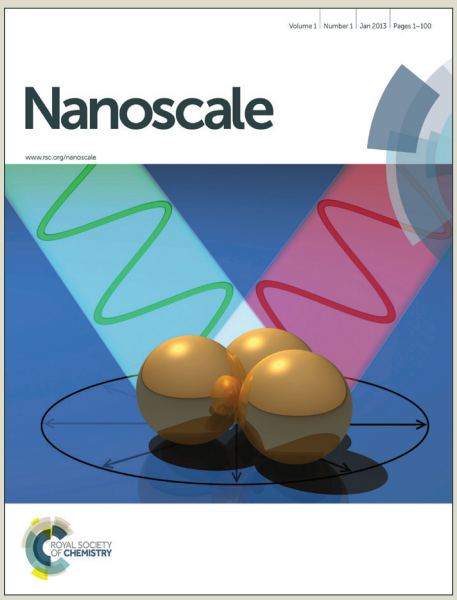
Nanoscale

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/nanoscale

Journal Name

RSCPublishing

REVIEW

Functionalization of protein-based nanocages for drug delivery applications

Cite this: DOI: 10.1039/x0xx00000x

Lise Schoonen[a] and Jan C.M. van Hest[a]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Traditional drug delivery strategies involve drugs which are not targeted towards the desired tissue. This can lead to undesired side effects, as normal cells are affected by the drugs as well. Therefore, new systems are now being developed which combine targeting functionalities with encapsulation of drug cargo. Protein nanocages are highly promising drug delivery platforms due to their perfectly defined structures, biocompatibility, biodegradability and low toxicity. A variety of protein nanocages have been modified and functionalized for these types of applications. In this review, we aim to give an overview of different types of modifications of protein-based nanocontainers for drug delivery applications.

1 Introduction

Conventional drug molecules diffuse throughout the body where they can affect normal cells next to their intended targets, leading to undesired side effects. In fact, only 5% of all new potential therapeutics have favourable pharmacokinetic and bio-pharmaceutical properties.[1] Recent nanotechnological advances have led to the development of smart nanoparticles which are able to selectively target tissues and deliver therapeutics. These nanosystems may be produced either by breaking down large structures into smaller pieces by techniques such as etching (top-down approach), or by assembling small components into supramolecular structures (bottom-up approach).

The main advantage of nanoparticles in drug delivery is the ability to combine multiple functionalities into one therapeutic (Fig. 1). Modifications to the exterior surface can impart a preferred distribution of drugs to the desired site of action compared to healthy tissues and thus drastically reduce side effects. To this end, targeting groups that bind specifically to the surface of the target cell type can be attached. The additional advantage of doing this on the surface of a nanoparticle is the potential increase of binding avidity through multivalency effects. In addition, a protecting layer (corona) can be attached to increase blood circulation times and reduce non-specific interactions. Simultaneously, multiple copies of a drug cargo can be delivered to a target tissue through encapsulation of the drug into the interior of the particles. Control over the time and location of cargo release can be obtained by integrating disassembly and degradation mechanisms that are activated under the desired conditions.

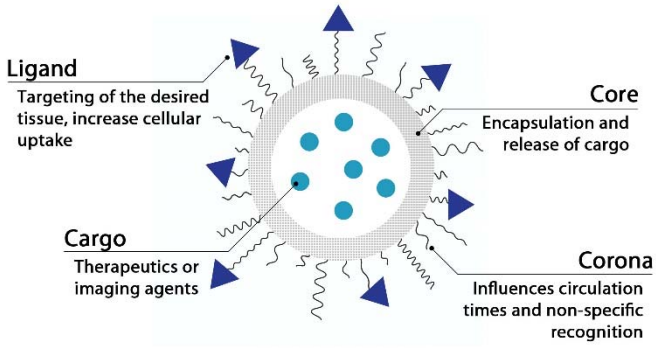


Fig. 1 Schematic of a nanoparticle platform for drug delivery, featuring a core, corona, cargo and targeting ligand.

Protein cage assemblies are symmetrical, monodisperse and robust assemblies consisting of a limited number of protein subunits. They are highly promising for drug delivery purposes due to their stability, low toxicity, biocompatibility and biodegradability. Moreover, due to the highly repetitive structure, one single modification is identically displayed in a controlled fashion around the entire particle. Protein cages are often spherical, creating the possibility of separate interior and exterior modification through biological and chemical approaches. This review will focus on the functionalization of protein-based nanocages, where in particular modifications which we deem valuable for drug delivery applications will be mentioned. The emphasis will be on genetic and synthetic modification strategies for the attachment of molecules relevant to drug delivery, for instance therapeutic cargo or targeting moieties. Also, we will evaluate the toxicity, immunology and biodistribution of the discussed nanoparticles, as these aspects are crucial for their application in drug delivery. For a more detailed description of the application of nanocontainers in addressing specific diseases, the reader is referred to other reviews on this topic.[2,3]

For other biomedical applications of proteins, for instance the use of antibodies in targeted delivery of therapeutics, the reader is referred to some excellent reviews on this topic.[4-6] Other types of nanoparticles that have been used for biomedical applications, including lipid-based (e.g. liposomes[7,8] and solid lipid nanoparticles[9]), polymeric (e.g. polymersomes and polymer micelles)[10-12] and inorganic nanoparticles (e.g. metal nanoparticles[13,14] and quantum dots[15,16]) are also outside the scope of this article.

2 Protein-based nanocages

In this section the different protein-based cages will be introduced that have been applied in a biomedical setting. A distinction will be made between nanocapsules of non-viral and viral origin.

2.1 Protein cages

In nature, a variety of proteins exist that self-assemble into capsule-like architectures. These protein cages function as carriers and storage devices for minerals and metal ions or hosts for biomineralization reactions. A well-known example is ferritin, an iron-storage protein, which is produced in almost all living organisms. Other proteins that self-assemble intro cages are the ferritin-like DNA-binding proteins from starved cells and small heat shock proteins.

Ferritin. Apoferritins consist of 24 subunits which assemble into nanoparticles of approximately 450 kDa[17], with outer and inner diameters of 12 nm and 8 nm, respectively (Fig. 2).[18] The natural functions of ferritin are binding of Fe^{II}, oxidation to Fe^{III} and biomineralization towards ferric oxide clusters.[19,20] Ferritins are robust proteins; they can withstand high temperatures (85 °C) and high pH values (8.5-9.0) for a relatively long amount of time without significant loss of structure. Also, ferritins are biocompatible and non-immunogenic. Therefore, these protein cages have been studied extensively as biomineralization scaffolds and MRI contrast agents.[21]

Small heat shock protein. Heat shock proteins are expressed in response to elevated temperatures and other types of cellular stress. Their main function is the facilitation of correct synthesis and folding of proteins, by forming stable complexes with folding intermediates of their protein substrates. One class of heat shock proteins consists of the small heat-shock proteins (sHsps). In this section we will focus on the sHsp from *Methanococcus jannaschii* (MjHsp). This protein cage consists of 24 subunits, which self-assemble into a cage of 16.5 kDa with octahedral symmetry and an outer diameter of 12 nm and an inner cavity of 6.5 nm (Fig. 3).[22-24] The major difference between MjHsp and ferritin is the presence of large 3 nm pores, which allow small molecules to diffuse in and out of the cage. This cage is also quite robust, as it is stable in the pH range 5-11 and at temperatures of up to 70 °C.

