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Authors

Stark, Marie
Cheng, R Holland

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Surface modulatable nanocapsids for targeting and tracking toward nanotheranostic delivery

Marie Stark[1] & R Holland Cheng*,[1]

[1]Department of Molecular and Cellular Biology, University of California, Davis, CA, USA

*Author for correspondence: rhch@ucdavis.edu

Nanoparticle diagnostics and therapeutics (nanotheranostics) have significantly advanced cancer detection and treatment. However, many nanotheranostics are ineffective due to defects in tumor localization and bioavailability. An engineered Hepatitis E Virus (HEV) nanocapsid is a proposed platform for targeted cancer-cell delivery. Self-assembling from HEV capsid subunits, nanocapsids retain the capacity to enter cells and resist proteolytic/acidic conditions, but lack infectious viral elements. The nanocapsid surface was modified for chemical activation to confer tumor-specific targeting and detection, immune-response manipulation and controlled theranostic delivery. Nanotheranostic molecules can be packaged in the hollow nanocapsid shell during *in vitro* assembly. Complementing the adapted stability and cell-entry characteristics of the HEV capsid, a modified nanocapsid serves as a tunable tumor-targeting platform for nanotheronostic delivery.

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Keywords: cancer targeting • nanotheranostics • viral capsid

General challenges in cancers' theranostics

Specific tumor cell targeting and delivery are primary avenues for developing cancer diagnostics and therapeutics (theranostics). Treatment at earlier stages in cancer progression is highly correlated with reduced treatment time and higher survival rate [1]. Diagnostic sensitivity and specificity are essential to early treatment, but early diagnosis is only half the battle as improved cancer treatment remains critical. Many chemotherapeutics are unstable in systemic circulation or do not specifically target tumor tissue, resulting in damage to healthy tissue. Current methods in tumor cell delivery are compromised by factors such as aggregation, degradation and nonspecific cell uptake. Such complications contribute to reduced tumor delivery and severe patient side effects. Inefficient theranostic transport is often compensated for with excess dosage of detection and/or therapeutic molecules, further increasing drug toxicity. Nanotheranostics have revolutionized cancer diagnostics and therapeutics, often with unparalleled safety and efficacy [2–5]. Still, many nanotheranostic formulations are susceptible to instability, lysosomal and immune degradation, and nonspecific cell uptake. By stabilizing and directing nanotheranostics to tumor sites, a nanocapsid platform could vastly improve tissue-specific delivery.

Recent approaches in cancer therapeutics emphasize individualized treatment. While carefully controlled trials establish the general efficacy of a chemotherapeutic agent, individual patient responses vary greatly. Among other factors, patient differences and heterogeneity within a tumor subtype contribute to this variability [6]. Accordingly, a chemically tunable nanocapsid construct could be an ancillary platform in methods of patient-specific modulation.

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A virus-derived nanocapsid delivery platform

To address barriers in diagnostics and chemotherapeutics, a virus-derived nanocapsid was modified for surface modulation and nanoparticle encapsulation. The self-assembling Hepatitis E Virus (HEV) capsid protein was isolated to form an HEV virus-like particle (HEV_VLP). Because HEV evolved mucosal stability as a feco/orally transmitted virus, the structurally similar HEV_VLP is resistant to low pH and proteolytic environments. While the VLP can resist harsh host–cell environments to bind and enter cells, it lacks genetic and nonstructural viral elements, forming a hollow noninfectious nanocapsid. The N-terminal residues associated with RNA-binding in the native virus are truncated from the HEV_VLP; hence, VLP capsid formation is independent of RNA encapsulation [7]. HEV_VLP (henceforth referred to as nanocapsid) was further modified to covalently bind tumor-targeting and/or -tracking molecules.

HEV is a ssRNA virus that forms a gut-stable icosahedral capsid with apparent protrusions on the icosahedron surface. The HEV-derived nanocapsid is encoded by amino acids 111–606 in the second open-reading frame, effectively excluding any infectious viral elements from the construct. The nanocapsid self-assembles 60 identical subunits composed of the S, M and P domains [8]. The S and M domains are responsible for icosahedral capsid formation, which acts as a scaffold onto which the P domain extends via a flexible hinge as an exposed surface protrusion. The P domain protrudes away from the icosahedral capsid shell, thus stabilizing its surface interactions. Consequently, the nanocapsid is highly flexible to modification of the P domain via insertion and conjugation.

