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# Cowpea mosaic virus nanoparticles for cancer imaging and therapy

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ABSTRACT

Nanoparticle platforms are particularly attractive for theranostic applications due to their capacity for multifunctionality and multivalency. Some of the most promising nano-scale scaffold systems have been co-opted from nature including plant viruses such as cowpea mosaic virus (CPMV). The use of plant viruses like CPMV as viral nanoparticles is advantageous for many reasons; they are non-infectious and nontoxic to humans and safe for use in intravital imaging and drug delivery. The CPMV capsid icosahedral shape allows for enhanced multifunctional group display and the ability to carry specific cargoes. The native tropism of CPMV for cell-surface displayed vimentin and the enhanced permeability and retention effect allow them to preferentially extravasate from tumor neovasculature and efficiently penetrate tumors. Furthermore, CPMVs can be engineered via several straightforward chemistries to display targeting and imaging moieties on external, addressable residues and they can be loaded internally with therapeutic drug cargoes. These qualities make them highly effective as biocompatible platforms for tumor targeting, intravital imaging and cancer therapy.



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## 1. Introduction

Many different scaffold and carrier systems have been co-opted from nature, or synthetically designed for use in tumor cell-specific imaging and drug delivery (reviewed in [1–7]). Naturally-occurring scaffold-carriers include viral nanoparticles (VNP) and self-assembling protein cages [7–13]. VNPs such as temperate filamentous bacteriophage (M13, fd), lytic capsid and tailed bacteriophage (T4, P22, λ etc.) or plant viruses; brome mosaic virus (BMV), red clover necrotic mosaic virus (RCNMV), potato virus X (PVX), tobacco mosaic virus (TMV), cowpea chlorotic mottle virus (CCMV), cowpea mosaic virus (CPMV) and mammalian viruses; canine parvovirus (CPV), influenza A and hepatitis B have been used as scaffolds and cargo-carriers in cancer research [7,9–11,14–18]. Supramolecular, self-assembled protein cages such as heat shock protein (Hsp), ferritin, and vault have also been utilized as drug delivery nanoparticles [8,19]. In addition, medically relevant, organic and inorganic synthetic nanoparticles have been designed; nanobombs, nanoworms, micelles, liposomes, dendrimers, dendrons, superparamagnetic iron oxide nanoparticles, gold nanoparticles and quantum dots [5,20–22]. Although these various carriers differ in shape and macromolecular components they share three essential features; (1) consist of uniform size distribution within their type, (2) act as molecular scaffolds to display different functional moieties, and (3) act as drug delivery vehicles due to their internal cargo carrying capacity [5,7]. Possibly the largest advantage of using any of these nanoparticles in cancer research, diagnosis and therapy is their multifunctionality. Nanoparticles, including VNPs like CPMV, can simultaneously display imaging probes, targeting or homing moieties and carry a chemotherapy drug as cargo for maximum anticancer activity [5]. For example, non-invasive, intravital, vascular imaging has been problematic as a tumor detection and diagnostic tool because of inadequate resolution and poor fluorescent dye tissue penetration [23]. However, the VNP scaffold multifunctionality allows for high density display of fluorescent dye molecules and targeting ligands simultaneously with the ease of VNP extravasation through leaky tumor blood vessels [24]. The fluorescent nanoparticles remain bright, without detectable quenching, which increases the resolution, and can target deep tissue vascular endothelial cells for up to 72 h [23,25].

Which carrier system to use for tumor imaging and or drug delivery depends on the physiochemical and pharmacokinetic properties of the carrier, biological distribution of the tumor, immunogenicity between the carrier and host, and the ratio of toxicity between host diseased cells to host healthy cells.

There are many features of CPMV nanoparticles that make them good candidates for both chemical and genetic engineering for development as cancer imaging and therapy tools. These features include their non-pathogenicity, biocompatibility, non-aggregation and biodegradability in mammalian systems, temperature and pH stability, ease of external functionalization by either single or multiple functional group display, cargo loading capacity, native tropism towards vimentin on endothelial cells and their native immunostimulatory effect within solid tumor cancer models. In addition, CPMVs have been fully characterized by X-Ray crystallography, electron microscopy and genome sequencing and protocols have been developed to produce CPMVs using in vitro and in vivo methods. This review discusses some of the uses of CPMV nanoparticles for the use in research and development of diagnostic, therapeutic and theranostic cancer tools. Table 1 lists some of the advantageous features of CPMV for use in tumor cell imaging and therapy. Table 2 highlights some examples of functional group classes that have been reported in the literature as being conjugated to CPMV.

**Table 1**
Advantageous features of CPMV for use in tumor cell imaging and therapy.

| Features | Measurement or description | Reference |
|---|---|---|
| Non-pathogenic and non-toxic | Dosages up to 100 mg/kg body weight in mice ($10^{16}$ CPMVs) are nontoxic. | [23,27] |
| Biodistribution | After dosing in mice, found in various organs, culminating in the liver and spleen. | [40,47] |
| Biocompatible and long retention | CPMV were retained for 72 h in chick embryo endothelium system and in mice for 1 to several days. | [23] |
| Biodegradable | CPMVs do not persist in vivo, therefore they are good candidates for therapeutic use. | [55] |
| Physical and chemical stability | Temperature: up to 60C, pH range 3 to 9, organic solvents such as dimethyl sulfoxide, resistant to proteolysis and gastric and intestinal conditions. | [7,33,40] |
| High resolution and non-aggregating | Dye-labelled CPMV nanoparticles did not aggregate, allowing for high resolution imaging. | [23] |
| Fully characterized | RNA-1 and -2 genomes and gene products sequenced and annotated. X-ray crystallography and cryogenic electron microscopy done on capsid | [32,57,118] |
| In vivo production of CPMV | CPMVs with encapsidated RNA genomes. High yield: 0.8 to 1.0 mg CPMV per g infected cowpea leave tissue | [7] |
| In vitro production of eCPMV | CPMV VLPs via trans expression of L-S subunit fusion protein and 24 K protease in cowpea protoplasts or insect cells. High yield: 1 g pure eCPMV per kg fresh-weight *N. benthamiana* leaf tissue | [28,82,89] |
| Ease of functionalization | Synthesis and purification of dye-labelled CPMV nanoparticles in one day, using either standard or click chemistry | [57] |
| External conjugation with, lysine residues | 60 asymmetrical protein units per capsid with 5 solvent-exposed lysine residues per protein unit, providing a total of 300 potential conjugation sites per nanoparticle. | [33,57] |
| Single functional group display | CPMV labelled with multiple copies of a single functional group (e.g. 120 copies of fluorescent dye) made with no detriment to the signal. | [57] |
| Multiple functional group display | CPMV labelled with single copies of multiple functional groups made with no detriment to targeting. | [57] |
| Native tropism | WT-CPMV nanoparticles bind to vimentin on endothelial cells | [112] |
| Internal conjugation | The thiol side chains of 2 cysteine residues are ligation handles for internal conjugation. | [112] |
| Cargo loaded capsid capacity via infusion | 130 to 155 dye or drug molecules per CPMV nanoparticle. | [27] |
| Cargo loaded capsid storage stability | Cargo-loaded CPMVs stored in buffered saline, pH 7 at 4 °C were stably encapsulated for weeks. | [27] |
| Deep tissue visualization | Intravital imaging of large vessels to a depth of 500 μm or microvasculature to 200–250 μm for up to 72 h. | [23,57] |
| In vitro studies | Yolk sac and embryonic vasculature (vascular endothelium and blood flow) of explanted mouse embryos. Various plate assays. | [107] |
| In vivo studies | Tumor cell xenografts of chick embryo chorioallantoic membrane and adult mouse vascular endothelium. | [23] |
| Immuno-stimulatory effect | CPMV can induce potent antitumor immune response after in situ vaccination in models of skin, ovarian, breast, and colon cancers CPMV may also eliminate M2 macrophages. | [107] |

## 2. Characteristics of the cowpea mosaic virus

The cowpea mosaic virus (CPMV) is a picorna-like virus of the order *Picornavirales*, family *Secoviridae* and genus *Comoviridae* that naturally infects the black-eyed pea plant *Vigna unguiculata*. CPMV is an isometric, icosahedral lattice capsid with pT = 3 quasi symmetry, approximately 30 nm in diameter, with a net negative surface charge and a molecular weight of $5.6 \times 10^6$ g mol$^{-1}$ (Fig. 1) [3,11,19,23,26]. The capsid shape provides a large surface area to volume ratio, which is advantageous for enhanced multifunctional group display and cargo (or payload) carrying capacity [27]. CPMV has a bipartite, positive-sense RNA genome comprised of RNA-1 (5.89 kb) and RNA-2 (3.48 kb) molecules that are encapsidated separately [19,27–29]. As

**Table 2**
Some examples of functional group classes conjugated to CPMV capsids.

| Class of functional group | Description | Reference |
|---|---|---|
| Shielding groups | | |
| Polyethylene glycol | Inert polymers that add an immunity-response shielding 'stealth layer' to the particles. | [7,19,23,33,73] |
| Carbohydrates, lipids and proteins | Alternative, nature-inspired shielding materials. | [75] |
| Imaging groups | | |
| Fluorescent dye molecules | Alexa Fluor 647, A555, Oregon Green 488 | [20,23,40] |
| PEG500f | Peptide with a fluorescein, monodisperse PEG polymer and hydrazido terminus. | [54] |
| Gadolinium ions | Contrasting agent platforms. CPMVs can be decorated with hundreds of $Gd^{3+}$ ions. | [55,64] |
| Targeting/homing groups | | |
| Vimentin | CPMV shows natural targeting of surface exposed membrane vimentin. | [54,99,109,110,112] |
| Folic acid | Folic acid ligand targets cancer cells in vitro. | [100,101] |
| Human-holo-transferrin protein | Targets transferrin receptors that are overexpressed on a variety of cancer cells. | [67] |
| Pan-bombesin peptides | Targets gastrin-releasing peptide receptors overexpressed on prostate carcinoma cells. | [20] |
| Peptide F56 | Targets specifically to VEGFR-1 receptors on tumor endothelial cells. | [54] |
| E7p72 peptides | Targets EGFL7 protein expressed by endothelial cells undergoing vascular remodelling and not by quiescent cells. | [25] |
| complex sugars, peptides | Variety of other molecules conjugated to CPMV. Reviewed in references listed. | [2,11,58,67,69] |
| Therapeutic groups | | |
| $C_{60}$ Buckyball | Photosensitizer. | [103] |
| Zinc ethynylphenyl porphyrin | Photosensitizer, conjugated to CPMV-dendron hybrid nanoparticle. | [84] |

well, approximately 10% of wildtype (WT), intact, viral particles can be found empty of RNA [19]. Density gradient centrifugation separates these CPMV particles into a top (T), middle (M) and bottom band (B) that are; empty particles (CPMV-T), RNA-2 carrying particles (CPMV-M) and RNA-1 carrying particles (CPMV-B), respectively [27,30].