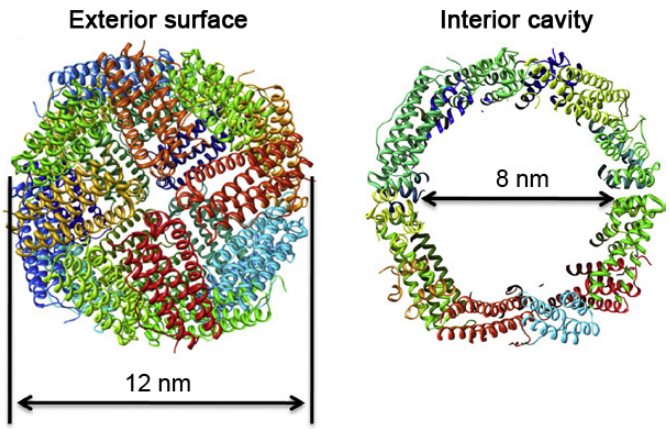


Fig. 2 Ribbon diagrams of exterior surface view and interior cavity of wild-type human heavy chain ferritin (HFn). Reprinted from Biochimica et Biophysica Acta (BBA) - General Subjects, 1800, M. Uchida, S. Kang, et al., The ferritin superfamily: Supramolecular templates for materials synthesis, 834-845, Copyright (2010), with permission from Elsevier.[21]

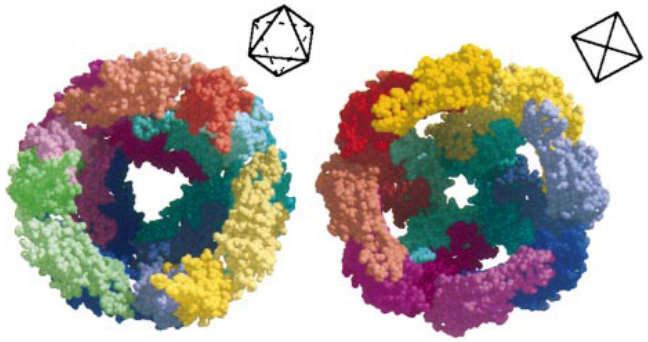


Fig. 3 Space filling model of the interior of MjHsp 16.5 viewed along the crystallographic three-fold axis (left) and four-fold axis (right). Obtained from the Protein Data Bank (PDB 1SHS). Reprinted by permission from Macmillan Publishers Ltd: Nature[22], copyright (1998).

2.2 Virus-like particles

Viruses in their most simplistic form consist of a protein coat (capsid), which stores and protects viral nucleic acids (DNA or RNA). Their natural purpose is to infect plants, bacteria or animal cells in order to replicate and assemble new particles. Viral capsids have appealing properties for use in nano-technology, one of which is their wide variety in sizes and shapes (Fig. 4). Icosahedral particles ranging from circa 18-500 nm and rod-shaped particles with lengths of > 2 µm exist. Their ability to form robust and monodisperse structures with a high degree of symmetry makes them unique nanocontainers.[25] For many viruses, it has been observed that their capsid proteins can self-assemble into stable virus-like particles (VLPs), in the absence of their corresponding genetic material. These particles are non-infectious and have proven to be very suited for nano-technological applications, such as vaccine development[26-28], biomedical imaging[29,30], synthesis of inorganic materials[31], gene therapy[32,33] and development of arrays and films for electronics and tissue engineering[34-36].

Icosahedral plant viruses. The Cowpea chlorotic mottle virus (CCMV) capsid consists of 180 identical 20 kDa capsid proteins 2which assemble into an icosahedral protein cage with T = 3 symmetry. The assembled cage has outer and inner diameters of 28 nm and 18 nm, respectively.[37] CCMV can be obtained from its natural host, *Vigna unguiculata*, in yields of about 1-2 mg per g of infected leaves. Its coat proteins can also be obtained using yeast- or *E. coli*-based expression systems, with comparable yields. The N-terminus of the capsid proteins are located on the inside of the viral capsid, providing a suitable location for functionalization. The residues on this terminus are predominantly positively charged, allowing the encapsulation of negatively charged species. An attractive feature of CCMV is its ability to undergo a reversible, pH-dependent swelling. This results in the formation of 60 openings of 2 nm in diameter.
Cowpea mosaic virus (CPMV) has a 28 nm capsid, composed of 60 subunits which are made up of a small (24 kDa) and a large (41 kDa) subunit.[38,39] CPMV has been widely used as protein-based nanoreactor due to its non-toxicity and stability in a wide range of pH, temperatures and solvents. It also has the natural capacity to bind and enter mammalian cells, which has already

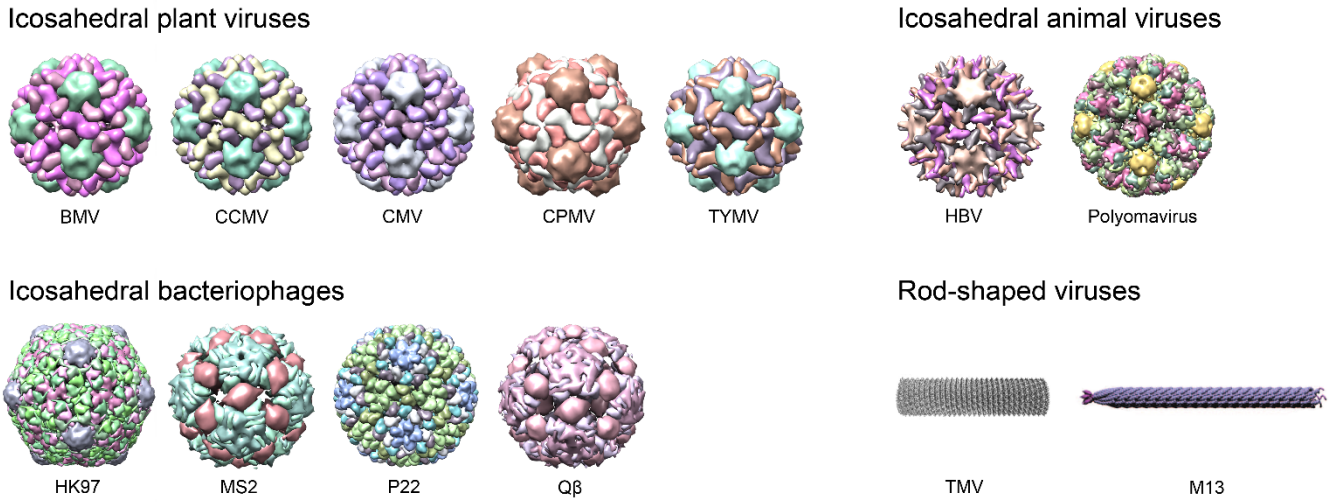


Fig. 4 Virus(-like) particles used in biomedicine. Icosahedral plant viruses: Brome mosaic virus (BMV), Cowpea chlorotic mottle virus (CCMV), Cucumber mosaic virus (CMV), Cowpea mosaic virus (CPMV), Turnip yellow mosaic virus (TYMV). Icosahedral animal viruses: Human Hepatitis B (HBV), Murine Polyomavirus. Icosahedral bacteriophages: HK97 (tail not shown), MS2, P22 (tail not shown), Qβ. Rod-shaped viruses: Tobacco mosaic virus (TMV), bacteriophage M13. Images of the icosahedral viruses were reproduced from the VIPER database (www.viperdb.scripps.edu). BMV: PDB 1JS9[41]; CCMV: PDB 1CWP[37]; CMV: PDB 1F15[42]; CPMV: PDB 2BFU[38,39]; TYMV: PDB 1AUY[40]; HBV: PDB 1QGT[44]; Murine Polyomavirus: PDB 1SID[43]; HK97: PDB 1OHG[47]; MS2: PDB 2MS2[45]; P22: PDB 2XYY[48]; Qβ: PDB 1QBE[46]. The structure of TMV was reprinted from Journal of Molecular Biology, 371, C. Sachse, J.Z. Chen, et al., High-resolution Electron Microscopy of Helical Specimens: A Fresh Look at Tobacco Mosaic Virus, 812-835, Copyright (2007), with permission from Elsevier.[50] The structure of M13 was reproduced from PNAS, 104, A.S. Khalil, J.M. Ferrer, et al., Single M13 bacteriophage tethering and stretching, 4892-4897, Copyright (2007), with permission from National Academy of Sciences.[49]

proved useful in intravital imaging of vascular development. CPMV is commonly produced from infected black eyed pea plant leaves in about 0.9 mg/g yields.

Red clover necrotic mosaic virus (RCNMV) is a T=3 icosahedral virus with an outer diameter of 36 nm and an inner diameter of 17 nm. Its capsid is composed of 180 identical capsid proteins of 37 kDa. RCNMV can be obtained by inoculation on *Nicotiana clevelandii* plants, followed by collection and purification. Similar to CCMV, it can undergo a structural transition resulting in the formation of pores in its capsid.

The icosahedral turnip yellow mosaic virus (TYMV) capsid is 28 nm in diameter and composed of 180 protein subunits which self-assemble into a capsid with T=3 symmetry.[40] It can be isolated from turnip or Chinese cabbage. TYMV is stable in a broad range of pH's, at high temperature and in various chemical environments.

