 cells. *In vitro* cell inhibition reached 100% at a 100 μg/ml concentration. *In vivo* tumor inhibition of up to 89.66% was reported compared with 69.9% in the control group in murine models (72). In a similar study, PEGA-pVEC was used to develop an MS and HA NS as a carrying vehicle for DOX and an siRNA directed against the BC overexpressed connective tissue growth factor gene. The NS was tested in drug-resistant MDA-MB-231 cells. *In vitro* inhibition increased from 59 to 80% compared with the control. *In vivo*, the tumor inhibition increased up to 60% compared with the control (73). A similar study developed a self-aggregating nano-gel based on protamine aggregates carrying EGCG as an anticancer agent to MDA-MB-231 cells and xenografts. *In vitro*, there was a 15-fold increase in cytotoxicity compared with the control, while *in vivo* tumor inhibition was 80% compared with the control (74).

Chimeric arginine-glycine-aspartate CPP (RGD and RGE RPPR). The chimeric arginine-glycine-aspartate CPP was evaluated in MDA-MB-231 cells using gambogic acid (GA) as an anticancer agent and nanostructured lipid nanocarriers. When cells were treated with a 2 μg/ml concentration, *in vitro* cell viability was decreased to <20% compared with that of the control. However, the best inhibition rate and tumor size decrease were achieved with the NS carrying RGERPPR alone, suggesting that only one CPP can benefit a single NS (75).

CPPs coupled to NSs for gene and recombinant therapy in BC gH625 CPP (HGLASTLTRWAHYNALIRAF). An NS, delivering a novel, non-disclosed siRNA consisting of superparamagnetic iron oxide NPs (SPIONs) functionalized with PEG, and cationic polymers, such as chitosan and L-arginine with CPP gH625, was designed and evaluated against the MDA-MB-231 cancer cell line. After 4 h of exposure, the NS penetrated the cells aided by the gH625 peptide, and the MDA-MB-231 cancer cell line genes were downregulated due to the interference mechanism of the siRNA. At 72 h, there was cell growth inhibition of nearly 80% (51). gH625 in an NS SPION couple was also tested in MDA-MB-231 cells as a theranostic system. Cell uptake increased 3-fold in the NS compared with that in the control assay (76).

R_9 CPP (RRRRRRRRR). The Twist gene is a transcription factor for an epithelial-mesenchymal transition that is heavily involved in the metastatic activity of tumors. Therefore, a nano-corona NS consisting of a DOPE NP carrying an siRNA against the Twist gene, the R_9 for tumor penetration, and coated with human serum albumin for immune system camouflage, was designed and evaluated in 4T1 cells. The nano-coronas were labeled with IR-780 dye for combination therapy: Twist gene silencing and tumor growth inhibition using photothermal therapy for thermal ablation. Cell uptake indicated a higher NS translocation in the cytoplasm after 24 h of incubation; the cell migration rate decreased up to 66%. The cell viability index was decreased by 90% when treated by photothermal therapy. *In vivo* experiments showed the ability of the NS to penetrate tumors; tumor inhibition progression was 83.6% and metastasis inhibition was 92.2% after 13 days of combination therapy (77).

TAT protein basic domain-derived CPP (RKKRRQRRR-Cys). This CPP was used to develop a lipid-based nanobubble system carrying an siRNA against an epidermal growth factor. The main objective was to evaluate the NS when applying ultrasound irradiation inside tumor cells to enhance the siRNA anticancer activity targeting MDA-MB-231 cells. Cell inhibition was increased from 48 to 72 h, while *in vivo* tumor growth was decreased by 42.08% (78).

Chimeric Tat-Mu CPP (YGRKKRRQRRRMRRAHHRRRR ASHRRMRGG). The fusion of a HER2 antibody mimetic-affibody and CPP Tat-Mu was used to deliver an anti-tissue factor using an shRNA. The NS was a cationic N,N-di-n-hexadecyl-N,N-dihydroxy ethyl ammonium chloride-based nanoliposome used as a vehicle to MDA-MB-231 cells and xenografts. *In vitro*, cell uptake increased 7-fold compared with that of the control, while there was a significant tumor size decrease, to <10% of the original size, *in vivo* (79).