Modifying the nanocapsid surface for chemical modulation serves many practical purposes in tumor-cell targeting/delivery: reduced production time, synthetic peptide conjugation and reduced background immune detection. Nanocapsid production is carried out through a US FDA-approved high-yield Bac-to-Bac® expression system in which self-assembled nanocapsids are released from cells for simple purification [8]. Because nanocapsids are exceptionally stable, large quantities can be placed in long-term storage for subsequent chemical activation. In this way, troubleshooting various surface compositions requires simple chemical modulation to ready-made nanocapsid vectors. Surface molecules that would otherwise degrade in storage can be stabilized on the capsid surface. Many theranostic ligands are synthetic or too large to be introduced into a vector through genetic recombination or DNA delivery. Chemical modulation vastly increases the diversity of targeting and tracking molecules compatible with the nanocapsid delivery vector.

Site-specific surface modulation

Nanocapsid modulation is primarily achieved through cysteine replacement with residues located in exposed variable loops on the capsid surface. Surface-modified cysteines can participate in several covalent-binding interactions including thiol coupling, thiol-ligand exchange and maleimide-thiol conjugation. Thiol conjugates may also participate in additional chemical reactions such as alkylation, acylation, arylation, succinylation, cycloaddition or oxidation. Previously, thiol conjugation was coupled with an initial alkyne–azide cycloaddition reaction to modulate the nanocapsid surface with a cancer-targeting peptide [9]. Alternative covalent conjugation methods have been employed to modulate the capsid surface, such as amine coupling, reductive amination and amide-bound formation. Though the nanocapsid can be modified with these methods, these reactions are often not site-specific and limit the capacity to finely tune the capsid surface [10]. For example, *N*-hydroxysuccimide ester-functionalized ligands can bind primary amines such as lysine. Biological platforms often have numerous primary amine-binding sites, such as the nanocapsid, which exposes 240 lysine residues, limiting site-directed modulation strategies. The alkaline conditions required for *N*-hydroxysuccimide-primary amine coupling often compromise protein/conjugate structures. Conversely, the native HEV nanocapsid does not contain surface cysteines, so modified cysteines are the only available residues for thiol conjugation. Site-directed cysteine modification helps to control binding ratios and heterologous-binding interactions. In addition, thiol conjugation can be carried out physiological buffer conditions. Most cysteine modifications are on residues in the P-domain and C-terminus variable loops.

Genetic insertions of foreign peptides in the nanocapsid P-domain, particularly the C-terminus, stably expose foreign epitopes on the capsid surface [8,11,12]. This follows that cysteine-modified nanocapsids genetically inserted with cell-targeting, tracking or antigenic epitopes can be chemically bound to a pharmaceutical excipient. Genetic insertion of foreign peptides is therefore an additional tool to enhance surface molecule exposure as well as nanocapsid targeting and immune stability.

Patent specifications

The patent depicts an HEV_VLP nanocapsid platform modified with a surface cysteine for site directed, cova-