Studies on several other icosahedral plant viruses, such as brome mosaic virus (BMV)[41], cucumber mosaic virus (CMV)[42] and hibiscus Chlorotic Ringspot Virus (HCRSV) have also been reported.

Icosahedral animal viruses. The family *Polyomaviridae* consists of many icosahedral animal viruses of 40-50 nm in diameter. They have been extensively studied as tumor viruses in humans and animals. Three species in particular have also been functionalized for biomedical applications: Simian vacuolating virus 40 (SV40), human polyoma JC virus and murine polyomavirus. The murine polyomavirus is composed of an outer layer, composed of 72 VP1 capsid proteins, and an inner layer, consisting of capsid proteins VP2 and VP3.[43] VP1 can be separately expressed and purified from *Escherichia coli* and spontaneously forms capsids in the absence of VP2 and VP3. The structure of SV40 is very similar to that of the murine polyomaviurs. The human polyoma JC virus consists of two viral proteins: the major coat protein VP1 and inner core protein VP2.

The rotavirus consists of a three-layered protein capsid of 77 nm in diameter. This surrounds its viral DNA, which is composed of 11 identical double helixes coding for 12 different proteins. Six structural proteins are involved in the formation of the capsid (VP1, VP2, VP3, VP4, VP6 and VP7), whereas six other non-structural proteins are only produced in cells after infection by the rotavirus.

The human hepatitis B virus (HBV) is a human-specific liver virus composed of three different types of proteins: the small, medium and large proteins.[44]

Icosahedral bacteriophages. MS2 is an RNA-containing bacteriophage with an icosahedral phage head of 27 nm consisting of 180 identical capsid proteins.[45] These subunits spontaneously self-assemble into genome-free, non-infectious hollow particles, independently of the phage tail. The 32 pores in MS2 of ~ 2 nm wide enable interior functionalization without disassembly of the capsid. The MS2 VLPs can withstand pH's from 3 to 10, making them a versatile platform for chemical modification. The viral capsid proteins of MS2 can be expressed in *E. coli,* after which the capsids can be assembled towards VLPs. Yields of 30 mg/L have been reported for this procedure.

E. coli bacteriophage Qβ is a 28 nm T=3 icosahedral particle composed of 180 identical coat proteins of 14 kDa, which can be obtained by bacterial expression.[46] The virus strongly resembles bacteriophage MS2. However, due to strong non-covalent interactions between the subunits, the viral capsid is more stable than MS2 under extreme temperature, pH and chemical reagent conditions.

Bacteriophage HK97, known to infect *E. coli*, is a large phage with a spherical phage head consisting of 415 capsid proteins with a double-stranded DNA genome.[47] A mature virus head is formed during a series of maturation steps, during which the capsid expands from 56 to 66 nm. A library of HK97 particles

with different sizes and stabilities can be created by trapping them in different maturation stages.
The *Salmonella typhimurium* bacteriophage P22 has a 60 nm icosahedral T=7 head and a short tail. The capsid is assembled from 415 copies of the 47 kDa coat protein.[48] Similar to HK97, the structure of P22 can undergo maturation. Additionally, maturation can be mimicked by extended heating. In this case a wiffle-ball capsid is formed, which is identical in structure to the mature capsid, except for the presence of 12 holes of 10 nm.

Rod-shaped viruses. Bacteriophage M13 is a commonly used rod-shaped display system, composed of five different proteins. It is 880 nm long and 7 nm in diameter.[49] The virus is composed of a piece of circular, single-stranded DNA surrounded by 2700 copies of major coat protein P8. Its ends are capped with 5 copies of 4 different minor coat proteins. These proteins are utilized in a technique called phage display, in which peptides that can recognize specific targets are presented on the virus ends.
The Tobacco mosaic virus (TMV) is a 300 nm rod-shaped virus with a helical capsid symmetry.[50] It is build up from 2130 identical proteins of 17.5 kDa, which assemble around the viral DNA. The resulting helix has an outer diameter of 18 nm and a 4 nm wide core. In 1999, TMV was used as a template for biomineralization for the first time.[51] Since then, TMV has often been employed for the production of conductive polymers and metal nanowires. TMV is commonly obtained from infected tobacco plants in high yields.
The Potato Virus X (PVX) has a rodlike structure of 500 nm in length and 13 nm in diameter composed of 1270 identical 25 kDa capsid proteins.

3 Covalent functionalization strategies

Several chemical methods exist which can be used for selective modification of certain amino acids (Fig. 5). In most cases, endogenous lysines, cysteines and glutamic and aspartic acids are targeted.
Lysines residues, which possess a primary amine residue, are mostly reacted with *N*-hydroxy-succinimide esters (NHS esters) (Fig. 5a). The advantage of this residue is that it's often solvent-exposed, due to its hydrophilicity. At the same time, targeting of specific lysine residues can be more complicated. N-termini can be derivatized using similar chemistry. However, these amines are not necessarily solvent-exposed, thus N-terminal modification with NHS esters is only applicable for some protein nanocages. A limitation of NHS chemistry in general is a side hydrolysis reaction in water, which competes with nucleophilic attack of primary amines.
Two popular modification strategies exist for solvent-exposed cysteine residues, which provide reactive thiols (Fig. 5b). Maleimide-thiol Michael-type couplings are most often used in cysteine-targeted functionalization, but halogen-substituted acetylamides have also been used as reaction partners.
Aspartic and glutamic acids provide free acid moieties, which can be addressed by primary amines in the presence of carbodiimides such as *N*-ethyl-*N*'-(3-dimethylaminopropyl)-carbodiimide (EDC) (Fig. 5c). The disadvantage of this type of functionalization is the use of primary amines, which are ubiquitous when working with proteins and/or in biological systems.
Tyrosine residues have been both utilized in their endogenous form, as well as after genetic introduction. Oxidative coupling of a phenylene diamine derivative with $(NH_4)_2Ce(NO_3)_6$ as the oxidant has been used to functionalize tyrosine residues (Fig. 5d). Alternatively, via a diazonium coupling, benzenediazonium derivatives have been obtained. The latter has also been done for genetically introduced tryptophan residues (Fig. 5e) and *para*-amino-L-phenylalanine residues (Fig. 5f).
Another useful approach for functionalization is the reaction between alkynes and azides, as these react in an orthogonal fashion with each other. Therefore, homopropargylglycine (HPG) (Fig. 5g) and azidohomoalanine (AHA) (Fig. 5h) have been incorporated into protein nanocages. Generally, copper(I) is used to catalyse the azide-alkyne cycloaddition (copper(I)-catalyzed azide/alkyne cycloaddition, CuAAC). Moreover, several types of strained alkynes have been developed recently which react with azides without the need of a copper catalyst (copper-free click chemistry). This approach can be useful when AHA is incorporated into the target protein.

4 Applications in drug delivery

In this section we describe the different modification strategies that have been followed to introduce biological function to protein cages for a biomedical purpose. This entails both genetic methods as the chemical strategies depicted in Fig. 5.

4.1 Protein cages

Ferritin. Douglas *et al.* were the first to introduce a modification onto the exterior of ferritin, while maintaining the overall cage architecture and the ability to form ferromagnetic iron oxide nanoparticles.[52] The authors attached the amino acid sequence RGD-4C (CDCRGDCFC)[53] to the exterior N-terminus of human heavy chain ferritin (HFn) via a genetic modification strategy. This peptide selectively binds integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which are known to play an important role in the initial phases of some human diseases such as rheumatoid arthritis and cancer.[54,55] The RGD-4C-HFn mutant showed increased specific targeting to amelanotic melanoma cells, compared to control HFn. In a separate study, the same authors proved that RGD-4C-HFn was able to specifically target THP-1 human acute monocytic-leukemia cells.[56] Additionally, it was found that the HFn mutant was internalized much more efficiently than the control. Xie *et al*. showed that a hybrid ferritin nanocage can be constructed by a pH dependent disassembly and reassembly of two differently functionalized protein cages.[57] This methodology was also used to construct RGD-4C-functionalized HFn.[58] The advantage of this method is the possibility to functionalize the exterior of ferritin with multiple functional groups. Recently, the authors showed that, after being precomplexed with Cu(II), the chemotherapeutic agent doxorubicin (Dox) can be loaded into RGD-4C-HFn with high efficiency.[59] Compared to free Dox, the drug-loaded capsules showed an extended circulation time and reduced cardiotoxicity, while maintaining integrin selectivity imposed by RGD-4C.
A melanoma-targeting HFn platform, functionalized with α-melanocyte-stimulating hormone (α-MSH), was developed by Ceci *et al*.[60] The nanoparticles were specifically taken up by melanoma cells and not by other types of cancer cells. By masking the ferritin cage with polyethylene glycol (PEG) molecules, non-specific recognition was significantly reduced and the circulation times were improved.
Another biochemically interesting target is the epidermal growth factor receptor (EGFR), which is overexpressed in a number of cancers, such as lung and breast cancer.[61] Cao *et al.* produced EGF-HFn nanoparticles, which were efficiently taken up by breast cancer MCF-10A and MDA-MB-231 cells.[62]