Lin TT1 CPP (AKRGARSTA). The TT1 CPP has specificity against p32, a mitochondrial essential tumor regulator protein. Iron oxide NPs were the vehicle for the TT1 CPP fused with the proapoptotic peptide [D(KLAKLAK)2] (inducing apoptosis through the activation of caspase-3) and tested in MCF10CA1a and 4T1 cell lines. *In vitro* cell uptake increased 4-fold, while a tumor size decrease of 50% was observed *in vivo* (80). Another study consisted of a cholesterol-based nano-micelle carrying TT1 plus two anticancer components, siRNA targeting PDL-1 and indoleamine 2,3-dioxygenase inhibitor (1-methyl tryptophan), that led to the activation of cytotoxic T lymphocytes against the 4T1 cell line. After 4 h, cell uptake was 16.3-fold higher than that of the control assay (81).

Penetratin-derived CPP (CKRRMKWKK). Ephrin type-A receptor 2 (EphA2) is a transmembrane protein whose overexpression has been linked to carcinogenesis, metastasis and a poor clinical prognosis. The YSA peptide, an ephrin mimetic bound with EphA2, was fused with a penetratin-derived CPP. DSPE-PEG nanobubbles were used as the vehicle for an anti-Myc siRNA acting as an anticancer agent. The *in vitro* cell uptake was higher with this NS treatment. *In vivo* tumor growth inhibition after 24 days of treatment was decreased to 31.2% compared to the control, while no significant body weight loss was found in the mice. The NPs with YSA peptides improve therapeutic effects *in vivo* (82).

uCendR CPP (RPARSGRSAGGSVA). A urokinase activatable CPP was developed to ensure tumor specificity for *in vivo* targeting of 4T1 cell tumors in murine models. In addition, silver NPs were coated with PEGylated neutravidin as a vehicle and for immune system stealthiness. However, *in vivo* distribution was determined only in tumor tissue, without fluorescence in healthy tissue (83).

Reversibly activatable CPP (RACPP). The difference in pH between the tumor microenvironment and the healthy

extracellular matrix has been exploited along with CPPs to improve the specificity of the NS. Nanopolymer micelles masked with PEG-lactic acid were used as a vehicle for the RACPP, containing a pH-sensitive sequence, and PTX as an anticancer drug targeting 4T1 cells and 4T1-BALB/c tumor xenografts models. Cell uptake was increased 3-fold, while *in vitro* IC50 was decreased from 1.595 to 1.035 μg/ml. *In vivo*, tumor size decreased by ~50% at 16 days post-injection, while the mouse survival rate increased 1.5-fold (20).

MAP CPP (KLALKLALKALKAALKLAY). In another case, the pH-sensitive MAP CPP was used with the highly pH-sensitive histidine-glutamate (HE) oligopeptide (HEHEHEHEH EHEHEHEHEHE) coupled to glutathione-S-transferase acting as a cargo protein. The delivery activity was measured in MDA-MB-231 cells and xenografts through fluorescence imaging. At an acidic pH simulating the tumor microenvironment, there was a 3-fold increase of fluorescence *in vitro*. *In vivo* results showed that at 6 h post-injection in mice, the system was localized mainly around the xenograft tumor. These results further show the advantage of using the pH of the tumor microenvironment for intelligent drug delivery systems (84).

NGR chimeric CPP (NGR-CKRRMKWKK). Using a different approach, a thermosensitive NS was developed using a heat-activatable CPP after mild thermal stimulus at temperatures ranging from 37 to 42˚C, which facilitates drug delivery. The NS consisted of a nanoliposome that immediately encapsulated drugs in heating tissue or organs. These were coupled with a penetratin-derived CPP, the NGR peptide and DOX as the anticancer agent against MCF-7. *In vitro* cellular uptake was increased 5-fold compared with the control, while *in vitro* cytotoxicity of the preheated NS showed an increase of 1.5-fold compared with the NS without preheating treatment. However, both cases showed increased cytotoxicity compared with that of free DOX. Similarly, *in vivo* tumor inhibition was increased in the preheated NS. Also, there was an ~9-fold tumor volume decrease with the preheated NS compared with the control, with no apparent body weight decrease (42).

7. CPPs coupled to NSs for PC

Considerably less research and NS development have been conducted for PC, providing an important opportunity in this area. Certain CPPs have been tested on PC with some success, similar to BC, due to an observed increase in cell specificity or uptake. The CPP-NSs described below were designed to improve drug therapy, for gene therapy or to couple with other therapies such as electromagnetic field and laser radiation.