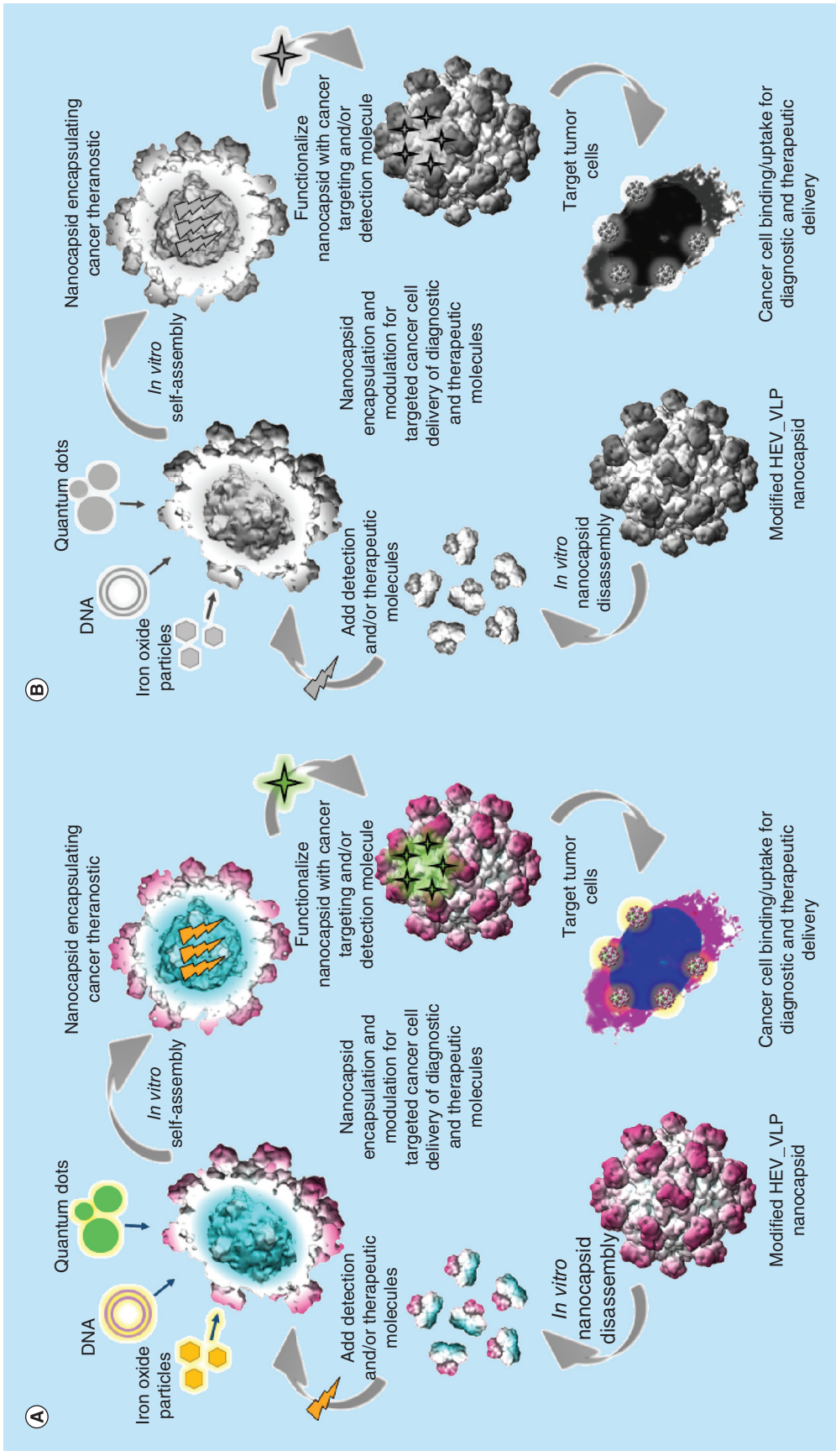

Figure 1. Schematic of nanocapsid modulation and delivery.

lent modulation and functionalization [13]. The invention comprises several constructs and applications of cysteine-modified nanocapsids (Figure 1). The patent is organized into eight specific aspects:

- The first aspect describes a proteolytic/pH-stable nanocapsid that self-assembles and contains at least one cysteine replaced on a protrusion domain variable loop or C-terminus. This includes variable loop/C-terminal cysteine modulation including alkylation, acylation, arylation, succinylation, cycloaddition or oxidation to a detectable label or bioactive agent;
- The second aspect depicts nanocapsid-anchoring bioactive/labeling reagents in conjunction with a pharmaceutical excipient, resulting in a heterologous multifunctional nanocapsid for detection and/or delivery and multicysteine site modifications and/or modulation;
- The third aspect includes the genetic sequence encoding the modified capsid protein;
- The forth aspect comprises a nucleic acid cassette and expression cassette with a heterologous promoter operably linked to the polynucleotide sequence encoding the modified capsid protein;
- The fifth aspect concerns the cell/organism containing the nucleic acid and/or expression cassette;
- The sixth aspect describes a method for high-yield production and purification of modified nanocapsids and postproduction modulation of functional groups;
- The seventh aspect encompasses a method for modification and modulation of nanocapsids for specific tumor targeting including at least one P-domain and/or C-terminal site for attaching a cancer cell-targeting moiety. Tumor targeting can coincide with heterologous-labeling strategies for multiligand conjugation such as fluorescent labels and metal/magnetic clusters;
- The eighth aspect addresses postproduction encapsulation of bioactive, detection and/or chemotherapeutic reagents such as DNA, nanometal clusters, magnetic nanoclusters, nucleic acids and/or fluorescent molecules. Encapsulation is a method to stabilize and delivering packaged theranostic molecules to a particular tissue type in coordination with chemically modulated VLPs for targeted delivery.