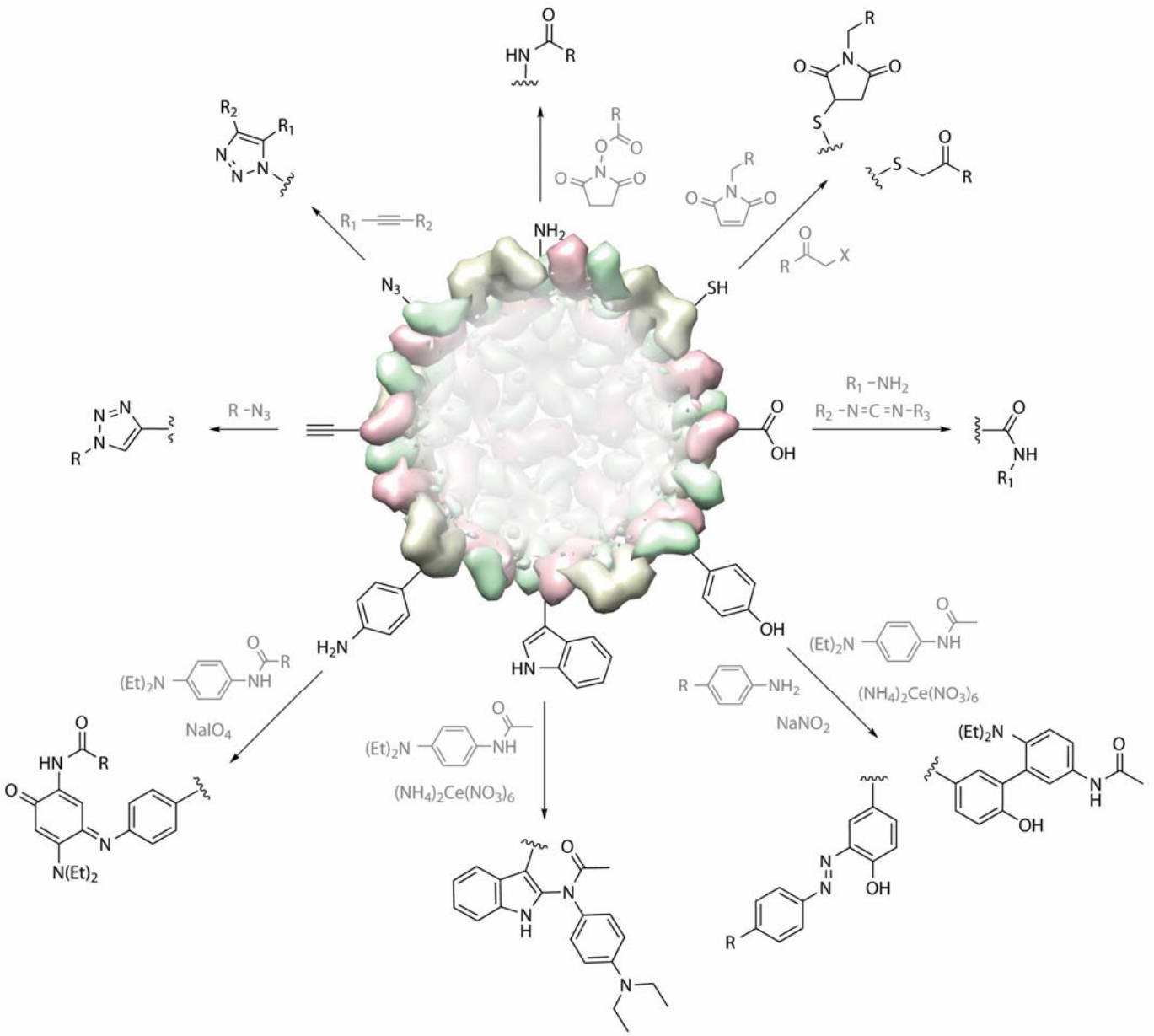


Fig. 5 Depiction of the most predominantly used internal and external chemical functionalization strategies of protein-based nanocages. (a) Lysines, N-termini: conjugation to *N*-hydroxysuccinimide esters. (b) Cysteine: thiol-maleimide coupling or coupling with halogen-substituted acetamides. (c) Glutamic and aspartic acidamide formation through activation with a carbodiimide. (d) Tyrosine: oxidative coupling of a phenylene diamine derivative or oxidation through a diazonium coupling reaction. (e) Tryptophan: oxidative coupling of a phenylene diamine derivative, similar to that shown for tyrosine. (f) *Para*-amino-L-phenylalanine: oxidative coupling of a phenylene diamine derivative, similar to that shown for tyrosine and tryptophan. (g) Homopropargylglycine: click chemistry between the alkyne and an azide. (h) Azidohomoalanine: click chemistry between the azide and an alkyne, similar to that shown for homopropargylglycine.

Kang *et al*. genetically modified HFn to provide reactive cysteine residues on its exterior surface. These were functionalized with 24 β-cyclodextrins through a thiol-maleimide addition followed by CuAAC.[63] The resulting β-CD-HFn complexes could form inclusion complexes with hydrophobic molecules such as adamantanes. These guest molecules were released slowly in a buffer solution, illustrating the potential of these systems for the delivery of hydrophobic drugs.

The same authors developed a stimuli-responsive delivery platform based on ferritin from hyperthermophilic archaeon *Pyrococcus furiosus* (PfFn).[64] The C-terminal glycine in the interior cavity could be substituted with cysteine (G173C) for site-specific conjugation of cargo molecules. Furthermore, a thrombin cleavage peptide was introduced on the outer surface, which could be selectively cleaved by thrombin resulting in the release of the C-terminal helix, including cysteine 173 and its attached cargo. Specific recognition by MDA-MB-231 cells was achieved by the attachment of 48 biotins per cage on average. Kang and co-workers realized tumor targeting of PfFn by genetically introducing the Fc-binding peptide of IgG into a flexible loop region between two helices and mixing the modified protein with the natural protein subunits.[65] Binding to

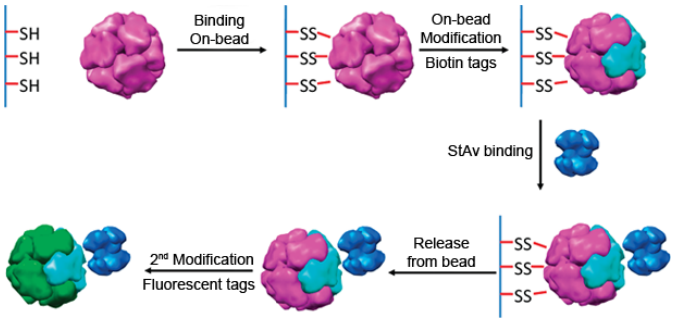


Fig. 6 Surface-masking approach for toposelective modification of the surface of LiDps towards Janus-like particles. Adapted with permission from J. Am. Chem. Soc., 131, Suci, P.A., Kang, S. Young, M. and Douglas, T., A Streptavidin-Protein Cage Janus Particle for Polarized Targeting and Modular Functionalization, 9164-9165. Copyright (2009) American Chemical Society.[67]

breast cancer cells and folate receptor overexpressing cells was demonstrated.