Polyarginine-cholesterol CPP (Chol-R_9). Cancer-associated fibroblasts (CAFs) are involved in microenvironment remodeling in PC. Therefore, a novel approach was constructed using a CPP-based amphiphilic peptide, Chol-R_9, an siRNA targeting chemokine ligand 12, and anti-human FAP-α for their involvement in cancer metastasis. As a result, there was an *in vitro* 7-fold increase in CAF uptake compared with that found using free siRNA, while *in vivo*, tumor weight decreased 53.4% (85).

Polyarginine, PSA-selective peptide and polyanionic shielding peptides (DGGDGGDGGDGGHSSKYQG-R_8). A DSPE-PEG-based nanoliposome was developed with high PC specificity carrying peptides. This NS included polyarginine CPP and the PSA-selective peptide (HSSKYQ), which possess a cleavable moiety by PSA to enhance further specificity towards PC cells. The anticancer agent was an siRNA targeting polo-like kinase 1. Two cell lines were evaluated, 22Rv1 and PC-3. The latter was used as the control since it is PSA negative. *In vivo* results showed a 54% increase in apoptosis and a 5-fold tumor volume decrease (86).

Poly arginine (R_{11}). Among the CPPs used in PC, the proline-rich sequence R_{11} has shown a high-efficiency uptake in PC cell lines coupled to therapeutic systems (87), making it a promising strategy. The R_{11} CPP and the anticancer agent tumor suppressor MIR145 were coupled in a polyethyleneimine polymeric NS to increase the blood circulating time by protecting against the host immune system. *In vitro* cell uptake was increased from 5.2 to 87.5%, while *in vivo* NS accumulation in the tumor increased 3.5-fold compared in both cases with free miRNA. Tumor size was decreased 7.5-fold, increasing the mouse lifespan up to 18 weeks (88).

CendR motif peptide (CRGDK). Another NS with a PC-specific CPP was developed with the CRGDK peptide targeting neuropilin-1 (Nrp-1) in glutathione-functionalized gold NPs, along with platinum IV as an anticancer agent. The NS was tested in PC-3 (Nrp-1 positive) and DU145 (Nrp-1 negative) cell lines. Cell uptake and *in vitro* cytotoxicity were increased 4- and 28.13-fold, respectively (89).

Polyarginine (R_{11}). An NS of iron oxide NPs, poly lactic-co-glycolic acid (R_{11}-Mn-PGLA), polyarginine CPP and the radio-sensitizer 8-dibenzothiophen-4-yl-2-morpholin-4-yl-chromen-4-one was tested in PC-3 and PZ-HPV-7 cells. Results showed the uptake of the NS by the PC3 cells in a dose-dependent manner. However, the R_{11} CPP increased cell uptake when an electromagnetic field was applied (90). Using a similar approach, R_{11} CPP was used to develop a NS consisting of iron oxide NP coated with poly(N-isopropylacrylamide-acrylamide-allylamine) (PMNPs). The R_{11}-PMNPs were biocompatible with normal cells up to 500 μg/ml. *In vitro* cell uptake for PC3 and LNCaP cell lines was higher when PMNPs carried R_{11}. *In vivo*, tumor R_{11}-PMNPs accumulation was higher than that of normal tissue (91).

Chimeric peptide (Ste-R_6L_2). Tripterine is a bioactive compound from Tripterygium wilfordii that is used in traditional Chinese medicine. Nanostructured lipid carriers coated with Ste-R_6L_2 CPP and loaded with tripterine were designed for targeting PC-3 cell lines. The IC_{50} reported was decreased from 0.88±0.08 to 0.55±0.07 μg/ml, while *in vitro* apoptosis increased from 0.71 to 14.15%. *In vivo*, tumor volume was decreased by 57.2%, while the tumor inhibition rate increased 1.96-fold to 72.68±6.7% (92).

Chimeric TAT-bombesin peptide (^{99}mTc-N2S2-Tat(49-57)-Lys_3-bombesin). The gastrin-releasing peptide receptor (GRP-r) is overexpressed in PC. Bombesin, a 14-amino acid

Table II. Summary of discussed CPPs in NSs targeting breast and prostate cancer.