Applications of chemically activated nanocapsids

Diagnostics

In the initial study depicting surface cysteine modulation, five nanocapsid constructs were generated, each of which maintained stable icosahedral structure for chemical activation [9]. The selected cysteine replacement sites were in P-domain-variable loops (Figure 2) distal from the dimer interface to preserve capsid formation [8]. Epitopes with high surface exposure, identified through immunogenicity and cell-binding studies, were selected for cysteine replacement [14–16]. All five cysteine-modified constructs were chemically bound to maleimide-biotin and compared for streptavidin reactivity. While disassembled constructs all bound streptavidin equally, one assembled nanocapsid construct, NC_573C, generated the strongest streptavidin-binding signal. NC_573C was then selected to covalently bind a breast cancer-targeting ligand, LXY30. LXY30 was synthetically developed to target $\alpha 3\beta 1$, an integrin overexpressed in several cancer subtypes including breast cancer [17]. MDA-MB-231 breast cancer cell binding was compared between a LXY30-activated nanocapsid (NC_LXY30) and the nonfunctionalized NC_573C using flow cytometry and confocal microscopy (Figure 3A & B). These assays revealed that binding of NC_LXY30 was higher as compared with NC_573C. These constructs were subsequently compared in mouse models (Figure 3C). NC_LXY30 and NC_573C were separately injected into MDA-MB-231 breast cancer-xenografted mice, after which mice were fluorescently imaged at various time points postinjection. The NC_LXY30 fluorescent signal localized to the tumor site and remained 24-h postinjection, displaying significantly stronger tumor fluorescence than that of NC_573C. As a proof of concept, this study demonstrated the capacity for nanocapsids functionalized with cancer-targeting ligands to efficiently and specifically target tumor tissue *in vivo* [9].

One or more nanocapsid sites can be modified with cysteine for multipurpose functionalization. Particularly in the case of diagnostics, sensitive detection is equally critical to tumor cell targeting. While fluorescent *in vivo* imaging has been difficult to employ clinically, more recent fluorescent detection molecules such as quantum dots and upconverting nanoparticles have a distinguished advantage over classic organic fluorophores [18,19]. Such advantages include broad stimulation spectra, small size, low surface area to volume ratios and high quantum yield. Other nanomaterials have unique detection properties such as superparamagnetic iron oxide par-

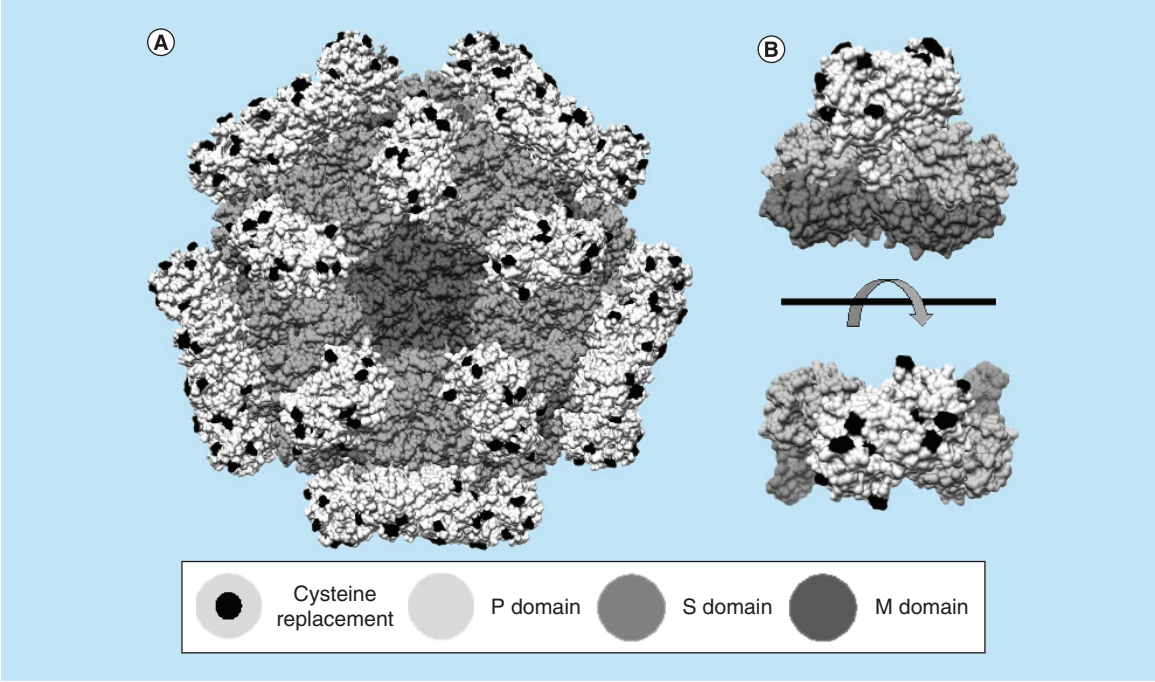

Figure 2. Thiol modification on nanocapsid surface.