RNA-1 encodes: proteinase K cofactor (ProC), helicase, virus genome-linked protein (Vpg), 24K proteinase and RNA-dependant RNA polymerase. RNA-2 encodes, 48/58 K movement protein and VP60 which is post-translationally cleaved by the RNA-1 encoded 24K protease to form the two capsid protein subunits; L and S (Fig. 2). Each linear RNA genome has a Vpg cap at the 5′ end and a polyadenylation tail at the 3′ end [31]. The two capsid protein subunits are called the small (S, 24 kD) and large (L, 42 kD) subunits, with one (A) and two jelly roll β-barrel domains (B and C), respectively [11,19,32,33]. This jelly roll β-barrel polypeptide structure of two twisted, antiparallel β-sheets, is common amongst Comoviruses and presents as externally and internally extended loops on the capsid [19,26]. Together, domains A, B and C, comprise one asymmetrical unit with each capsid containing 60 units total (Fig. 1) [11].

In nature, beetles from the Subfamily Galerucinae and Family Chrysomelidae that feed on the leaves and stems of virus-host legumes such as *V. unguiculata* act as vectors to transmit CPMV from host plant to host plant, with the severity of plant host infection positively correlating with the beetle feeding rate [34]. There is no evidence that the carrier beetles contract disease from CPMV. Viable CPMV infection of *V. unguiculata* is only mediated when both B and M nanoparticles are introduced into plant leaves or stems together, and the empty particles are non-infectious [27].

### 2.1. CPMV biocontainment, biodistribution and pathology

The approval of the use of viral-based therapeutics in humans in North America has been slow due to concern that the viruses, even attenuated versions, could develop higher virulence, adapt to new hosts or revert to wildtype because of their high mutation and recombination rates [35]. Mammalian viruses like adenovirus, herpesvirus and lentivirus have been developed as successful therapeutic agents against multiple different diseases in humans (reviewed in [36]). However there have been examples of these viral-based therapies causing harm or even death in treated patients [37]. Concerns have also been raised about using RNA-virus based therapeutics due to the potential for activation of human proto-oncogenes or inactivation of tumor-suppressor genes via mutagenesis from viral RNA integration into the human genome [6]. Bacteriophage and plant virus-based VNPs, unlike mammalian viruses, are non-infectious towards mammals and so are considered nontoxic to humans and safe for use in intravital tumor cell imaging and drug delivery. However, CPMV is a picorna-like virus similar in genetic synteny and protein capsid structure with animal picornaviruses such as polio viruses, coxsackie viruses and Theiler's murine encephalomyelitis virus [38]. Certainly, there are some viruses that have host ranges that include plants and animals [39]. As well, CPMV particles will bind to the surface of vertebrate endothelial cells and become internalized into the cells, although they cannot replicate. This feature allows the virus to be used as an imaging and drug delivery nanoparticle [20]. CPMV particles also remain intact after administration in vivo, even through the gastrointestinal tract, therefore there is a potential path for CPMV leakage from experimental animals or patients to plants and the environment [28,40–43]. Although CPMV particles recovered from mice tissues or incubated with plasma or serum were unable to cause an infection cycle in plants [43]. Taken together, although the risks are very low, it is considered prudent to protect patients, humans and the environment from inadvertent mutation, new host adaptation, off-target effects and escape into the environment from therapeutic use of CPMV therefore bio-containment measures should be employed with in vivo CPMV therapy [6,44].

CPMV is resistant to most of the common viral attenuation protocols, however, exposure to 254 nm UV radiation for short time doses was sufficient to crosslink the encapsidated RNA genome, and therefore inactivate the virus, while still retaining protein structure and function [19,42,45]. However, since *in planta* CPMV infection necessitates the presence of the bipartite RNA genomes within the capsids to allow for expression of the viral gene products needed for replication and capsid production, removing the RNA genomes would render CPMV non-replicative [46]. The use of non-replicative, CPMVs would obviate the need for bio-containment and therefore allow therapeutic usage of medically effective CPMVs in humans. Of course, these non-replicative CPMV versions need to maintain the capsid structure, function and chemical reactivity of the WT-CPMV nanoparticles [42,45].

The plasma clearing, biodistribution and toxicity of CPMV in mice dosed either orally or intravenously and in chick embryos intravenously has been studied with varying results [23,40,47]. CPMV inoculated into chick embryos was detected and internalized within the vascular endothelium system [23]. In mice intravenously dosed with 100 μg of fluorescently labelled CPMV, the nanoparticles were detected in the spleen, kidney, liver, lung, stomach, small intestine, lymph nodes, bone marrow, vascular endothelium and brain for several days after administration [40]. Oral dosage of the same amount and labelling of CPMV in mice

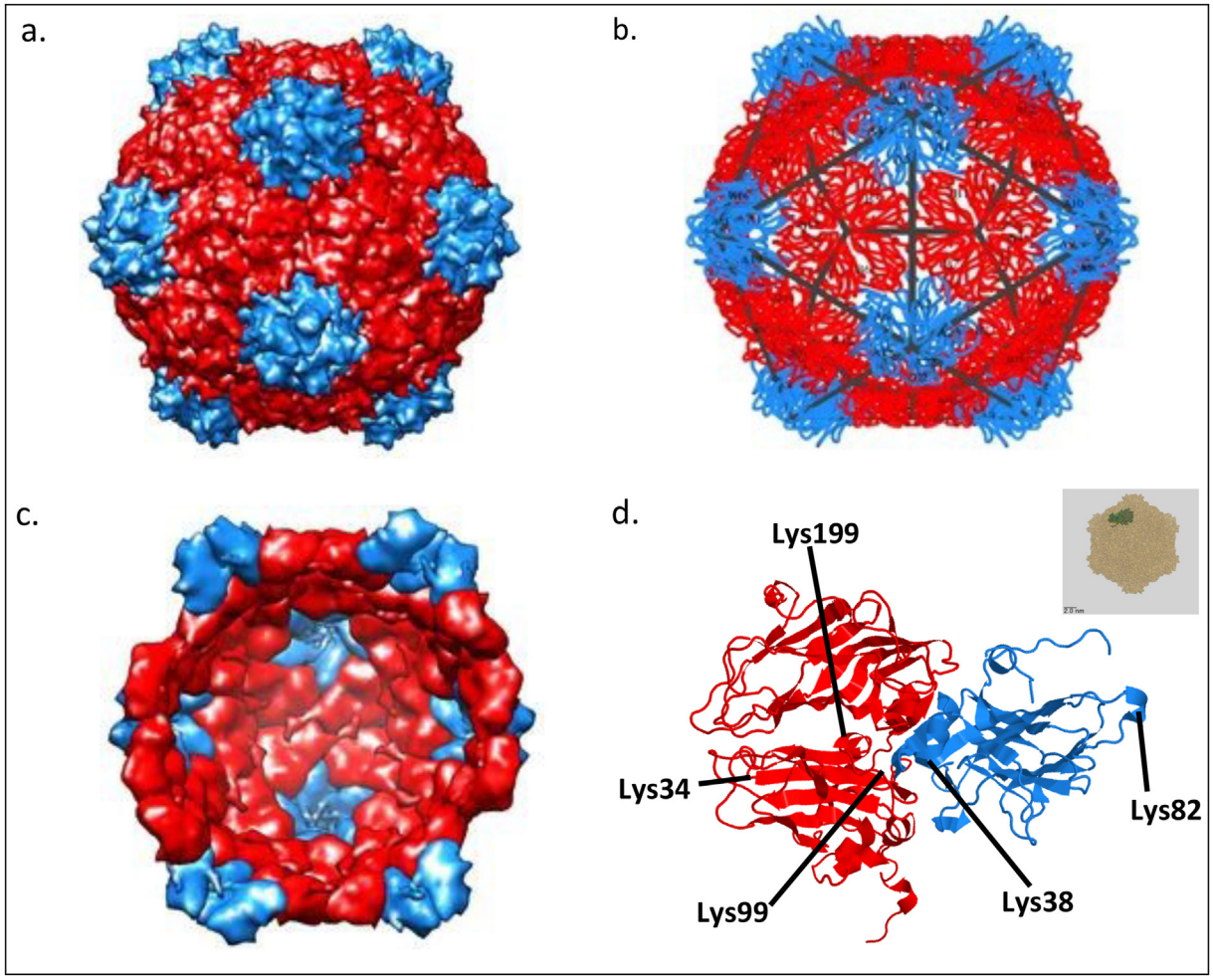



**Fig. 1.** CPMV images. (a) Subunit organization of capsid at 3 Angstrom resolution, (b) CPMV T = pT3 lattice scaffold, (c) Cut-away inside view of CPMV, (d) the one domain of the S (small) subunit (blue) and the two domains of the L (large) subunit (red). The S and L subunits comprise one asymmetrical unit. The CPMV capsid is made of 60 asymmetrical units. The inset image shows the location of one asymmetrical unit on the capsid. The five addressable lysine residues that are commonly used as ligation handles are labelled. The figures/data were obtained from VIPERdb (http://viperdb.scripps.edu). [119].