Another member of the ferritin superfamily is the DNA-binding protein from *Listeria innocua* (LiDps). This nanocontainer is composed of 12 identical 18 kDa subunits and has inner and outer diameters of 9 and 5 nm, respectively. Janus-like particles were created with this protein cage by toposelective modification of its surface by a masking/unmasking method on a solid support (Fig. 6).[66] Two different moieties could be attached to different sides of the cage, allowing for sophisticated drug delivery through a polarized orientation with respect to the cell surface. As a proof of concept, the particles were loaded with monoclonal antibodies against *Staphylococcus aureus*.[67]

Small heat shock protein. MjHsp has been proven to be a versatile platform for interior and exterior modification, pioneered by Douglas *et al.* They first demonstrated that specific amino acid residues could be genetically replaced for the display of thiol and amine groups.[68] This methodology was used to encapsulate and release Dox in a controlled and selective manner.[69] Mass spectrometry confirmed the attachment of 24 molecules of Dox on the interior surface of MjHsp. A hydrazone linker was used, which could be hydrolyzed under acidic conditions, similar to the conditions in the endosome. Subsequent studies showed that the MjHsp nanocontainer could be functionalized with cell-targeting peptides and antibodies.[70] The attachment of abovementioned $\alpha_v\beta_3$ and $\alpha_v\beta_5$ targeting RGD peptides imparted cell-specific targeting capabilities.[70,71]

Douglas *et al*. also utilized LyP-1, a small peptide which targets tumor-associated lymphatic vessels and macrophages, as a targeting moiety.[72,73] Macrophage-rich atherosclerotic lesions in mice were targeted by MjHsp cages with LyP-1 genetically attached to the C-terminus.[74]

Hashizume *et al*. used MjHsp to target hepatocellular carcinoma (HCC) cells, by functionalization with HCC-binding peptide SP94 (SFSIIHTPILPL).[75] The authors reacted amine groups on the exterior of MjHsp with a bifunctional PEG linker and the SP94 peptide.[76] By fluorescence microscopy, it was shown that the nanocages bound specifically to HCC cells and not to other cell lines tested. Furthermore, the authors optimized the affinity of the cages for HCC cells by varying the amount of immobilized SP94, the conjugation site of SP94 and linker length. The most effective binding was found for conjugation of the N-terminus of SP94 to the protein cage with high SP94 levels and a longer linker length. The same authors also published a method by which the SP94-functionalized MjHsp cage can be made genetically.[77] This way, the density of the SP94 peptide on the outer surface was easier to control, compared to the multi-step chemical process. Dox was conjugated to the interior of this cage through a hydrazone linker and its cytotoxic effects towards Huh-7 HCC cells were demonstrated.

Furthermore, liver cell specific targeting nanocages were developed by genetically modifying the outer surface of MjHsp with the preS1 peptide.[78] This peptide is derived from the hepatitis B virus and is known to have a high affinity for liver tissue. The functionalized protein cages showed lower cytotoxicity and higher specificity towards human hepatocyte cell lines than other cell lines *in vitro*.

4.2 Virus-like particles

CCMV. One of the first examples of chemical modification of VLPs was reported for CCMV.[79] Douglas *et al.* addressed natural amines and carboxylic acids present on the surface for the attachment of functional molecules. In addition, the authors genetically engineered a mutant with two surface exposed thiols per capsid protein since the wild type virus does not have reactive cysteine residues. It was found that up to 540 amines, 560 carboxylic acids and 100 thiols could be labelled with a fluorescent dye, without disrupting the cage structure. Moreover, surface exposed thiols were chemically linked to an antitumor 24 amino acid peptide sequence.

In order to control the attachment of different groups to the same VLP, Douglas *et al.* constructed CCMV capsids through mixed self-assembly.[80] Two populations of capsids were created by attaching different ligands to exterior lysine residues. The capsids were disassembled *in vitro* and the resulting subunits were separately purified by size exclusion chromatography (SEC). Then, the purified capsid proteinss were mixed in defined ratios in order to precisely control the composition of the final capsids (Fig. 7).

An application of the functional modification of CCMV came with the development of dual-functionalized CCMV capsids for the treatment of bacterial infections via photodynamic therapy (PDT).[81] In PDT, specific wavelengths are used to excite photosensitizers to generate reactive oxygen species, thereby

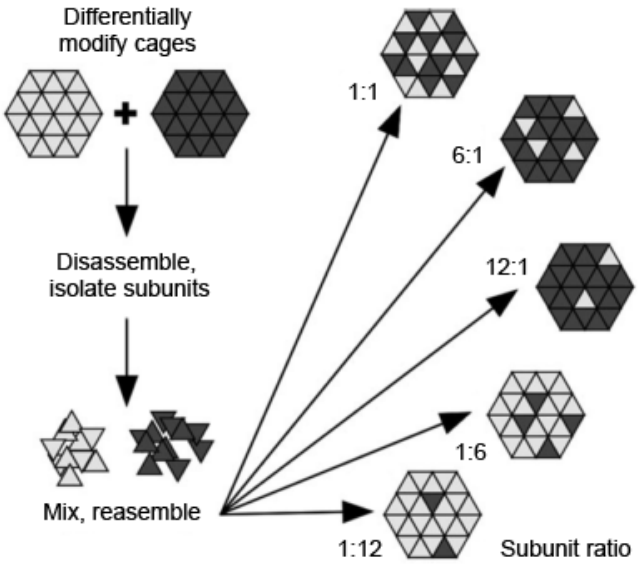


Fig. 7 Schematic presentation of the assembly of mixed protein cages. Reproduced with permission from John Wiley and Sons (Copyright © 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim).[80]

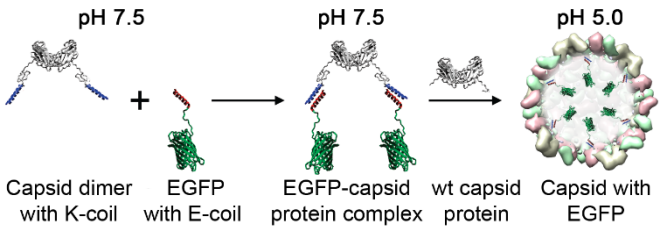


Fig. 8 Schematic presentation of the formation of the protein-capsid complex with leucine zippers.[82]

killing the target cells. A ruthenium complex, which acts as a photosensitizer, was chemically attached to exterior cysteine residues. To improve the PDT, specific antibodies were also linked to the capsid to target *S. aureus* cells. Killing of targeted cells increased up to 100-fold relative to the non-targeted cells or the free ruthenium complex.

In contrast to the abovementioned examples, methods have also been developed to selectively modify the interior of CCMV. Cornelissen *et al.* developed an encapsulation method based on non-covalent interactions between the capsid proteins and the cargo, as this eliminates the need for chemical reactions.[82] The N-terminus of the capsid protein was modified with a positively charged leucine zipper and its negative complementary counterpart was attached to enhanced green fluorescent protein EGFP (Fig. 8). By varying the ratio between wild-type and modified capsid protein, the amount of encapsulated EGFP could be precisely controlled and up to 15 EGFP molecules were encapsulated.

CPMV. A wide variety of targeting molecules have been attached to the CPMV capsid by chemical conjugation to either lysines or cysteines using for instance NHS and maleimide conjugated ligands, respectively.[83-92] Specific interior modification of the CPMV capsid has been performed by addressing the naturally occurring reactive interior cysteines.[93] Fluorophores, biotin affinity tags, PEG polymers and various peptides were conjugated to the capsid interior. Since native CPMV does not display reactive cysteines on its exterior surface, cysteines have been genetically inserted on the surface. A variety of ligands have been shown to react with the inserted cysteines, including carbohydrates[85], antibodies[84,86], oligonucleotides[88], organic dyes[84,94], biotin[94], peptides[95] and proteins[89]. A problem of genetic introduction of thiols however, is the possibility of disulfide bond formation, followed by aggregation. Therefore, a chemical method was developed for the introduction of protected thiols to CPMV VLPs.[96] Surface-exposed lysines were modified to thioacetate groups using *N*-succinimidyl-*S*-acetylthiopropionate. The protecting group could be easily removed with hydroxylamine hydrochloride. Surprisingly, no aggregation of the particles was observed after deprotection, probably due to the positioning of the thiol groups. Further mutagenesis studies showed that the exterior solvent accessible lysines are addressable in a different degree.[97] A series of CPMV mutants was produced where specific lysines were replaced by arginine residues, in order to tailor the locations of the reactive groups on the surface. This knowledge was applied for the specific attachment of a component of HER2 tyrosine kinase receptor to a single lysine, after replacing the other four lysines with arginines.[89] Additionally, histidines have been genetically introduced at various locations on the CPMV capsid.[98] Their relative reactivity was confirmed by labelling of the histidines with nanogold.