CPP	Sequence	NP type	Target cells	NS size, nm	Anticancer agent	Effect of the NS
PVF	PFVYLI	Nanoliposome	MCF-7 MDA-MB-453S	100	DOX	2.25-fold tumor weight decrease
HIV-derived TAT	YGRKKRRQRRRTAT	Metallic	MDA-MB-231	23.4	DOX	4.8-fold increase *in vivo* cell uptake
TAT peptide fragment	RKKRRRQRC	Metallic	BT-549	123	Protoporphyrin IX (PpIX)	Cell viability decrease
Angiopep-2	TFFYGGSRGK RNNFKTEEY	Polymeric	4T1	Reduction 185.7 to 55.6 (intelligent NS designed to reduce its size by action of the MMP-2)	DOX	3-fold tumor volume and 4-fold tumor weight decrease
Arginine-rich amphiphile	lauryl-PPPPRRRR	Nanoliposome	MCF-7	95.26	DOX and PTX	50% *in vitro* cell viability decrease
Not named	QLPVM	Nanoliposome	MCF-7	96.93	DOX and TAM	7-fold tumor volume decrease
Chimeric polyarginine	$AVPIR_8$	Polymeric	MCF-7 Dox-resistant	<50	DOX	97.9% decrease *in vitro* cell viability
R_7 Polyarginine	RRRRRRR	Polymeric	MCF-7	235.8	Vincristine sulfate	Cell viability decrease to 30.65%
R_9 polyarginine tumor	RRRRRRRRR	Nanoliposome	4T1	13	Cabazitaxel	1.73-fold increase in localization at
Chimeric iRGD	CRGDK/RGPD/EC	Nanoliposome	4T1	150	PTX	5.6-fold decrease in tumor growth rate
Pentapeptide CALNN	CALNN	Metallic	MCF-7	<10	Linalool	80% increased *in vitro* cytotoxicity
Chimeric peptide	CREKA-CN2-CKDEP QRRSARLSAKPAPPK PEPKPKKAPAKK-NH2	Polymeric	MDA-MB-231	N/A	Erlotinib	2.8-fold tumor size decrease
tLyP-1	CGNKRTRG	Polymeric	MDA-MB-231	110	DTX	3.5-fold tumor size decrease
PEGA-pVEC	CPGPEGAGC-LLIIL RRRIRKQAHAHSK	Polymeric	MCF-7	180	Epigallocatechin-3	89.66% tumor inhibition
Chimeric arginine-glycine-aspartate	RGD and RGERPPR	Nanoliposome	MDA-MB-231	25.81	Gambogic acid	20% higher tumor size decrease
gH625	HGLASTLTRW AHYNALIRAF	Metallic	MDA-MB-231	79	Non-disclosed siRNA	3-fold increased cellular uptake
Not named	RKKRRQRRR-Cys	Nanoliposome	MDA-MB-231	582	siRNA targeting epidermal growth factor	Tumor growth decrease by 42.08%

Table II. Continued.

CPP	Sequence	NP type	Target cells	NS size, nm	Anticancer agent	Effect of the NS
Not named	RKKRRQRRR-Cys	Nanoliposome	MDA-MB-231	582	siRNA targeting epidermal growth factor	Tumor growth decrease by 42.08%
Chimeric Tat-Mu	YGRKKRRQRRRMRRA HHRRRRASHRRMRGG	Nanoliposome	MDA-MB-231	N/A	shRNA anti-tissue factor	7-fold tumor size decrease
Lin TT1	AKRGARSTA	Metallic	4T1	175	Lin TT1 CPP targets p32 tumor regulation protein	50% tumor size decrease
Penetratin-derived	CKRRMKWKK	Nanoliposome	MCF-7	203	Anti-Myc siRNA	Tumor growth inhibition by 31.2%
uCendR	RPARSGRSAGGSVA	Metallic	4T1	50	N/A	*In vivo* distribution only in tumor tissue
Not named	RACPPKLALKLAL	Polymeric	4T1	25.3	PTX	Tumor size decrease by 50%
MAP	KALKAALKLAY	Polymeric	MDA-MB-231	N/A	N/A	3-fold increased accumulation in xenograft tumor
Chimeric NGR	NGR-CKRRMKWKK	Nanoliposomes	MCF-7	89.23	DOX	9-fold increased tumor volume decrease
Polyarginine-cholesterol	Chol-R_9	Polymeric	Cancer-associated fibroblasts	100	siRNA targeting CXCL12	53.4% tumor weight decrease
Chimeric polyarginine	DGGDGGDGGD GGHSS KYQG-R_8	Nanoliposome	PC-3 and 22v1	200	siRNA targeting PLK1	5-fold tumor volume decrease
CendR Motif	CRGDK	Metallic	PC-3 and DU145	5.2	Platinum IV	4 and 28.13-fold increase *in vitro* cell cytotoxicity in PC-3 and DU145, respectively
Polyarginine	RRRRRRRRRR	Metallic	PC-3 and LNCaP	100	N/A	5-fold increase *in vivo* tumor accumulation
Not named	Ste-R_6L_2	Nanoliposome	PC-3	126.7	Tripterine	Tumor volume decrease by 57.2%
TAT-Chimeric bombesin	^{99}mTc-N_2S_2-Tat (49-57)-Ly_{s3}-bombesin	Metallic	PC-3	8	Laser thermal ablation	98% *in vitro* cell inhibition
Ypep	YTFGLKTSFNVQ	Bacteriophage		930	N/A	1.5-fold *in vitro* cell uptake