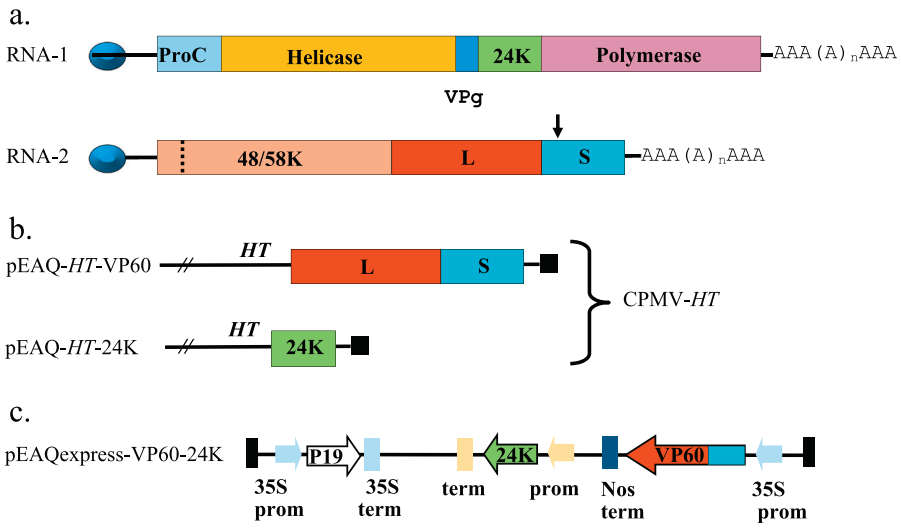



**Fig. 2.** (a) The CPMV bipartite RNA genome organization. The arrow in RNA-2 points to the location in the nucleic acid sequence that encodes the βB-βC loop in the S subunit, which is a site for insertion of a nucleic acid sequence encoding a peptide to be externally displayed on the capsid. (b) Two vectors that were designed to allow high expression levels of the VP60 and the 24K protease genes *in planta*, to produce eCPMV, together these vectors are termed CPMV-*HT*. (c) pEAQexpress-VP60-24K is a vector designed for transformation into *N. benthamiana* plants for transient expression of the L and S subunits in *N. benthamiana* leaves, allowing for in vitro production of eCPMV nanoparticles.

showed a similar biodistribution as the intravenous route except for a lower level in brain tissue. CPMV labelled with gadolinium (Gd) chelate or $Gd^{+3}$ and $Tb^{+3}$ ions complexed directly to the capsid were quantitatively measured in mice after intravenous administration of one, ten and 100 mg CPMV per kg body weight [47]. By 20 min after injection there were no detectable CPMV particles in the plasma and by 30 min most of the CPMV particles were found in the liver and spleen. Encouragingly, even after administration of the highest dose of decorated CPMV, the mice showed no toxicity or behavioral changes, although the hematology analysis showed that the mice were slightly leukopenic [47].

### 2.2. Chemical bioconjugation

Capsid virus nanoparticles like CPMV can be chemically functionalized on three surfaces of their capsid structure: inside, outside or the subunit interface [4] (reviewed in [48,49]). Functionalization requires the presence of chemically-reactive ligation handles on the capsid and complementary chemically reactive functional group (s) that will allow for an efficient conjugation reaction to proceed [48,50]. There are a few external, solvent exposed, amino acid residues per asymmetric unit that have been experimentally proven to be addressable sites for chemical derivatization on the CPMV capsid; 5 lysine residues (300 per capsid), 8 to 9 aspartic and glutamic acid carboxylate groups (480–540 per capsid) and 2 tyrosine residues (120 per capsid) [11]. The number of internally exposed addressable residues is limited to 2 cysteine residues per asymmetrical unit, one on the L subunit (Cys 295 and one on the S subunit (Cys 4) [11,51]. It is important to remember that although viral nanoparticles like CPMV are drawn as inflexible shells, they are actually dynamic structures that can undergo reversible transitions [51]. The addressable lysine residues (Lys 38, 82, 99, 34 and 199) have been used extensively in cancer research as ligation handles by derivatizing the side chain amino group with activation reagents and so will be the focus of this review (Fig. 1) [33,52,53].

There are two general chemical strategies employed to introduce ligation handles to the side chains of selected addressable residues; standard (also called traditional) chemistry and Click chemistry. These chemistries derivatize the amino acid residue with a reactive chemical group that adapts them to become ligation handle sites for functional moiety decoration of the capsid [14,54]. Some commonly used standard chemistry activation reagents include N-hydroxysuccinimidyl ester (NHS), maleimide, isothiocyanate and carbodiimide [11,55,56]. The chemical nature of the activation reagent used depends on the chemistry of the amino acid residue to be used as the ligation handle (Fig. 3) [23,55,57]. Standard chemistry activation reagents have slow kinetic properties, therefore excessive amounts of the reagent must be used to ensure that all of the addressable residues are derivatized and can participate as ligation handles, which limits the functional groups to low molecular weight compounds like dyes [14,58]. The position of the lysine in the asymmetric unit also influences derivatization density in that some lysine residues are preferentially derivatized before others.

Click chemistry is a term that describes a set of versatile, covalent organic reactions that are used to “click” chemical fragments together in a way reminiscent of LEGO pieces to form complex molecules from simple primary chemical components (reviewed in [59,60]). Click reactions are orthogonal because although the reagents are highly reactive with each other and form quantitative yields of product, they are also highly selective and therefore unreactive with a broad range of other functional chemical groups under mild conditions [61]. One of the most often-used types of click reactions for the derivatization of CPMV nanoparticles is the copper (I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC) that makes a thermally stable triazole linkage that is inert to oxidation, reduction and hydrolysis [62–65]. CuAAC has a high thermodynamic driving force which catalyses an irreversible conjugation reaction, even with low concentrations of high molecular reagents [11,66,67]. Therefore, high molecular weight functional groups can be conjugated to addressable capsid residues. The external solvent exposed CPMV residues are first converted to active ligation handles by a chemical reaction with N-(4-pentynoyloxy) succinimide to derivatize them with an alkyne group. Then the azide-containing functional moiety is reacted with the alkyne to perform the click chemistry reaction. Alkyne and azide containing molecules are virtually absent from biological systems, so the CuAAC reaction can be considered bioorthogonal since these two reactive partners will not chemically interact with other chemicals found within the cell [59]. The allows for CuAAC reactions to be done under physiological conditions and in vivo without the production of toxic by-products [59]. Another type of click reaction prevalent in CPMV derivatization is hydrazone ligation between aldehyde groups and either hydrazide or alkoxyamine groups. This derivatization strategy has been successfully used to conjugate a vascular endothelial growth receptor-1 targeting peptide to CPMV nanoparticles, which is discussed further in Section 4.5 [54].

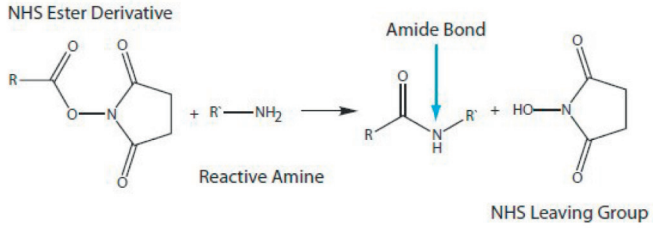
Multi-functionalized CPMV, also termed ‘smart’ tools, allow for the design of nanoparticles as theranostic tools for use in both diagnosis and therapy [20]. These capsids may be conjugated with functional moieties for imaging and targeting and may also carry a drug payload for delivery to tumor cells (reviewed in [48]). There are multiple different approaches to making a CPMV nanoparticle smart tool, which really highlights the versatility of these VNPs. The concentration of activation reagent dictates the number of ligation handles that are activated and available for conjugation, therefore one approach is to activate a fraction of the lysine residues at a time by using a low concentration of the activation reagent and then conjugating the chemically reactive functional groups to these ligation handles in sequential steps [58,68]. Another method is “one-pot” chemistry that takes advantage of the orthogonal nature of click chemistry to combine two or more click reactions simultaneously, such as CuAAc and hydrazone ligation [60]. A totally different approach to multi-functionalization was described in Section 2.5. with the cell-free protein synthesis platform.

Overall, the activation reagent kinetics, the varying degree of lysine derivatization and the use of orthogonal linkage chemistries have been used advantageously to conjugate the addressable lysine residues with more than one type of functional group in a graduated, stepwise derivatization procedure [54,60].

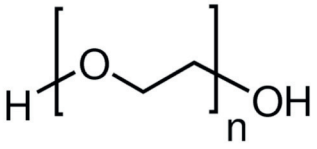
CPMV conjugation reactions are done in aqueous solutions, therefore if the peptide ligand to be displayed on the CPMV external surface is hydrophobic in nature its solubility will be low, resulting in low bioavailability, poor conjugation efficiency and unstable CPMV-peptide ligand display [20]. However, the conjugation efficiency of hydrophobic ligands can be maximized by conjugating the ligands to the terminal ends of PEG polymers so that the hydrophobic ligands are distal to the nanoparticle surface. In addition to this tethered distancing, conjugating the hydrophobic ligands to PEG first and then the hydrophobic ligand-PEG group to the capsid at the addressable lysine residues using click chemistry, also maximizes the conjugation efficiency.

CPMVs reported in the literature for cancer research have been functionalized with a variety of different sizes of PEG, ligands, peptides, epitopes, carbohydrates, metal cofactors, synthetic polymers, photosensitizers, and dyes (some of these are depicted in Fig. 3) [2,66]. CPMVs have also been designed and tested for uses other than cancer diagnosis and therapy, such as controlled fabrication of CPMV solid support arrays for receptor-ligand recognition and binding and organometallic moiety CPMV decoration for nanoscale electricity [69,70].