Native CPMV also possesses 180 accessible carboxylate groups which can be addressed, as shown by Evans *et al.*[99] This method of modification is very valuable, as it provides chemists with coupling chemistry which can be independently used without affecting amines and cysteines on the CPMV surface.

Derivatization of the CPMV exterior has also been performed using CuAAC, which allows conjugation with different types or more challenging ligands, compared to abovementioned strategies. Another advantage of using click chemistry over direct functionalization of the lysines or cysteines is the selectivity. Both reaction components (azide and alkyne) are non-reactive, unless they are mixed in the presence of a Cu(I) catalyst. Finn *et al.* showed that azides and alkynes can be introduced on the CPMV capsid surface at both reactive lysine and cysteine residues.[100] These handles were then used to incorporate a wide variety of ligands, including organic dyes, carbohydrates, peptides and PEG polymers.[100-103] For tumor-targeted delivery, transferrin (Tfn) and glycopolymers were conjugated to CPMV using click chemistry, as the Tfn receptor and carbohydrate receptors are overexpressed on several types of tumor cells.[101,104] Azides were also installed on the CPMV surface by addressing accessible tyrosines, followed by a click reaction to conjugate an organic dye.[105] This was only possible however, if an inhibitor was added to prevent crosslinking of tyrosines after oxidation.

The abovementioned methods for CPMV derivatization have been applied in several studies towards CPMV drug carriers. For instance, PEGylation has been shown to reduce the interaction of CPMV with cells *in vivo*.[106] When a targeting moiety is combined with PEG on the surface of CPMV, the desired cells are targeted while eliminating any background binding of the VLP to other cells. This was shown by Manchester and co-workers, who used folic acid (FA) in combination with PEG to target tumor cells.[107] Uptake of FA into cells is mediated by the folate receptor, which is overexpressed on the surface of many human cancers. PEGylation can also be applied to modulate binding of ligands to the surface op CPMV. For instance, the binding of Lerner-Janda blue fluorescent antibody to its antigen (stilbene), which was bound to CPMV, could be blocked and controlled by PEGylation of the surface.[86]

CPMV decorated with peptide ligands have also been used for tumor targeting. Peptide F56 was conjugated to the surface of CPMV, which specifically binds vascular endothelial growth factor receptor 1.[91] First, exposed lysine residues were converted into benzaldehyde groups, after which the peptide was ligated via a stable hydrazone linkage. *In vitro* specificity for tumors overexpressing the appurtenant receptor was observed. Poly-arginine cell penetrating peptides were attached to CPMV via a similar hydrazone ligation strategy.[108] Studies *in vitro* showed effective uptake by HeLa cells. Bombesin peptides were attached to CPMV via NHS acylation and click chemistry for targeting of tumor cells overexpressing the gastrin-releasing peptide receptors.[92] Targeting and uptake in human prostate tumor cells was demonstrated *in vitro*. The same strategy was used for the attachment of a cyclic RGD-containing peptide.[109] Selective binding and internalization into several cancer cell lines expressing RGD-binding integrin receptors was shown *in vitro*.

A useful biomedical application of CPMV in PDT was described by Manchester *et al*. Derivatives of C_{60} are excellent photosensitizers. However, C_{60} is very hydro-phobic, which limits its application in PDT. By attaching C_{60} to CPMV through its native lysine residues, the solubility of the photosensitizers was significantly enhanced.[90] Furthermore, cellular uptake by

HeLa human cancer cells was not inhibited by the attachment of C_{60}.

Utilizing both native carboxylic acid and amine groups on the exterior of CPMV, Evans and co-workers attached Dox to the capsid.[110] The carboxylic acid groups were modified with NHS, followed by a reaction with Dox to create a stable amide linker. Lysines were attached to Dox through a linker containing a labile disulfide bond. The ability of CPMV to target cancer cells via surface-expressed vimentin[111] was used to target the VLPs to HeLa cells. A higher cytotoxicity toward HeLa cells was observed compared to free Dox, when the amide linker was used. The VLP with disulfide linker was approximately as active as free Dox. This is the first example of a promising application of CPMV as a drug delivery vehicle.

Bacteriophage MS2. The Stockley group was the first to chemically modify the MS2 capsid for encapsulation of cargo molecules. They utilized the natural affinity of the coat protein for a specific fragment in the viral RNA sequence for drug encapsulation. The specific RNA stemloop was attached to a plant toxin, ricin A chain and packaged into the VLP.[112] To achieve targeting, surface-exposed lysines were modified to thiols with *N*-succinimidyl S-acetylthioacetate for the attachment of Tfn.[113] *In vitro* studies with the dual-functionalized MS2 capsids demonstrated cell-specific targeting and cytotoxicity.

Exterior lysines have also been used to attach the SP94 peptide for HCC cell targeting.[114] The authors showed that VLPs modified with the targeting ligand exhibited a 10^4-fold higher avidity for HCC than for control tissues. Furthermore, these conjugates were used to deliver a variety of cargo molecules to HCC cells.

Cell targeting was also achieved through functionalization of the exterior of MS2 capsids with the cell penetrating HIV-1 Tat peptide.[115] Amine groups were subsequently reacted with a heterobifunctional crosslinker and the N-terminal thiol side chain of the Tat peptide. The capsids were successfully delivered to human hepatoma Huh-7 cells. Covalent attachment of human transferrin to the MS2 surface induced targeting to HeLa cells.[116]

Other exterior residues have also been used to create functional handles onto the MS2 surface. For example, Francis and co-workers explored the reactivity of electron-rich amino acids tryptophan and tyrosine for exterior capsid functionalization.[117] The majority of these native residues are not solvent-accessible and thus unlikely to be prone to modification. One exception is tyrosine 85, an interior amino acid. However, the authors envisioned that functionalization of this residue would not be possible when large coupling partners were used. Tyrosine and tryptophan residues were thus introduced on external positions 19 and 15. As a proof of principle, PEG and RGD derivatives were attached to the surface of MS2. Swartz *et al.* used a cell-free methionine replacement approach to introduce surface-exposed AHA and HPG residues containing reactive side chains.[118] These could be reacted with alkyne- and azide-containing molecules, respectively. Proteins, nucleic acids and PEG chains were conjugated in a controlled fashion. This technology was applied to both bacteriophages MS2 and the bacteriophage Qβ (see also next section). Peabody showed that an exterior threonine could be substituted by cysteine.[119]

An alternative functionalization approach was introduced by Francis and co-workers. They implemented the procedure for unnatural amino acid incorporation, developed by Schultz *et al.*,[120] to introduce an alternative orthogonal functional handle in MS2. A *para*-amino-L-phenylalanine (pAF) was introduced in each capsid monomer using the amber codon suppression

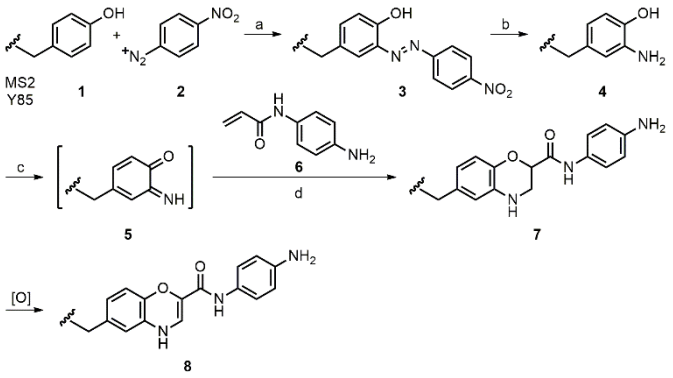


Fig. 9 Four-step methodology for the attachment of olefin-containing substrates to tyrosine residue 85 on the interior of MS2. (a) **2** (5 equiv.), pH 9, 4 °C, 15 min; (b) $Na_2S_2O_4$ (100 mM), pH 7.2, rt, 2h, 80-85% protein recovery, 2 steps; (c) $NaIO_4$ (100 μM), pH 6.5, followed by (d) 6 (10 mM), 2 h, 75% protein recovery. Adapted with permission from J. Am. Chem. Soc., 126, Hooker, J.M., Kovacs, E.W., Francis, M.B., Interior surface modification of bacteriophage MS2, 3718-3719. Copyright (2004) American Chemical Society.[125]

method.[121] In this method, the target codon is mutated to an amber stop codon in the mRNA. Suppressor tRNA aminoacylated with the unnatural amino acid recognize the amber codon as a sense codon, allowing for the incorporation of any unnatural amino acid. The resulting 180 copies of the aniline group could then be reacted with specific cell targeting peptides and DNA aptamers.[121,122] Subsequently, the aptamer-conjugated MS2 cages were loaded with porphyrins for PDT and targeted to Jurkat leukaemia T cells.[123] The dual-functionalized particles were able to kill the Jurkat cells selectively in only 20 minutes by PDT. Recently, Francis *et al.* reported a more efficient version of the aniline oxidation reaction under similar conditions.[124] High levels of conversion and equivalent chemoselectivities were achieved in < 2 minutes, using aminophenols instead of phenylene diamines for the coupling with aniline side chains.