ticles, positron emitting isotopes, gold nanoparticles and other MRI contrast agents that are useful for cancer theranostic applications [20–22]. Because of their documented benefits in cancer-cell detection, many functionalized compositions of these detection molecules, including maleimide/thiol functionalization, are commercially available. Attaching functionalized nanoformulations to a delivery platform is often necessary to prevent degradation and nonspecific cell uptake. A nanocapsid is conveniently sized to stably anchor both cancer-targeting ligands and synthetic detection molecules to the capsid surface. The resulting stabilized construct could provide a highly specific, highly sensitive cancer detection system. For example, gold nanoparticle formulations, such as $Au_{102}(pMBA)_{44}$ and $Au_{102}(C_6$-Maleimide), have been bound to the nanocapsid surface through previously described conjugation methods [13,23,24]. Many of the nanoformulations described above are multifunctional in diagnostic applications. Surface modulation can be extended to drug delivery in therapeutic applications.

Therapeutics

A modified nanocapsid can be reversibly disassembled and reassembled through chemical reduction and chelation, providing a means for *in vitro* encapsulation of theranostic nanomaterials. While the nanocapsid does not encapsulate viral RNA during production, plasmid DNA can be encapsulated in the capsid *in vitro*. Specifically, HEV_VLPs will disassemble into dimeric building blocks in a calcium-depleted solution composed of reducing and chelating reagents such as dithiothreitol (DTT) and ethylene glyco-bis(β-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA). Subsequent dilution of these reagents with a calcium-containing buffer will reverse the process, causing the nanocapsid to re-self-assemble. Plasmid DNA can then be added to the disassembled VLP solution, such that upon reassembly, DNA will be packaged in to the nanocapsid core. DNA-packaged nanocapsids can be delivered to cells for plasmid DNA expression [7]. It has previously been shown that plasmid DNA encoding an antigenic epitope for HIV Env protein was encapsulated in a modified nanocapsid construct. Oral administration of the nanocapsids packaged with HIV *env* DNA in mouse models elicited significant levels of IgG and IgA. Furthermore, the immunized mice exhibited a cytotoxic T-cell response specific to HIV Env [25]. These studies suggest the nanocapsid's potential as a nucleic acid delivery vector for gene therapy strategies in cancer treatment.

While virus-derived vectors have been studied as cancer therapeutics for over a century, interest in virus vectors surged following the discovery of oncolytic viruses (viruses that preferentially infect cancer cells) [26]. Recent pursuits in oncolytic viral therapy have implemented vectors such as herpes simplex virus, vaccinia virus, adenovirus and lentivirus. Oncolytic viruses, though highly effective in binding and transducing tumor cells, are nevertheless