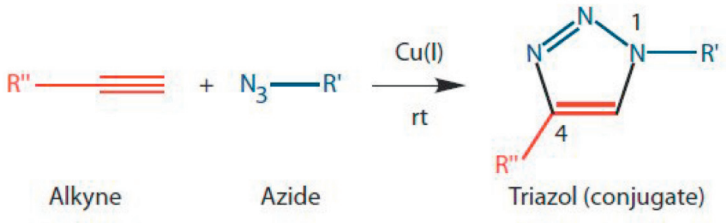
Fluorescence labelling of CPMVs is verified using UV–vis absorption spectroscopy and the number of dye molecules per capsid (degree of labelling) is calculated using the Beer-Lambert law and the specific extinction coefficients for the CPMV nanoparticle and the fluorophore [12]. Unlabelled CPMVs show UV–vis absorption peaks at 170 nm and 270 nm. The fluorescently labelled CPMV will be detected by the presence of the absorption peak for its particular fluorescence, for example AF647 dye molecules have one absorption peak at 660 nm. CPMV-AF647 will therefore show three peaks in the UV–vis spectra; 170 nm, 270 nm and 660 nm [71]. Functionalized CPMVs can be characterized



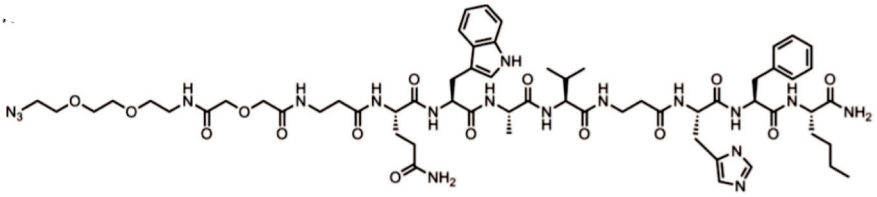

a. N-hydroxysuccinimidyl ester (NHS) activation of lysine on CPMV



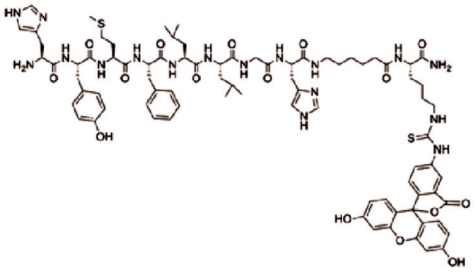
b. Polyethylne glycol (PEG) monomer



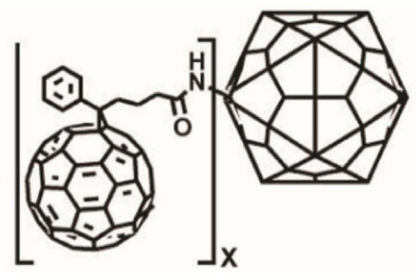

c. Click chemistry: Cu catalyzed coupling reaction of an azide and alkyne forming the triazol conjugate.



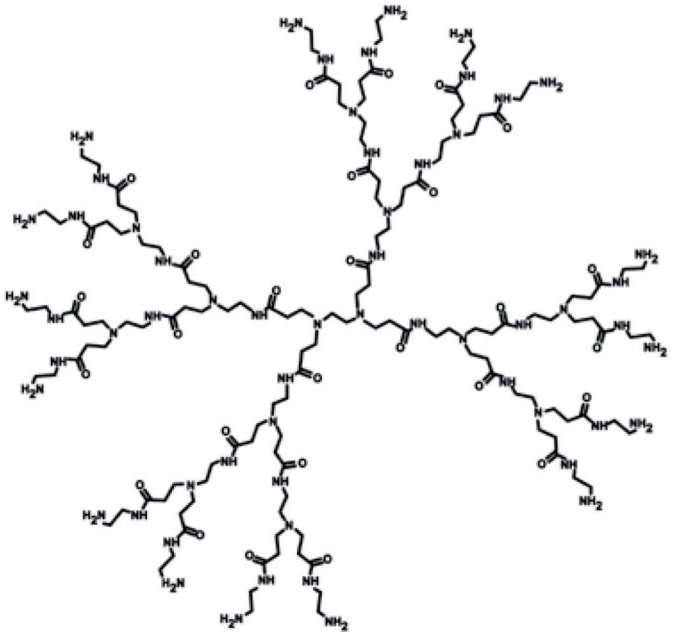
d. Modified pan-bombesin peptide targeting gastrin-releasing peptide receptors



e. Epidermal growth factor-like domain 7 binding peptide; E7p72



f. $C_{60}$ fullerene conjugated to CPMV



g. Polyamidoamine (PANAM) dendrimer

**Fig. 3.** (a) Traditional NHS activation reagent to derivatize the amine groups of addressable residues for conjugation, (b) ethylene glycol monomer, (c) copper catalyzed Click Chemistry to derivatize addressable residues for conjugation, (d) chemical structure of the modified pan-bombesin peptide, (e) chemical structure of the epidermal growth factor-like domain 7 binding peptide, (f) the C60 (Buckyball) fullerene modified to CPMV and (g) the polyamidoamine dendrimer conjugated to CPMV.

via indirect methods like banding patterns with sucrose gradient centrifugation and size exclusion chromatography and they can be visualized using transmission electron microscopy (TEM) to determine if the capsid structure is intact after chemical modification [14,43]. Denaturing SDS-PAGE is also often used to measure the molecular weight of purified labelled CPMVs so as to confirm that the functional labels are covalently attached [14]. For PEGylated CPMV nanoparticles either optical and chromatographic methods or gravimetric analysis is generally used to determine the degree of PEGylation [58,72,73].

### 2.3. Shielding of CPMV nanoparticles for increased bioretention

In vivo studies show that CPMVs elicit an immunogenic response in humans and that the mammalian reticuloendothelial system clears protein-based nanoparticles from the circulation system before reaching the target cells [53,73–75]. To mitigate these undesirable outcomes CPMVs are coated with inert polymers that add an immunity-response shielding 'stealth layer' to the particles [7,19]. Polyethylene glycol (PEG) polymers are commonly used as the stealth layer by conjugating the polymers to external addressable residues on the capsid, a process called PEGylation (Fig. 3) [23]. The water soluble, biocompatible, non-ionic PEG polymers comprise different numbers of straight or branched chain ethylene glycol monomers with various terminus chemical caps, and therefore differ in size, shape and molecular weight [53]. PEGylation has been shown to increase the in vivo half-life of CPMV in plasma, extend their circulation time and therefore increase accumulation in tumors, a process that is called the enhanced permeability and retention effect (EPR) [74,76]. These characteristics of CPMVs enable them to be effective tumor imaging and drug delivery tools. Further enhancement of these beneficial characteristics has been successfully done by coupling with longer chain or branched PEG polymers, increasing the density of PEG polymerization on the capsid surface or changing the terminus moiety [73,77]. PEGylation stealth layers can also reduce CPMV off-target actions [8]. The targeting ligands on functionalized CPMVs can also be bound to the PEG polymer at either the distal or proximal capsid side to increase target recognition or uptake by the target cell [53,73].

Recently there have been reports of PEG-specific antibodies found in human sera [75]. PEG antibodies would stimulate the hosts' immune system to tag the nanoparticles, leading to accelerated blood clearance (ABC) and reduction of PEGylated nanoparticle efficacy [75]. Alternative, nature-inspired shielding material like carbohydrates (heparin, hyaluronic acid and other glycosaminoglycans, sialic acid polymers), lipids (red blood cell membranes, white blood cell membranes, platelet membranes, synthetic membrane wraps), and proteins (serum albumin, CD47 cell membrane glycoprotein, elastin-like peptides, synthetic peptides, zwitterionic peptide) have been conjugated to CPMVs using click chemistry [75]. These decorated nanoparticles were tested in a variety of systems for immune recognition avoidance and showed positive results as camouflage agents [75].

### 2.4. Genetic engineering to introduce functionality

CPMV separately encapsulates the RNA-1 and RNA-2 genomes that carry the genetic code for the viral proteins, therefore this technology allows for a genotype-phenotype linkage similar to phage display technology. DNA encoding the recombinant peptide is cloned into cDNA vectors of the RNA genomes (Fig. 2). Cloning sites for DNA insertion have been engineered into the VP60 gene so that the translated functional peptide moiety is displayed on externally-exposed β-barrel loops, as a chimera [19,26,78]. Functional group exposure via genetic mutational insertion can either display the peptide on the highly surface-exposed βB-βC and βC'-βC" loops on the S subunit or the less surface-exposed βE-βF loop on the L subunit [20,79]. The genetically-modified, chimeric viral capsid retains form, infectivity and yield comparable to WT-CPMV if the recombinant peptide sequence is 40 amino acids or less and has an isoelectric point of 9 or less [20,26]. These externally exposed recombinant peptide sequences have been tested experimentally as targeting peptides, receptor-association peptides and epitopes for antibody production [26].

The CPMV VP60 gene sequence has also been genetically modified to introduce mutant, surface exposed cysteine residues for use as addressable thiol sites on the capsid [20]. Thiols are reactive groups that can be easily functionalized by conjugation with a variety of chemical moieties. These genetically modified CPMV nanoparticles, called $CPMV_{CYS}$, have been produced that display thiol-reactive fluorescent dyes. However, undecorated, externally exposed thiols tend to form disulfide bridges with each other, therefore a disadvantage to these mutant $CPMV_{CYS}$ is the ease of which they form interparticle aggregates [20]. Another series of genetically modified amino acid residue insertion mutant CPMVs are called $CPMV_{HIS}$. because the modified capsids display 6 sequential histidine residues in either the L subunit βE-βF loop or the S subunit βB-βC loop or carboxy terminus. The his-tags are surface exposed and addressable and can be derivatized with a variety of functional moieties [11].

### 2.5. Production of CPMV and CPMV virus-like particles

For in vivo production of CPMV, the leaves of ten-day old *V. unguiculata* plants are infected using a mechanical protocol where the leaves are lightly dusted with a mixture of the abrasive carborundum and CPMV particles carrying RNA-1 and RNA-2 separately. After 10 days of plant growth, the CPMV-infected leaves are harvested and stored at −80 °C [58,80].

The CPMV particles are purified from the aqueous fraction of the homogenized leaves via multiple centrifugation cycles, NaCl-PEG 8000 treatment and ultracentrifugation to form a crudely purified CPMV pellet. The CPMV sample is purified to homogeneity via sucrose gradient ultracentrifugation and size-exclusion fast protein liquid chromatography (FPLC) [58].