The Francis group was the first to modify the MS2 interior, utilizing the same strategy which had been previously applied for the interior modification of MjHsp[36], CCMV[59] and CPMV[83]. They developed an orthogonal four-step methodology for the attachment of olefin-containing substrates to modified tyrosine residues in the capsid.[125] Key steps were a diazonium-coupling reaction, followed by a hetero-Diels Alder reaction (Fig. 9). A similar strategy was also applied for the attachment of 50-70 dyes inside MS2, demonstrating the ability to encapsulate large drug molecules.[126] In the same paper, the authors showed that the particles could be subsequently functionalized with PEG chains to improve the circulation times *in vivo*. Interior modification was also achieved by mutating an asparagine residue to a cysteine.[127] To this handle, taxol was attached, which is a potent chemotherapeutic agent used in the treatment of several cancers. Upon incubation with breast cancer MCF-7 cells, the linker cleaved, thereby releasing the drug. Surprisingly, this was achieved without the use of targeting groups.

Bacteriophage Qβ. The field of Qβ functionalization was pioneered by the Finn group. Their first paper on this topic comprised the replacement of an exterior methionine for AHA and HPG via the sense-codon technique.[128]

They also showed that a functional handle inside the capsid could be introduced by mutation of Thr93 to an azidohomoalanine.[129]

Using click chemistry, both of these functional groups could be addressed without loss of the capsid stability.

Cell targeting was achieved for the first time when human transferrin was conjugated to Qβ particles.[130] The authors used a strategy they previously had used for the attachment of Tfn to CPMV.[101] Only in this case, Tfn was not derivatized at the cysteine residues, allowing for controlled derivatization and preserving protein function. Here, the sialic acid residues on Tfn were oxidized, followed by introduction of a reactive alkyne functionality. Exterior lysine residues on the Qβ capsid were exposed to a succinimidyl ester activated alkylazide to introduce the complementary reaction partner. The Qβ-Tfn conjugate, formed by CuAAC, showed specific recognition and internalization by Tfn-receptor-bearing cells. The uptake rate turned out to be proportional to the ligand density and was inhibited by the presence of free Tfn.

Finn *et al.* also modified Qβ such, that its exterior displayed 5-12 copies of EGF for cancer cell targeting.[131] A two-plasmid system was used to produce a mixture of a C-terminal truncated version of the native coat protein and a version with EGF fused to the C-terminus (Fig. 10). The functionalized particles were still prone to bioconjugation by click chemistry, without disruption of the ability to interact with the EGF receptor. Furthermore, apoptosis of A431 epidermoid carcinoma cells could be induced by interaction with EGFR.

In another application, cell targeting was achieved by conjugation of Qβ to a modified sialic acid which selectively binds to CD22.[132] The imparted targeting capabilities were combined with cytotoxic activity through the attachment of a metalloporphyrin derivative for PDT. Both ligands were provided with an azide functionality and alkynes were attached to the surface through acylation of the 720 surface exposed amine groups for functionalization by means of a CuAAC reaction. Successful targeting, internalization and phototoxicity were detected for the modified particles.

Photodynamically active VLPs have also been obtained by attachment of photosensitizer C_{60} to Qβ.[90] Conjugation of fullerenes to VLPs was already mentioned above for CPMV.[90] In this case, C_{60} was attached to Qβ via two different methods: directly to the native lysine residues and via click chemistry with azides conjugated to the Qβ surface via a linker. The authors showed that the Qβ-C_{60} complex was internalized by breast cancer cells. It was also proven that cell uptake and cell killing in prostate cancer cells is possible using white light therapy.[133]

Qβ nanoparticles were also used for the display of cationic motifs that act as heparin antagonists. Heparin stimulates thrombin inhibition by antithrombin and is therefore often used as an anticoagulant. Normally, high doses are administered to prevent thrombosis, which can lead to uncontrolled bleeding in some cases. Protamine, which is highly positively charged, is the only FDA approved agent for heparin neutralization, but its adverse effects are significant. Therefore, in order to develop an alternative for Protamine, several point mutations on the exterior surface of Qβ were used to introduce a spectrum of particles with different surface charges. The VLPs appeared to bind and block the anticoagulant function of heparin more effectively than Protamine in a biochemical assay.[134] In patient samples, the modified Qβ capsids also showed heparin reversal, even if high doses of heparin were administered to the patients.[135] In comparison to Protamine, more consistent results were obtained for the VLPs.

In order to improve the pharmacokinetic properties of Qβ, Finn and co-workers also prepared polymer-coated Qβ VLPs.[136] Atom-transfer radical polymerization (ATRP) initiators were attached to the exterior surface of Qβ via click chemistry, after which oligo(ethylene glycol)-methacrylate and its azido-functionalized analogue were polymerized on the surface. The resulting particles retained a low polydispersity and allowed conjugation of i.a. Dox via CuAAC.

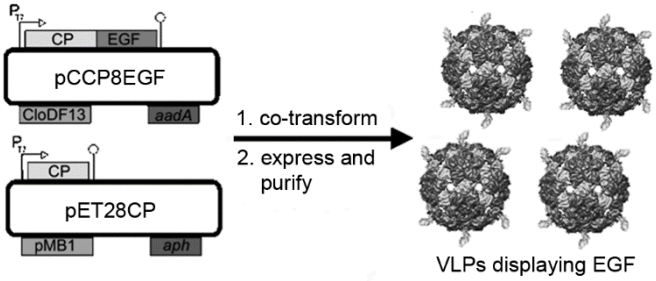


Fig. 10 Schematic presentation of the production of Qβ particles partially displaying EGF at the exterior C-terminus. Reproduced with permission from John Wiley and Sons (Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim).[131]

TMV. Mutants of TMV displaying lysine and cysteine residues on the outer surface have been made for functionalization via selective amine and thiol chemistry.[137,138] Francis *et al.* utilized naturally occurring external tyrosine residues for an efficient diazonium coupling/oxime formation sequence, similar to the abovementioned strategy for MS2 functionalization.[139] This approach was used to decorate the surface with > 1000 copies of PEG MW 2000 and 5000. In addition, glutamic acid residues on the interior could be modified via a carbodiimide coupling reaction with several amines. [139] The Wang group used the same diazonium chemistry in combination with CuAAC to modify the exterior tyrosines.[140,141] Instead of using diazonium salts bearing an ester-group, the authors used alkyne-functionalized diazonium salts, which could be clicked to a wide range of compounds.

Miscellaneous VLPs. Several other types of virus-like particles have been applied in studies on the development of drug delivery vehicles. Below, we will discuss three examples which we deem the most valuable in addition to the abovementioned VLPs. Less commonly applied VLPs are bacteriophage λ[142], BMV[143], HBV[144-146], CMV[147], HCRSV[148], HK97[149], PVX[150,151], RCNMV[152], rotavirus[153], TYMV[154,155]. The reader is referred to the given referenced for more information.

Bacteriophage P22 was utilized by Prevelige *et al.* as a molecular display platform by genetically introducing a cysteine residue on its exterior.[156] This cysteine proved reactive towards maleimide reagents. Non-covalent attachment of cargo to P22 was achieved using a stepwise addition protocol with the phage decoration protein (Dec).[157] This protein binds selectively to the surface of mature capsids.[158] Both termini of the bound Dec appeared available for cargo binding. Recently, the Douglas group found that the C-terminus of P22 extends towards the capsid exterior.[159] They used this knowledge to i.a. introduce a cysteine on the exterior, which can serve as a handle for functionalization. The same group also investigated different strategies for interior modification of the P22 capsid. Genetic introduction of cysteine residues, followed by site-specific attachment of a maleimide-biotin complex proved successful for both the empty capsid and the wiffle-ball.[160] Anchoring of streptavidin to the biotin molecules was possible for the wiffle-ball owing to its holes, providing a method for cargo loading. Site-selective initiation of ATRP on the interior of P22 was used to introduce handles for

the attachment of small-molecule cargo as well.[161] A new single point mutation was used to introduce an interior thiol, to which the ATRP initiator could be attached. After polymerization, the primary amine groups present on the side chains of the polymer were found reactive towards for instance a fluorescent dye. Finally, the authors used metal-ligand coordination to load cargo into P22.[162] Again, cysteine residues were genetically introduced in the interior, followed by reaction with 5-iodoacetamido-1,10-phenanthroline, binding of metal ions and addition of phenanthroline ligands. By attaching a small molecule to the ligand, cargo loading could be achieved.