NP, nanoparticle; CPP, cell-penetrating peptide; NS, nanosystem; DOX, doxorubicin; PTX, paclitaxel; TAM, tamoxifen; DTX, docetaxel; MMP-2, metalloproteinase-2; N/A, not available.

peptide, strongly binds to GRP-r. For this reason, an NS consisting of bombesin and TAT CPP was coupled with gold NPs for PC-specific targeting. The NS also carried radiopharmaceuticals ^{99}mTc and ^{177}Lu. Laser thermal ablation was performed as an anticancer treatment. *In vitro* cell uptake was increased 52.5% compared with that of the control in the PC-3 cell line. Once the cells were laser-irradiated, cell inhibition was 98.64±0.4% (93).

Ypep CPP (YTFGLKTSFNVQ). Bacteriophages have also been studied as NSs for the treatment of PC. The M13 bacteriophage was engineered to express the Ypep CPP targeting PC-3 cells. As a result, *in vitro*, cell uptake increased ~1.5-fold, with nearly 100% cytotoxicity. Unfortunately, no *in vivo* assays were performed, which could increase the therapeutic potential of this approach. However, the size of the phage could be an important limiting factor, since it had a width of 6 nm and a length of 930 nm (94).

8. Conclusions and outlook

The present review described NSs coupled to CPPs as guidance tools towards BC and PC cells and tumors. These NSs increased their specificity against cancer cells and tumors, leading to less cytotoxicity against healthy tissue. A summary of the components of the NSs discussed in this article and the effect they have on cancer cells and/or tumors can be found in Table II. One of the most relevant achievements of several of these NSs is a decrease in the anticancer drug used. This decrease, coupled with increased specificity, could lower the risk of adverse effects in patients. Several advantages and disadvantages should be considered to increase the specificity for the NS in order to reach clinical application consistently.

Assays evaluating the efficiency in cell uptake and drug delivery of each type of CPP could provide knowledge and insight on this topic. In addition, the proposed assays could be particularly useful for upcoming scientists developing different NSs that target BC or PC to increase reproducibility and as a baseline to compare their results.

Besides specificity and safety, NS design should consider production costs, development time and processing time. A cost-benefit analysis should be established to compare the specificity needs while keeping complexity at manageable levels. CPPs significantly improved the delivery of anticancer agents towards cancer cells while showing little effect on healthy tissue both on *in vitro* and *in vivo* assays. Cell uptake is one of the major contributions to the success of NSs in anticancer therapy. Still, several concerns exist with nanomedicine, with the main issue being the clearance of the NPs from the system once their therapeutic potential has been achieved. Further studies and clinical trials should provide a better understanding of the mechanisms involved in developing NS therapy for both PC and BC.

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Authors' contributions

SLG, CNSD and HLGB designed the theme of the review. SLG searched and retrieved the relevant literature and wrote the first draft. CNSD and HLGB reviewed and suggested corrections. All authors have read and approved the final manuscript. Data authentication is not applicable.

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Competing interests

The authors declare that they have no competing interests.

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