The CPMV RNA-1 and RNA-2 genomes have also been separately inserted into an *Agrobacterium tumefaciens*-based plant vector infiltration system used to infect *Nicotiana benthamiana* leaves [81]. Infection with *A. tumefaciens* carrying either RNA genome individually did not produce recombinant CPMV particles within the leaves. However, when both genomes were agro-infected into the plant leaves at an equal ratio, recombinant CPMV was produced and purified from the leaves with a yield similar to that from mechanical inoculation of plant leaves with CPMV. In addition, every inoculated plant became infected with CPMV showing that the agro-infection method was very efficient at CPMV production [81].

Non-replicative CPMV particles, termed virus-like particles (VLPs) can also be produced by taking advantage of the RNA genome-independent, in vitro self-assembly characteristics of the L and S subunits that make up the capsid, via two very different approaches; *trans* expression with high expression plasmids or mixed re-assembly of purified subunits in a cell-free protein synthesis platform [28,41,51,82–85].

The CPMV genes encoding the capsid proteins, as either the full-length RNA-2 sequence or as the partially-processed 60 kDa L and S subunit fusion protein VP60, and the 24K protease processing enzyme have been cloned into plasmid vectors that allow for high expression of the proteins in either plants or insect cell lines (Fig. 2) [28,78,86]. CPMV VLPs can be produced by *trans* expression of the virally encoded RNA-2 sequence or by VP60 and 24K protease genes in either *Spodoptera frugiperda* insect cells or cowpea plant cell protoplasts. The L and S subunits are efficiently processed and released by 24K protease cleavage from either the RNA-2 vector or the VP60 vector and they can self-assemble in either the plant cell protoplasts or insect cells to form structurally intact, RNA-empty VLPs (termed eCPMVs here but an equivalent term used in the literature is CPMV eVLPs, reviewed in [79]) [27,28,87–89]. The VP60 and 24K protease encoding genes can also be transiently expressed in *Nicotiana benthamiana* leaves, mediated by

the *Agrobacterium tumefaciens* binary vector transduction system, using either the two vectors that together are termed CPMV-*HT* or using a single vector designed for high expression levels called pEAQexpress-VP60-24 K that carries the VP60 and 24K protease genes along with P19 gene which reduces gene silencing in the plant (Fig. 2) [28,78,89]. One week after the *N. benthamiana* leaves have been transformed with the CPMV-HT vectors, the eCPMV particles can be harvested and purified. Yields of i*n planta* produced eCPMV are very high, at 1 g pure eCPMV per kg fresh-weight leaf tissue (Table 1) [89]. Genetically modified eCPMV nanoparticles can also be produced this way by insertion of DNA sequences encoding peptides into the VP60 gene prior to the transient *N. benthamiana* leaf transformation [78,89]. Genetic modification of CPMV RNA is discussed further in Section 2.4.

CPMV VLPs can also be made using an in vitro mixed re-assembly assay, or cell-free protein synthesis platform, that was originally designed with CCMV subunits [85]. In this approach two populations of capsid are functionalized with different display ligands; the capsids are disassembled, and the subunits are isolated. Then the subunits displaying the different ligands are mixed in a specific ratio and allowed to re-assemble together to form a mosaic capsid that is now displaying both functional ligands [85]. There has been much research published recently on the production and use of synthetic, mosaic or hybrid virus-like particles that are a combination of two or more self-assembling viral capsid proteins and chemically reactive polymers [90]. Mosaic capsids have also been made from mixing CPMV subunits and synthetic polymers like the dendrimer polyamidoamine (PAMAM) that is described in Section 3.4. These hybrid VLPs can exhibit enhanced scaffold and cargo vesicles properties.

Comparison of the crystal structure of purified eCPMV to the crystal structure of RNA-containing WT-CPMV and to the cryo-electron microscopy structure of eCPMV shows that they are identical in form [41].

eCPMV nanoparticles offer more benefits to their use than just their non-replicative nature; they may have improved capsid surface modification chemistries and they can take up a larger range of cargo molecule types [89]. Some examples of the design and use of CPMV VLPs in cancer research are discussed in this review.

## 3. Therapeutic and theranostic uses of CPMVs

There are different types of cancer treatments available currently, dependent on the location and type of cancer with the most common therapies involving highly toxic or invasive methods of chemotherapy, surgery and radiotherapy. Improvements have been made to these traditional treatments that include laser surgery and fluorescent imaging guided surgery, and new anti-cancer drugs have been developed, such as doxorubicin and STAT3 inhibitors [88,91]. VNPs, such as RCNMV, have also been developed to deliver doxorubicin to cancer cells for therapeutic procedures [92,93]. As well, novel tumor homing peptides are being discovered, experimentally validated and developed that can target different cancer cell types for imaging and therapy [94,95]. Currently, nanotechnology cancer therapies are being designed and studied extensively because they show great promise as improved drug delivery vectors with high specificity and high sensitivity in preferentially targeting cancer cells, even within deep, hard-to-reach tissues [95]. Additionally, multifunctional nanoparticles, CPMV included, are being designed as theranostic tools that combine imaging and therapy moieties in one device to provide effective detection of, and drug delivery to, cancer cells in vivo [88].

The bioavailability of CPMVs after administration via intravenous injection or orally in mice models have also been tested and the nanoparticles were found in nearly all tissues after both delivery methods [23,43,96]. In particular, with oral delivery, CPMV nanoparticles were stable under gastric conditions and because they were detected throughout the mice, CPMV appeared to have crossed the gastrointestinal epithelium via interactions with Peyer's patches [43]. CPMV nanoparticles lend themselves well for oral delivery because their natural hosts are edible legumes, therefore offering the potential to design edible therapeutics [40].

Researchers developing CPMV nanoparticles for human cancer therapy use eCPMV nanoparticles as the therapeutic agent. As described in Section 2.5 and Table 1, eCPMV retain all the well-characterized capsid proteinaceous structural features of CPMV, without the RNA genomes present. This ensures that eCPMV is non-replicative and therefore non-infectious in vivo [97].

### 3.1. Tumor homing peptides

One of the greatest advantages to using nanoparticles in cancer therapy is the ability to decorate the nanoparticle with a functional group, such as a peptide that will guide the nanoparticle directly to the cancer cells and efficiently differentiate cancer cells from healthy cells in vivo. There are many peptides described in the literature that have been studied and tested as targeting molecules and over 700 experimentally validated tumor homing peptides and covered 23 types of tumors have been compiled in a database called TumorHoPe that is freely available to researchers that want to design peptide-based drugs and drug-delivery systems [94].

Although CPMV nanoparticles are plant pathogens, they can naturally home towards mammalian cells; targeting and interacting with a 54 kD non-glycosylated membrane protein initially called CPMV binding protein (CPMV-BP) and recently identified as surface exposed-membrane bound vimentin [98,99]. Native tropism towards vimentin is discussed in more detail in Section 4.3.

Many types of human cancers, such as those found in ovaries, brain, kidney, breast, myeloid cells and lung, express and display high levels of folate receptors on their surfaces [100,101]. For some cancers, such as ovarian, folate receptor overexpression is linked with a higher histologic grade [100]. Folic acid-PEG decorated CPMV nanoparticles were able to differentially target tumor cells versus healthy cells in vivo. Research is ongoing into developing folate-conjugated CPMV nanoparticles that can target and internalize high grade ovarian cancer tumors in order to deliver anticancer drugs [100].

### 3.2. Cargo loading

CPMVs can stably carry molecules within its protein capsid, differentiate between healthy and cancerous host cells, be taken up and accumulate inside of tumor cells and release their cargo molecules once they have been internalized [27]. These features make CPMVs very effective at delivering toxic molecules into tumor cells while avoiding the healthy host cells and so these particles are promising therapeutic vectors in cancer treatment.

The CPMV capsid naturally has funnel shaped channels, that are 7.5 A in diameter at the external, narrow end, at the 5-fold axes points on the scaffold that appear to be permeable to various molecules [26,87]. CPMV naturally encapsulates one of the two viral RNA genomes. These nucleic acids have been used to help load cargo molecules with a reversible affinity for nucleic acid, for example dyes such as DAPI and proflavine that intercalate with nucleic acid or positively charged molecules with an electrostatic attraction to RNA. The non-covalent cargo load infusion capacity rate of RNA-containing CPMV particles was determined to be 10,000 fluorophores per nanoparticle for 1 h. Empty CPMV nanoparticles (i.e. no RNA genome) were not able to load the tested fluorophores into the capsid. The infused CPMVs were dialyzed and centrifuged through spin filter columns to remove excess, externally-associated cargo molecules, and the load capacity was measured at 130 to 155 dye or drug molecules per CPMV nanoparticle, dependant on the cargo molecule size [27]. This equated to a 50–70% recovery yield of released cargo relative to the amount of starting material used.

RNA genome-empty CPMV (eCPMV) can be infusion-loaded with cargo that does not require nucleic acid interaction for payload trapping

[87]. In particular, eCPMV nanoparticles have been used to load cobalt or iron oxides in a benign process that does not alter capsid size or monodispersion properties and the external, addressable amino acid residues can still be conjugated with functional moieties [87]. The CPMV interior has a net negative charge due to glutamic and aspartic acid residues, which entraps the metal ions via electrostatic interactions [87]. This technology has been propelled forward by improvements to generating large amounts of eCPMV nanoparticles in plants without viral infection, which would require the bipartite genome [28,87]. The electrostatic interaction between the capsid interior and metal ions also make the cargo stably loaded, even after 6 h of dialysis against buffers [87]. The internally-loaded cobalt or iron oxide CPMV nanoparticles are detectable by transmission electron microscopy (TEM) without staining, while the non-loaded eCPMV are not [87]. The metal ion cargo is also chemically reactive still with cobalt participating in reduction reactions enabling them to act as possible metal particle-nucleation sites and iron oxide able to undergo autocatalytic hydrolysis [87,102].

Cargo loading via covalent conjugation of the payload to the internal side of CPMVs has also been attempted [88]. CPMVs covalently loaded with the therapeutic drug doxorubicin resulted in successful tumor penetration, tumor cell internalization and targeting of the loaded CPMVs to the cellular endolysosome, but cargo release only occurred as the internalized CPMV particles were slowly metabolized, over a few days, by the tumor cells [88]. In contrast, the non-covalently loaded fluorophores were released from the internalized nanoparticles within 60 min [27].