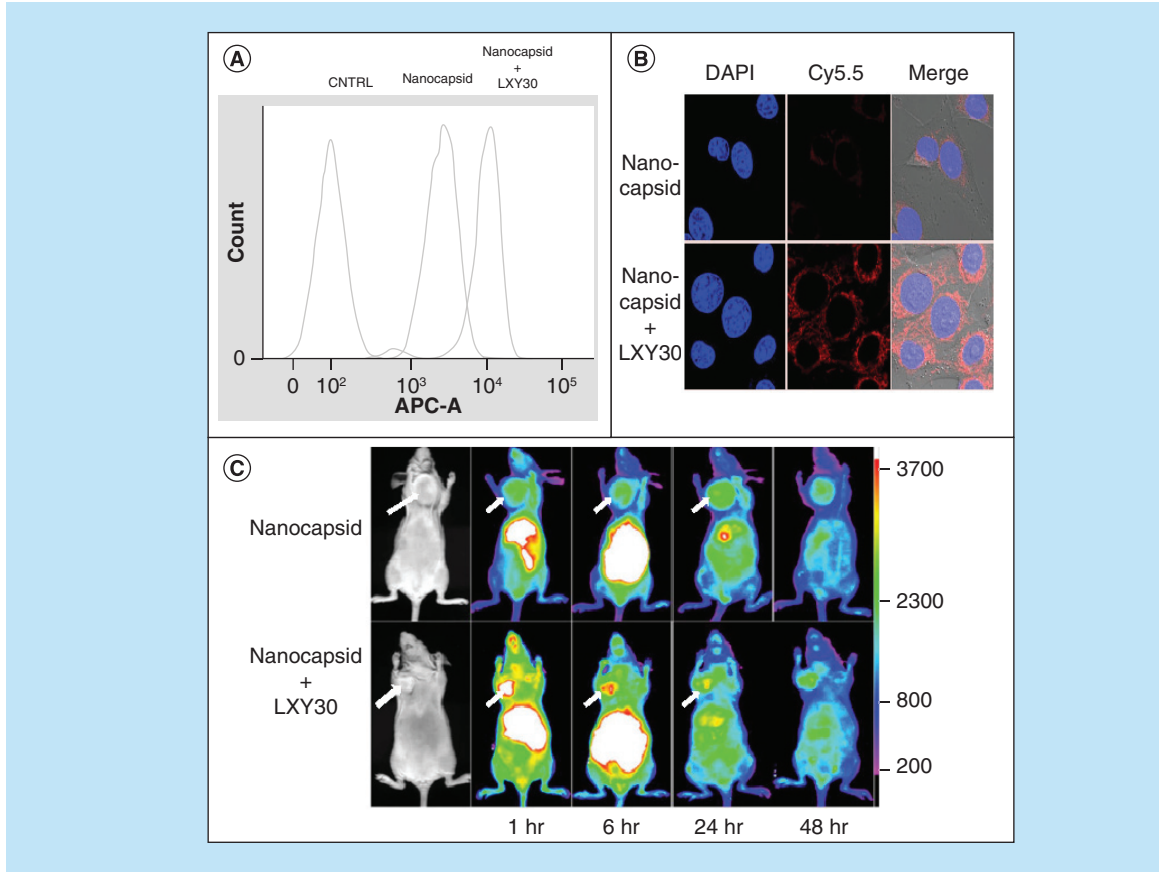

Figure 3. Breast cancer targeting with surface modulated nanocapsids.

infectious vectors, which can be a concern for toxicity and biosafety [27–29]. Conversely, nanocapsids harness mechanisms of mucosal stability, cell binding and cell entry evolved from the native virus but cannot cause infection or acquire pathogenic mutations. As such, nanocapsids combine the successful delivery properties of a virus without the side effects of infection.

In some cases of cancer delivery, nonpathogenic viral platforms, such as adeno-associated virus (AAV), that preferentially transduce tumor cells have been indicated in cancer gene therapy. AAV immunogenicity is a primary limitation to this delivery system, contributing to AAV vector degradation and low gene expression of AAV-transduced cells [30–32]. If immunotherapy is not the goal, this is problematic for effective gene delivery. Furthermore, AAV vectors have been associated with oncolytic mutagenesis insertion in hepatocellular carcinomas, presenting a potentially large risk to clinical use of these vectors ([33]). Nanocapsids are not infectious and cannot cause insertional mutagenesis. In addition, post-production modulation decreases background HEV immune detection as immunogenic sites on the HEV nanocapsid were intentionally modified for chemical activation. Genetic insertion and chemical modulation on the nanocapsid surface diminishes HEV-specific antibody recognition (Figure 4). In particular, genetic insertion into the protrusion domain depletes antibody recognition to immune-dominant sites on the nanocapsid surface [11]. In a previous study, binding maleimide-biotin to surface cysteines prevented high-affinity HEV antibodies from recognizing the nanocapsid [9]. Conjugation to cancer-specific ligands serves to both target cancer cells and reduce background immune response. Moreover, the antigenic surface of the nanocapsid could be chemically altered over the course of sequential treatments to avoid premature degradation.

Because of the complications described above, nonviral vectors have been extensively explored as potential alternatives for cancer therapeutics [34,35]. In addition to premature immune degradation and risk management concerns, many of the current viral vectors suffer from nonspecific cell uptake, despite high transduction in cancer cells. The opportunities in chemical modulation, mass production, immune evasion and biosafety provided by synthetic platforms have steered much cancer therapeutic development toward nonviral vectors [36]. Thus far, many of

these platforms have not been implemented clinically largely due to problems with aggregation and efficient theranostic transport. For example, polymeric micelles are self-assembling lipid structures that be attached to cancer-targeting ligands and package DNA for tumor-targeted delivery [37,38]. Though some micelle-derived platforms have been developed clinically, many exhibit passive cell uptake, physiological instability and heterogeneity [39]. Micelles have also been proposed for oral drug delivery, such that pH-controlled micelle degradation releases an encapsulated therapeutic for systemic circulation [40]. It is critical to note that oral micelle delivery is not compatible with specific tumor targeting, as therapeutics are released from the micelle vesicle in the GI tract. Surface modulatable nanocapsids take advantage of chemical modulation and ease of production, without losing the stability and cell-entry capacity of the native virus capsid. Importantly, orally delivered nanocapsids retain their structure for targeted delivery of an encapsulated therapeutic.