Besides the obvious advantage of a fast release from non-covalently bound, infusion-loaded CPMV payload, the other advantages to this method include; expansion in the chemical diversity of the payload from amino acid containing cargo to virtually any type of molecule, neither the cargo or the capsid require chemical or structural modification upon loading and no system is needed for efficient cargo release [27].

### 3.3. Photodynamic therapy using CPMV-$C_{60}$ fullerene conjugates

Photosensitizer chemicals will produce reactive oxygen species (ROS) after they are irradiated with a specific wavelength of visible light that matches the absorption spectrum of the specific photosensitizer [103]. ROS are strong oxidizing agents that quickly oxidize (remove electrons) DNA, proteins, lipids and carbohydrates, ultimately causing cell death. Photodynamic therapy (PDT) is a minimally-invasive therapy used to treat localized, shallow tumors like highly resistant and aggressive melanoma, leukemia T cells, prostate cancer cells and CD22 + human lung cancer cells [84]. In PDT, a photosensitizer (PS) is administered to the tumor tissue, then the patient is irradiated with visible light to catalyze the ROS production. PDT has been shown to kill cells via the ROS formation, but it also stimulates an immune response by the host, both of which help to kill the cancer cells. These excellent outcomes of PDT are unfortunately tempered by limitations of the PS. These chemicals tend to be inefficient at tumor-targeting, leading to off-target effects, have low accumulation within the tumors, low solubility under physiological conditions leading to formation of colloidal aggregates and low to moderate light absorption properties [84,91].

The Buckminsterfullerene carbon allotrope $C_{60}$ (buckyball), is an excellent free radical scavenger and therefore highly useful as a PS, but it is also hydrophobic and bio-incompatible in physiological conditions [103]. CPMV nanoparticles are highly biocompatible, can effectively target and accumulate in tumors and tumor cells. Researchers theorized that conjugating these two structures could result in an enhanced PS with improved bio-compatibility and targeting characteristics. $C_{60}$ buckyballs were successfully conjugated to CPMVs using click chemistry (CPMV-$C_{60}$) and cellular uptake of these functionalized capsids were measured using the HeLa human cancer cell line [103]. When the degree of derivatization was analysed the $C_{60}$ moiety was only found linked to lysine residue 38 (K38) on the large subunit, which was previously determined to be a highly reactive, solvent exposed, lysine (Fig. 1). Even with the low degree of $C_{60}$ decoration, CPMV-$C_{60}$ showed PS characteristics, improved water solubility and biocompatibility as well as evidence of accumulation within HeLa cells in an in vitro assay when imaged by confocal microscopy [103].

Zinc ethynylphenyl porphyrin (ZnEpPor) is a novel PS in the same chemical family as the often-used clinical PS, Photofrin. In vitro macrophage staining assays and murine melanoma studies showed that ZnEpPor is a more potent PS than Photofrin because of its enhanced accumulation in tumor tissues and better binding efficiency, due to its positive charge and zinc (II) cation, respectively [84]. When ZnEpPor is used as a PS in PDT treatment, macrophage and tumor cells are eliminated, however enhancing this PS would greatly improve the ZnEpPor-based PDT treatment. CPMV addressable lysine residues were derivatized with an azide linker, then conjugated via click chemistry to alkyne derivatized carbonyl dendrons [84]. Carbonyl dendrons are synthetic poly-branched polymers with carbonyl termini that allow for conjugation with carbohydrates. They were designed for use in diagnostic tools, regenerative medicine and nanobiotechnology applications, in order to exploit the highly specific recognition processes between glycans and their receptors [84]. Glycoconjugates can be easily produced on dendrons via carbonyl chemistry. The CPMV-dendron conjugate (CPMV*) has many more addressable sites for ligand display than CPMV alone. CPMV* and ZnEpPor PS were mixed to allow electrostatic interactions to form PS-CPMV*. PS decorated CPMV* showed improvements in both macrophage and cancer cell elimination compared to ZnEpPor [84].

### 3.4. Use of CPMV as a vaccine in cancer immunotherapy

One reason for tumor proliferation throughout the body is because the tumor cells mediate a tumor-localized suppression of the immune system. This de-sensitizes the host immune system to tumor specific antigens which allows the tumors to grow and metastasize unchecked. Immunotherapies designed to reverse tumor-mediated immune suppression have been studied to determine their potential efficacy as anti-tumor treatments [71,83,97].

Both wildtype and RNA genome-free CPMVs, especially non-PEGylated capsid particles, will elicit an immune response when introduced into mammalian tissues. These nanoparticles show a natural tropism towards immune cells; in specific, the M2 subpopulation of macrophages, although the mechanism of the attraction is not understood yet [97]. These features, along with the ability of CPMV and eCPMV to penetrate tumors and specifically target cancer cells, make them good candidates for use as in situ immunostimulatory nanoparticle vaccines aimed at re-sensitizing the host immune system to tumor specific antigens without needing to know what antigens are present [71]. eCPMV anti-tumor vaccines are intravenously injected into an already identified tumor where they stimulate the hosts' innate and then adaptive immune infiltration and activation; in effect priming the hosts' immune system against the tumor and turning it from a “cold” to a “hot” tumor [97].

Non-modified eCPMV nanoparticles were able to trigger the mouse innate and adaptive immune system, in particular the activated neutrophil population was stimulated, after in situ injection into the intraperitoneal (IP) space next to disseminated tumors at multiple anatomical sites [83]. The eCPMV vaccination was administered to a variety of mouse metastatic cancer tumors in vivo, including B16F10 lung metastatic-like melanoma, dermal B16F10 melanoma, 4T1 BALB/c syngenic breast cancer model that metastasizes to the lung, intradermal CT26 colon tumor and disseminated peritoneal serous ovarian carcinoma [83]. In all of these tumor models the tumors were either significantly reduced in size or even eliminated altogether (dermal B16F10 melanoma) after in situ eCPMV administrations. The eCPMV-mediated anti-tumor effect was experimentally determined to be due to immune stimulation and not from tumor cell cytotoxicity because in vitro application of the eCPMV particles to the tumor cell lines showed

no effect on cancer cell proliferation or viability [83]. However, the in situ eCPMV vaccination needed to be repeated weekly to replenish the nanoparticles to a therapeutic concentration to continue resensitizing the mouse immune system towards the ovarian tumors [83]. Although eCPMV nanoparticles are very effective in situ immune-stimulatory agents, especially with the metastatic ovarian cancer model, the administration of weekly, repeated IP vaccinations into human tumors, especially in deep, hard-to-reach locations like ovarian cancer tumors, is problematic because they require hospitalization.

In order to improve the in vivo retention time of the eCPMV vaccine after tumor-site in situ administration, and reduce the number of putative in-hospital injections, a mosaic eCPMV particle was developed [71]. The mosaic nanoparticles were generated by mixing the L and S subunits from CPMV and PAMAM dendrimer polymers together and allowing them to re-assemble into a novel nanoparticle called eCPMV-G4. These mosaic eCPMV-G4 nanoparticles showed increased retention time in vivo which allowed the nanoparticle concentration to remain at therapeutic levels for much longer than eCPMV without G4 [71]. PAMAM dendrimers are positively charged, nanoscale polymers that consist of an ethylenediamine core with radiating, branched amidoamine arms that terminate with primary amine groups [71]. They are used in medicine and industry as synthetic nanoparticles [5]. One administration of eCPMV-G4 to the IP cavity in a mouse disseminated ovarian cancer model showed the same level of efficacy as an antitumor vaccine as the weekly administration of the soluble CPMV [71]. This confirmed that the combination of the PAMAM dendrimer and eCPMV produces a novel mosaic nanoparticle that acts as a reservoir of eCPMV in the IP cavity. eCPMV is therefore slowly released to provide a continuous supply of immunostimulatory nanoparticles into the ovarian cancer tumor, which triggers the host immune response to recognize the tumor specific antigens immunogenic [71]. Reducing the number of vaccine administrations while maintaining a high concentration of the immunotherapeutic agent at the tumor site is essential for beneficial patient care and quality of life.

Researchers have also compared the efficacy of in situ injected tobacco mosaic virus nanoparticles (TMV) to stimulate the hosts' immune response against melanoma tumors versus the efficacy of eCPMV [104]. The eCPMV nanoparticles outperformed short-rod, long-rod, spherical, assembled-protein and free protein TMV variants, as measured by reduction of tumor volume after administration. This differential potency may be because eCPMV is more effective at recruiting monocytes into the tumor microenvironment which also attracts tumor infiltrated neutrophils and natural killer cells plus the production of effector memory cells [104].

The development of thermostable and chemical-resistant viral biological agents would greatly benefit their use as vaccines. CPMV nanoparticles have been successfully biomineralized into calcite crystals that can protect the spherical capsid from harsh chemical environments [105].

## 4. Intravital vascular imaging for non-invasive cancer detection

Ultrasound (US) and computed tomography (CT) are the conventional imaging technologies used for detection, diagnosis, triaging and prognosis of organ cancers and tumor metastasis [76]. Although these technologies have been proven to be very effective cancer research and clinical tools, they lack the resolution required to image small primary lesions at the microscopic level [20,76]. Magnetic resonance imaging (MRI), positron emission tomography (PET) and spinning disk confocal fluorescence microscopy are non-invasive, in vivo imaging tools that are used together with a contrasting agent, such as gadolinium ion tracers, to generate bright images with high sensitivity [50]. CPMV nanoparticles have been successfully developed as contrasting agent platforms that increase the density and allow for targeting of the specific cells. Due to their large surface area to volume ratio, CPMVs can be decorated with hundreds of gadolinium ions (Gd) by derivatizing the addressable lysine residues, therefore enabling CPMV-Gd particles to present with high relaxivity values [55,64]. High sensitivity and high-resolution imaging of tumor neovasculature and blood flood using CPMV nanoparticles functionalized with fluorescent dyes and with, or without targeting ligands, have been done using a spinning disk confocal fluorescence microscope, capturing three-dimensional Z stacks as well as time-lapse images [20,23,76,106].

The multivalent display capacity of CPMV nanoparticles provides the sensitivity and specificity needed to detect these cancers, while their nanoscale size allows these particles to extravasate through the leaky tumor vasculature, penetrate into deep tissue locations and ultimately be taken up by, and accumulate in, the targeted cancer cells, via the EPR effect [76,107].

The biological models used to determine the efficacy of experimental CPMVs in vivo include the chick embryo chorioallantoic membrane (CAM), mouse embryo and adult mice.

### 4.1. Chick embryonic chorioallantoic membrane models

Imaging cancer cell proliferation, invasion and mobility in live animal models is a powerful method to study tumor growth and experimental diagnostic and therapeutic targets. Intravital imaging has been limited however by the lack of efficient probes and animal models. Recently, both WT and fluorescent dye and target peptide-decorated CPMVs have shown to be excellent imaging probes [23,25,54,80]. The chorioallantoic membrane (CAM) of chick embryos is a very useful tissue model for nanoscale, in vivo, non-invasive, real-time, intravital imaging of tumor growth. Human tumors from cell lines; HEp3 (squamous carcinoma), HT29 (colon adenocarcinoma), HT1080 (fibrosarcoma), MDA-MB-231 (breast carcinoma), and PC3 (prostate cancer), have been successfully grafted onto the CAM of shell-less chick embryos [58]. High-resolution images of these tumors were obtained via confocal microscopy because the human tumors proliferate laterally and shallowly on the membrane, to an average depth of 200 nm. The CAM model is relatively inexpensive to use, the xenograft procedure is done without the need for anesthetic or surgery and a fully vascularized human tumor can be developed on the CAM within 7 days [76].

CPMV particles of varying sizes and ligand chemistries have been injected into the CAM at sites distal to the tumor xenograft where they flow through the bloodstream and can extravasate from the leaky tumor vasculature and also be quickly taken up by endothelial cells. This allows for the tumor vasculature to be labelled from both within and outside of the tumor. Additionally, CVMP nanoparticles can be stably maintained within tumor grafted CAM for up to 72 h for imaging and quantification measurements.

### 4.2. Deep tissue imaging using CPMVs decorated with multivalent fluorescent dyes

It is essential to use non-invasive techniques to visualize tumor endothelial cells and tumor remodelling of vascular tissue in vivo, to better understand where and when tumor neovascularization occurs. In vivo penetration of deep tissues with fluorescent dyes for imaging tumors is very challenging [23]. CPMVs conjugated with fluorescent dye molecules can show a high signal to capsid ratio which allows for imaging of rare or deep cellular targets. CPMV nanoparticles, displaying up to 120 fluorescent dye molecules per capsid (i.e. CPMV-A555), injected into adult mice were found to localize to the mouse vascular endothelium and internalize, via endocytosis, within endothelial cells as monodispersed particles (Fig. 4) [23]. This preferential targeting and internalization of endothelial cells allows for easy identification of the tumor-associated venous vessels, which in turn helps determine the vascular origin and directionality [23]. However, the exact mechanisms that allow for specific cell targeting and internalization between CPMVs and the targeted antigen-presenting cells (APCs) are not fully

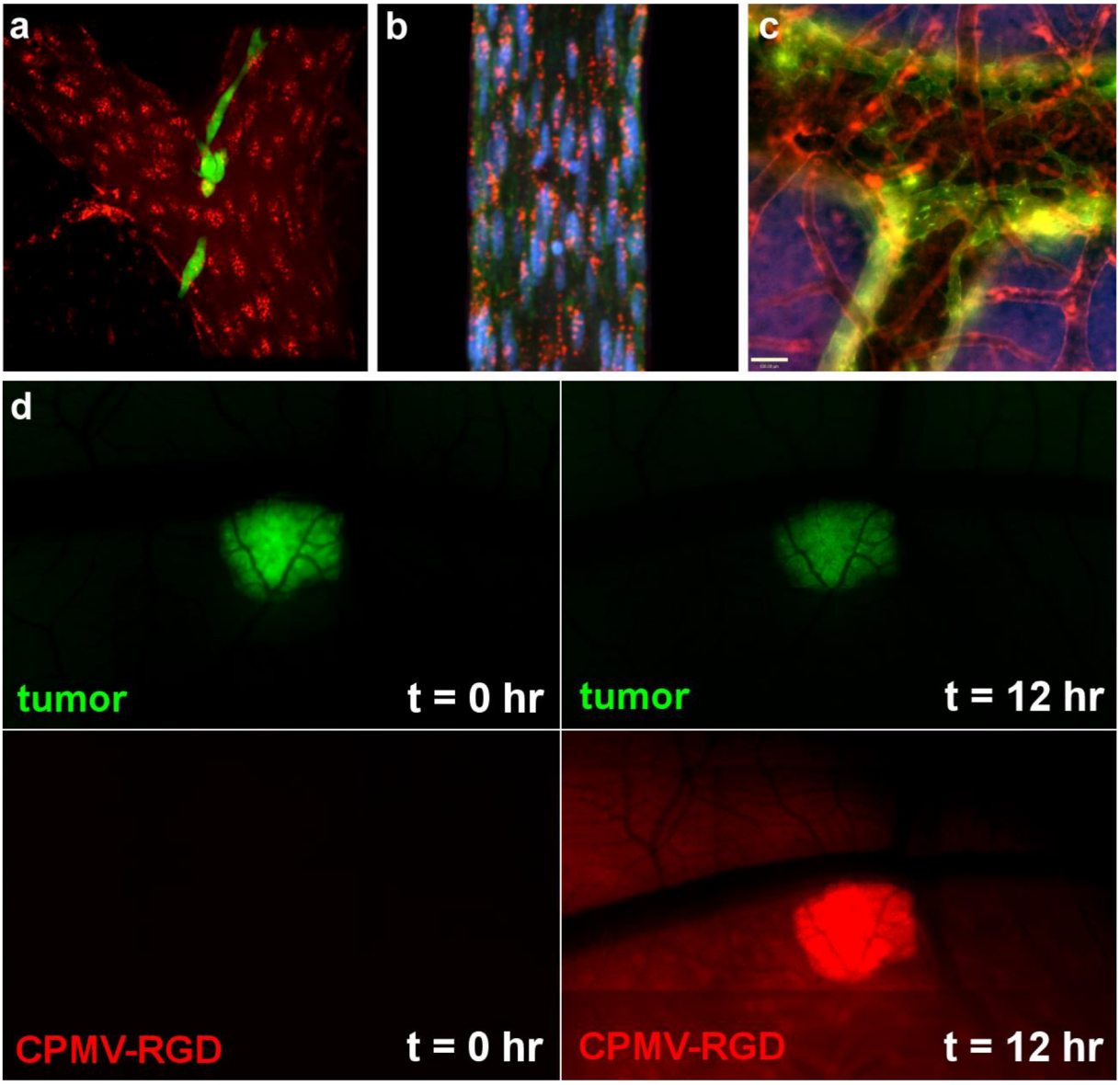



**Fig. 4.** Intravital imaging using engineered CPMV nanoparticles. (a) Visualization of individual head and neck HEp3 cancer cells (green) invading along a blood vessel (red) labelled with CPMV-A555 in the avian embryo CAM. (b) Intravital imaging of a mature blood vessel in the avian embryo CAM with CPMV-A555 (red) and CPMV-A488 (green) injected at distinct time points. Endothelial cell nuclei are visualized using Hoechst 33342. (c) Dual fluorescent CPMV labeling of blood vessel with CPMV-A555 (red) and surrounding lymph vessels with CPMV-A488 (green) in the avian embryo CAM. (d) Intravital imaging of human breast cancer tumors (MDA-MB468, green) in the chorioallantoic membrane using PEGylated CPMV VNPs functionalized with RGD peptides (red) over 12 h.

understood [108]. Research aimed at elucidating these processes will greatly benefit the development of CPMV nanoparticles as vaccines and tumor cell-targeting tools. CPMV used as vaccines is discussed further in Section 3.4 and CPMC tropism for vimentin-displaying cancer cells is outlined in Section 4.3.

As mentioned in the previously, CPMVs are stable within tissues for up to 72 h, therefore long-term, real-time intravital imaging with fluorescently labelled CPMV nanoparticles can allow for quantification of the endothelial cells and mapping of the neovasculature (Fig. 4) [23,57]. This differential cellular targeting and long-term stability could allow researchers to determine which tumor vascular system is the source of tumor cells metastasizing into the hosts circulatory system [23]. PEGylated CPMVs that were also conjugated to the fluorophore FITC were blocked from internalization within the endothelial cells, suggesting that the capsid structure that interacts with the cancer cell receptor, possibly vimentin, is obstructed when PEG polymers decorate the capsid externally [23]. The resolution of macro and microvasculature by CPMV-A555 during in vivo intravital imaging of both mouse and chick embryo models was superior to other dye-displaying nanospheres (Fig. 4) [23].

### *4.3. Native tropism of CPMV for vimentin on host cells*

Vimentin is a type III intermediate filament mainly expressed in the cytoplasm of mammalian mesenchymal cells. In wildtype cells, cytoplasmic vimentin is a cytoskeleton protein filament that takes part in wound healing, cell-cell interactions, motility, contraction, proliferation and molecular functions like transcription, translation, signal transduction and apoptosis [20,55]. Cytoplasmic vimentin is also a part of the cell adhesion-matrix adhesion complex which makes it an important protein for migrating cells, suggesting that cytoplasmic vimentin plays a role in tumor metastasis [109,110]. Vimentin is a mesenchymal cell marker and a potentially important component in epithelial–mesenchymal transition (EMT) events, which has been linked to

tumor invasiveness and aggressiveness [111]. Wildtype and cancerous mammalian cells also express surface-displayed vimentin for an, as yet, unknown function but inflamed endothelial cells associated with tumors can be differentiated from wildtype cells because they over-express surface-displayed vimentin [111]. In clinical studies, vimentin overexpression by breast cancer cells is linked to invasiveness and poor prognosis for patients [20]. The experimental and clinical evidence on vimentin and cancer cells points to surface-displayed vimentin as a good target for tumor cell localization and imaging.