Oral delivery of chemotherapeutics has many clinical benefits including modulated drug release, maintenance of plasma drug concentration, practical convenience particularly for more chronic treatment regimens, reduced discomfort for patients with advanced-stage cancer and increased immunotherapeutic efficacy [41]. Many synthetic vectors, such as lipid-based platforms, lack mucosal stability and cannot effectively traffic nanotheranostic molecules in the gastrointestinal system. Derived from a feco/orally transmitted virus, the nanocapsid has been shown to withstand the proteolytic and low-pH environment of the mucosal system. Other modified nanoparticles, such as Cowpea mosaic virus (CPMV), have been proposed as potential cancer therapeutic vectors. However, compared with nonoral delivery methods, oral delivery of CPMV vectors dramatically reduced the level of CPMV particles in antigen-presenting cells by 10- to 100-fold [42]. In contrast, oral delivery of HEV nanocapsids have shown increased immune stimulation compared with nonoral delivery methods [25]. Surface cysteine modification has been carried out on CPMV nanoparticles as well; however, the initial cysteine-modified particles often formed disulfide bonds resulting in aggregation. Consequently, CPMV conjugation reactions require controlled reducing conditions or cysteine replacement on less exposed domains [43]. The smooth CPMV structure may contribute to aggregation, whereas surface conjugation to the hinged protrusion domain on the HEV nanocapsid structure does not appear to cross-link nanocapsids [9].

Conclusion

HEV-derived nanocapsids are highly stable, self-assembling vectors with multi-functional modulation capacity. The nanocapsid, while devoid of infectious viral elements, retains critical traits of HEV including gastrointestinal stability, epitope exposure, cell binding and entry. The combination of these biological characteristics with chemical modulation for specific cell targeting, immune manipulation, detection and *in vitro* encapsulation results in an exciting nanotheranostic platform for tumor homing and delivery.

Future perspective

MRI detection & thermotherapy

Nanocapsid encapsulation is not a sequence-specific interaction; rather, it is a charge interaction such that nonviral or other unrelated plasmid DNA can be encapsulated for therapeutic applications. Molecules for encapsulation are not limited to nucleic acids, as other negatively charged nanomaterials can also be encapsulated such as heavy metals and quantum dots. In addition to applications in MRI detection, encap-

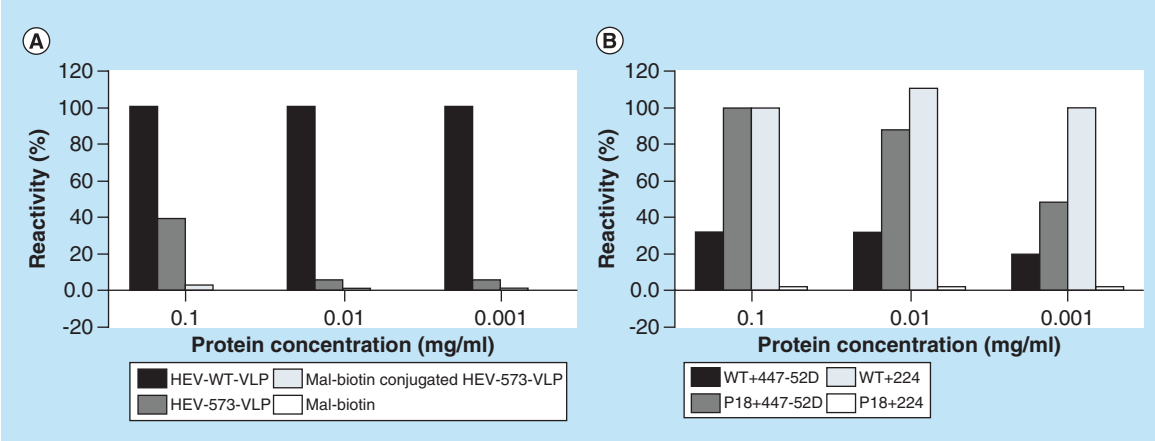

Figure 4. Antibody recognition of nanocapsids modulated to expose foreign peptides.