Wildtype CPMV displays natural tropism towards vimentin-expressing cells in vitro [1]. Specifically, CPMV shows a high affinity for surface displayed vimentin but this affinity is significantly reduced after labeling CPMV with PEG2000 [20,108]. Despite this low affinity, CPMV labelled with either the fluorescent dye A647 (CPMV-A647) or both A647 and PEG2000 (P2-A647) were able to target and be taken up by vimentin-expressing human colon adenocarcinoma cells (HT-29) in vivo after a longer incubation time. In fact, P2-A647 uptake by the tumor cells was three times higher than CPMV-A647. Other recent studies have shown that low affinity ligands appear to target tumor cells better than their high affinity counterparts and this may be due to the EPR effect [112]. It was theorized that P2-A647 showed high uptake by vimentin-expressing cancer cells despite its low affinity for two reasons; because it extravasated in the tumor-associated blood vessels and more importantly, the circulation half-life was increased due to the PEGylation stealth layer [20]. Therefore, fluorescent dye-labelled CPMVs have been effectively used to find and visualize tumor-associated endothelial cells and tumor neovasculature by targeting to surface-displayed vimentin [12,20].

### 4.4. CPMV targeted to gastrin-releasing peptide receptors

Gastrin-releasing peptide (GRP) receptors are over-expressed on many different tumor cell types and so its efficacy as a highly specific target for tumor detection and imaging was tested using CPMVs as the delivery tool. CPMV was multivalently functionalized using the externally addressable lysine residues and a combination of standard conjugation chemistry and click chemistry to display Alexa Fluor (AF) 647, PEG and a 14 amino acid peptide analogue of pan-bombesin [20]. The PC-3 prostate cancer tumor was grafted onto the CAM and the CPMV-AF647-PEG-bombesin nanoparticle was introduced at the distal side of the tumor. The CPMVs accumulated in 5 mm wide tumors in the CAM test. The observation that nanoparticles can home onto rare target cells using the EPR effect gives credence to the theory that the EPR effect occurs with small tumors and metastatic lesions [20].

### 4.5. Neovascular imaging via vascular endothelial growth factor receptor (VEGFR) targeting

Angiogenesis, the de novo formation of new blood vessels, is a complex, multi-pathway process that in normal, adult physiology occurs when the body repairs tissue after wounding or during placental formation with pregnancy [25,113]. The formation of new blood vessels is highly regulated at the transcriptional level in wildtype cells, in part by a multitude of different growth factors and their respective receptors, such as the well-studied vascular endothelial growth factor (VEGF) and VEGF receptors 1, 2 and 3 (VEGFR-1, -2 and 3) [54,114,115]. VEGFR-2 and -3 are over-expressed in tumor vasculature and so have been popular targets, both individually and in combination, for non-invasive imaging and targeting of solid tumor endothelial cells in the neovasculature [113,115]. VEGFR-1 has been identified as a tumor-specific vascular endothelial cell surface protein by subtractive proteomic mapping and is expressed in tumor cells in breast cancers, gastric cancers and schwannomas [54]. Therefore VEGFR-1 may also be a useful target for tumor imaging and targeting. Tumor angiogenesis is characterized by abnormal vasculature and a poor cancer prognosis with high mortality. During cancerous tumor growth, angiogenic pathways are deregulated so that new blood vessel production is always on. This has the effect of nourishing the tumor cells with the elements and molecules required for cell growth and increasing the likelihood of metastatic tumor cell dispersal to other parts of the body. Inhibition of tumor angiogenesis (tumor neovascularization) would stop the tumor cell nutrient supply and the dispersal of tumor cells throughout the body, therefore this process is considered an important anti-tumor therapeutic target [54,113].

A report was recently published that outlined the use of a humanized anti-VEGF monoclonal antibody (called Bevacizumab) in an anti-angiogenic immune-therapy that has been approved for treatment of glioblastoma, non-small cell lung cancer, metastatic colorectal cancer and metastatic renal cell cancer, as either a stand-alone drug or in combination with other drugs [116]. In an effort to selectively target VEGFR-1, a high affinity VEGFR-1 interacting peptide was found (called F56) using phage-display and was conjugated to CPMV to produce VEGFR-1 targeting nanoparticles [54]. The CPMV-F56 nanoparticles (called FP3) was generated using hydrazine ligation chemistry and is an excellent example of a 'smart' multifunctional nano-platform because not only does it display 133 copies of the VEGFR-1 selective peptide, it also was decorated with over 140 fluorescent dye molecules for imaging and over 50 PEG polymers to lengthen the plasma circulation time [54]. The in vivo targeting and imaging capability of FP3 was measured in HT-29 tumor-bearing mice, known to express high levels of VEGFR-1. Two hours after administration of FP3, the tumors were immune-fluorescently stained and imaged using confocal microscopy and FP3 was found accumulated throughout the tumor tissue but not on the endothelial cells that line the blood vessels [54]. Therefore, VEGFR-1 appears to be expressed on both endothelial cell and tumor cell surfaces but due to FP3 extravasating via the EPR effect from the leaky tumor blood vessels to the tumor cells, decorated CPMVs were not detected on the endothelial cells [54].

### 4.6. Neovascular imaging via epidermal growth factor-like domain 7 (EGFL7) protein targeting

Despite the VEGF anti-angiogenic therapies under development or available for therapeutic use, clinical evidence is mounting to suggest that targeting VEGF may not be effective in inhibiting tumor neovascularization [25]. The 30 kDa epidermal growth factor-like domain 7 protein (EGFL7) is only expressed by endothelial cells undergoing vascular remodelling and not by quiescent cells and has been identified as a key regulator of several angiogenic pathways [25]. Evidence has accumulated to implicate EGFL7 as a key factor in tumor angiogenesis and therefore this protein may be a good candidate as an anti-angiogenesis target. In mice studies, over-expression of EGFL7 led to abnormal vasculature remodelling; similar to tumor-related abnormal blood vessel remodelling. Solid tumor in vivo studies detected high amounts of EGFL7 protein, which have also been positively co-correlated with high tumor grades and poor prognosis in breast cancer, hepatocellular carcinoma, laryngeal squamous cell carcinoma, malignant glioma, ovarian cancer and pancreatic cancer [25].

CPMVs displaying EGFL7-targeting peptide ligands, called E7p72, were developed to determine the validity of EGFL7 as a target for the detection and imaging of tumor-related, active vasculature remodelling [25]. Similar to the FP3 nanoparticle described above, the E7p72 decorated CPMV nanoparticle was developed as a multifunctional platform [25]. The resulting CPMV displayed Alexa-Fluor 647 near infrared dye molecules and PEG-E7p72 polymers conjugated with E7p72 displayed on the distal side of the capsid (CPMV-PEG-E7p72). These nanoparticles were tested for targeting and imaging efficacy using an in vitro endothelial cell plate assay with confocal microscopy and an in vivo mouse model and chick embryo-CAM study and, both xenografted with the HT1080 fibrosarcoma cell line. The xenografted tumors were assayed to positively determine that the tumor neovasculature associated endothelium cells expressed high levels of EGFL7 [25]. ex vivo staining of mouse tumor cryo-sections with CPMV-PEG-E7p72 showed that

the decorated nanoparticles were bound to the tumor blood vessel cells. Time-lapse intravital imaging of intravenously injected CPMV-PEG-E7p72 CAM xenografted tumors revealed that the decorated nanoparticles accumulated in endosomal compartments of the tumor endothelium cells over a 90-minute time duration but not within non-tumor endothelial cells [25]. E7p72 peptide labelled with Gd ions also showed targeting to EGFL7 via PET imaging [25].

These studies suggest that using EGFL7 expression as a biomarker for tumor angiogenesis would be beneficial since it is expressed in cells associated with tumor blood cell remodelling and not by mature blood vessels [25].

## 5. Conclusions and future directions

Not only can the CPMV capsid display functional groups that include (but is not limited to) targeting ligands, imaging dyes, photosensitizers and epitopes, it can also carry a payload of molecules like drugs or dyes to tumor cells. This allows for drugs toxic to both the healthy and the cancerous host cells to be preferentially released inside the cancerous cells only, thereby mitigating off-target toxicity. Where CPMVs show their greatest advantage as tumor targeting, imaging and cancer cell therapy tools is when they are dually modified to both display targeting and imaging moieties on external, addressable residues and infusion loaded with therapeutic drug cargo. CPMVs naturally use their native tropism for tumor cell-surface displayed vimentin and the EPR effect to extravasate through tumor neovasculature to efficiently penetrate tumors and target and be internalized by tumor cells. Infusing ligand-decorated CPMVs with cargo molecules that offer a complementary tumor therapy would potentially produce a targeted, tumor-killing therapy. For example, infusion loading CPMV-PEG-E7p72 particles with an anti-angiogenic drug could allow for effective drug delivery targeted directly to the blood vessel cells undergoing tumor-associated active remodelling.

Integrating the use of proteinaceous scaffold structures like CPMVs along with ligands aimed at targeting the molecular mechanisms behind complex cancer-associated processes like tumor cell migration and metastasis are promising future cancer therapies. This therapy method has the potential to target these processes in multiple synchronized ways that would reduce formation of multi-drug resistance [20,117].

CPMV nanoparticles have shown great efficacy at targeting tumor cells by natural tropism for surface-vimentin, over-expressed in tumor endothelial cells, and via a variety of different conjugated ligands. This efficacy is greatly improved by the ability of CPMVs to extravasate by the EPR effect. CPMVs also make excellent fluorescent dye carriers that can be used for deep tissue intravital imaging of in vivo cancer models like the chick embryo CAM. PEGylation of the nanoparticles also extends the anti-immunity stealth mode of CPMV so that they can remain in the circulatory system for longer time periods than otherwise.

The RNA encapsidated CPMV also makes an excellent cargo delivery vessel via noncovalent interaction of the payload with the already-present native nucleic acid cargo. The CPMV particles are naturally internalized into the targeted tumor cells where they then release the cargo. This means that drugs that would be toxic to healthy host cells as well as tumor cells can now be delivered directly to tumor cells only.

Overall, the potential of CPMV for multifunctionality of display molecules, intravital real-time imaging of tumor neovascularization and targeted cargo delivery is as great as our ability to imagine the various possibilities inherent to this nanotechnology.

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## Competing interests statement

The authors declare no competing financial or non-financial interests.

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