sulation of superparamagnetic iron oxides could serve in chemotherapeutic applications. The preliminary results described in the patent suggest the capacity for nanocapsids to package negatively charged quantum dots and Fe_3O_4 [13]. Magnetic nanoparticles are attractive in cancer therapeutics because of their biocompatibility and thermal activation behavior. Particularly in the case of FDA-approved iron oxide nanoparticles, a substantial amount of research has undergone into investigating *in vivo* circulation and heat activation [21,44]. Hyperthermia, a method of heat-induced cell death, is already established as a cancer treatment – this method exploits the increased heat susceptibility of tumor tissue compared with that of healthy tissues. However, healthy cells still incur damage during hyperthermia treatment due to passive delivery resulting in generalized heat dissipation. Much effort has gone into investigating applications of magnetic nanoparticles to localize heat dissipation to a tumor site; however, avoiding nanoparticle degradation and aggregation as well as targeting particles to cancerous tissue remain a challenge [45]. Magnetic particles encapsulated in nanocapsids would be detectable through MRI and could be activated to induce tumor-localized hyperthermia. Hence, encapsulating tumor-targeted nanocapsids with ferrite nanoparticles could prevent degradation and aggregation to of magnetic particles trafficked to the tumor site.

Immonotherapy

Developments in cancer immunotherapy are focused on harnessing the immune system to kill cancer cells.

Executive summary

Abstract

- This review highlights a recent patent that depicts a modified nanocapsid with chemical surface modulation for targeted cancer delivery.

General challenges in cancer theranostics

- Some limitations in cancer diagnostics and therapeutics (theranostics) include insufficient tumor specificity and vector instability.
- Nanoparticle theranostics (nanotheranostics) have advantages in unique detection and controlled activation.
- Many current nanotheranostic platforms are vulnerable to premature degradation and nonspecific cell uptake.

Background: virus-derived nanocapsid platform

- The Hepatitis E Virus (HEV) virus-like particle (HEV_VLP) forms a self-assembling, noninfectious nanocapsid.
- The modified HEV_VLP is highly stable and can anchor ligands on the capsid surface.
- The capsid surface can be chemically functionalized for cell targeting/delivery/detection.
- The empty capsid can encapsulate nanomaterials *in vitro*.

Patent specifications

- Eight specific aspects in the current patent outline modified VLP production as well as methods and applications in surface modulation, encapsulation and activation for cell targeting.

Site-specific surface modulation

- Cysteine substitutions on the capsid surface provide site-specific covalent-binding sites via thiol-coupling, maleimide-thiol and ligand exchange interactions.
- Modulation and modification can obstruct HEV-specific immune recognition.

Applications of chemically activated

- Diagnostics
  - A nanocapsid functionalized with a tumor-binding molecule, LXY30, targeted cancer cells in tumor xenograft mice detected through *in vivo* fluorescence imaging.
  - Heterologous molecules can simultaneously bind capsid surface for dual-purpose delivery such as targeting and tracking.
- Therapeutics
  - The various ligands that stably bind to protrusion domain demonstrate the flexibility of attaching theranostic materials to the capsid surface.
  - *In vitro* disassembly and reassembly can package nucleic acids in cavity of the nanocapsid.
  - Nucleic acid encapsulation and delivery by nanocapsids could serve as in cancer therapeutics without requiring virus infection.
  - Alternative delivery platforms and their comparative advantages are detailed in this section.

Future perspective

- Negatively charged nanotheranostics can also be packaged in nanocapsids, such as ferrite oxides, for applications in cancer theranostics.
- Cancer immunotherapy is of significant interest for the nanocapsid platform. This section revisits the immune-stimulatory potential of the VLP for cancer therapeutic applications.

Similar to drug resistance displayed in certain tumor tissues, immunological resistance also contributes to unregulated cancer cell growth [46,47]. Tumor cells often manipulate their local environment to suppress and evade immune recognition. Advances in cancer immunotherapy are considered a major breakthrough in cancer therapeutics. Because of the previously mentioned risks and limitations of oncolytic virus vectors, recent developments have focused on nonpathogenic and/or noninfectious virus vectors in cancer immunotherapy. Inherently immunogenic viruses and virus-mimetics serve as antigen-presenting vectors to stimulate an antitumor and/or tumor-localized immune response [48,49]. These vectors do not kill tumor cells directly through infection, but instead, act as adjuvants to mount an inflammatory response at the tumor site. Cancer-targeting nanocapsids could similarly trigger a tumor-localized immune response. One of the primary applications of these nanocapsids is in exposing foreign epitopes for vaccine development [11,12,25].

Particularly because of its mucosal stability, bioavailability and repeated surface epitopes, nanocapsids can induce a strong humoral and cellular immune response. Ideally, cancer-specific antigens covalently bound to the nanocapsid would further stimulate an antitumor immune response. The capacity to chemically modulate the capsid surface makes it possible to diversify the immune function of the nanocapsid vector.

Financial & competing interests disclosure

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