Bacteriophage M13 was first used by Chen *et al.* for drug delivery.[163] They genetically produced chimeric phages by displaying RGD-4C and a streptavidin-binding peptide for tumor targeting and tracking, respectively. *In vivo* tumor targeting of the chimeric phage complexed with streptavidin-quantum dots was achieved. Chemical methods have also proven useful for the attachment of chemotherapeutic agents Hygromycin and Dox to surface-exposed carboxylic acids.[164,165] Chloramphenicol-loaded M13 phages have been used as antibacterial targeted delivery vehicles.[166] Wang *et al.* explored the chemical modification of three reactive groups on M13: amino groups of lysine residues and the N-terminus, carboxylic acid groups of aspartic and glutamic acid residues and phenol groups of tyrosine residues.[167] Fluorescent molecules and cancer cell targeting motifs such as FA could be attached. The reactive groups could also be addressed separately, creating new methods to selectively produce dual-functionalized M13 phages. Next, the authors used the folic acid-conjugated M13 to create a core shell structure where the phage formed the shell and copolymer poly(caprolactone-*b*-2-vinylpyridine) the core, incorporated with Dox.[168] FA was in this case conjugated to free amines on the surface of M13 via an EDC coupling reaction. It was shown that the Dox-loaded particles were internalized more efficiently than free Dox, due to the presence of FA targeting groups.

As mentioned above, three types of polyomaviruses have been applied in biomedical applications: murine polyomavirus, SV40 and human polyoma JC virus. The VP1 capsid of murine polyomavirus has been genetically modified by Böhm *et al.* to display the sequence of a WW domain in its β-turns.[169] WW domains are small protein domains named after the two conserved tryptophan residues which are essential for its folding and function.[170] They specifically bind proline-rich peptide sequences. It was shown that one variant of the WW domain maintained its high selective binding affinity for proline-rich ligands. This allows for a short-term coupling of cargo to the exterior of the VP1 capsid. The same authors similarly introduced a WW domain in the interior of the VP1 capsid.[171] Assembly of the VP1-WW particles in the presence of proline-rich cargo resulted in an efficient encapsidation into the particles.

Other methods of VP1 modification are based on the specific interaction between VP1 and the inner core proteins VP2 and VP3. Encapsulation can in this case be achieved by fusing the desired cargo to (parts of) VP2 or VP3. Following this strategy, green fluorescent protein (GFP)[172], antimetabolite methotrexate[172] and EGFP[173] were enclosed in murine polyomavirus. In all cases, targeted drug delivery was achieved. However, the particles were irregularly shaped and showed poor solubility. SV40 has been was utilized in for the encapsulation and delivery of EGFP and cytosine deaminase.[174] In a separate study, several peptides were inserted into VP1 surface loops through mutagenesis.[175] It was shown that introduction of foreign peptides, such as Flag-tags, on the surface can reduce natural virus-cell interactions. Also, the ability to bind new target tissues was introduced by insertion of RGD motifs. The same authors conjugated human EGF to the exterior of a cysteine-containing mutant of SV40 through a thiol-maleimide coupling.[176] They showed increased selectivity for cells displaying the EGF receptor. Human polyoma JC virus has been used to create a pH-responsive drug release vesicle.[177] VP2 was modified to display His_6 tags inside the VLPs, which offered specific and reversible attachment of drug molecules containing a His_6 tag targeting segment. As proof of concept, a fluorescent small molecule was encapsidated and released in mouse NIH 3T3 cells. A similar system, in which drug release is triggered by glutathione, was developed by the same authors.[178] In this case, cyclodextrins were coupled to the inside of VP1 through a disulfide bond so that hydrophobic drugs could be encapsulated. Release of different types of dyes was shown to increase with glutathione concentration. Furthermore, anticancer drug paclitaxel was released inside the cytoplasm of NIH 3T3 cells, as the glutathione concentration in the cytoplasm is approximately three times higher than outside cells.

5 Biodistribution, toxicity and pharmacokinetics

Most of the abovementioned plant and animal VLPS are not human pathogens, which implies that these particles are less likely to interact with human surface receptor proteins, leading to toxicity. These viruses are considered much safer than for instance human adenoviruses, which are often used for gene delivery purposes and are associated with severe toxic effects.[179] Unfortunately, there are only few studies describing the characteristics of plant and animal VLPs *in vivo*. Before testing VLPs for nanomedical applications, a better understanding of the influence of size, shape, composition, surface chemistry and physical properties of their *in vivo* behaviour should be obtained.[180,181]

Animal studies have been carried out with bacteriophages M13, MS2 and Qβ and plant viruses CCMV, CPMV, HCRSV, PVX and TMV. Biodistribution studies showed that CPMV[182], M13[183], MS2[184,185], Qβ[129], PVX[151] and TMV[186] accumulate mainly in the spleen and liver. Accumulation in these organs is expected, since they are part of the reticuloendothelial system and their function is to remove antigens from circulation, including nanoparticles. CCMV is distributed to a broad range of tissues throughout the body, including the bladder, thyroid gland, liver, spleen and salivary gland.[187] Surprisingly, no signs of toxicity were observed for CCMV, despite its broad distribution.[187] Likewise, no apparent toxic effects were reported *in vivo* for CPMV[182,188], Qβ[189] and TMV[186]. HCRSV showed no cytotoxicity towards OVCAR-3, CCL-186 and CNE-1 cells.[148]

In addition to biodistribution and toxicology, pharmacokinetic properties of VLPs are also important, as their needs to be a good balance between tissue penetration and clearance from the body. Longer circulation times allow drugs to accumulate into the targeted tissue, but the chance of toxic side-effects increases. It has been shown that circulation times depend on the surface chemistry, for instance surface charge. Positively charged nanoparticles exhibit longer half-lives than negatively charged particles. The same trend is observed for VLPs. CCMV, which has a negative outer surface, displays a short blood half-life of 12 min. and within 24 h after administration, a high percentage (57-73%) is excreted.[187] In addition, a test on the mouse urine suggested *in vivo* degradation of at least a part of the capsids. Rapid blood clearance is also observed for CPMV (4-7 min)[182], MS2 (4.5 min)[184] and TMV (3 min)[186]. Long circulation times (> 4 h) are observed for Qβ, which has a positive outer surface.[129]

M13 has a half-life of 4.5 h, which was reduced to several minutes for receptor-targeted phages.[183] In case of MS2, substantial amounts of VLP remain in the bloodstream.[185]
Even though abovementioned VLPs are not human pathogens, it has been shown that the regular structure of VLPs promotes immune responses.[190] In particular, B-cells are activated which will produce virus-specific antibodies. A possible explanation for this phenomenon is that the regular array of capsid proteins can contact a bivalent B-cell receptor efficiently, which already causes a response without the aid of T-cells. Many strategies are now being developed to overcome these immunogenic responses *in vivo*, one of which is masking of the particle surface by PEGylation. This approach has already proven successful for adenoviruses.[191,192] PEGylated versions of VLPs have already been generated for CPMV[86,87,92,93,101,106,151,193], MS2[117,118,124,126], PVX[150,151], Qβ[118] and TMV[139,140]. Studies with CPMV have shown that introduction of a PEG coating can inhibit the anti-VLP response in mice[87], as well as prevent internalization by several cell types *in vitro* and *in vivo* through nonspecific binding[91,106,107,193,194]. Also, the plasma circulation time increases upon PEGylation.[182] Shielding efficiency was found to be dependent on polymer length, the number of PEG chains attached, the density on the surface and the location of conjugation.[193] A higher efficiency was obtained for more and longer PEG chains. Also for MS2 and PVX, reduction of cellular uptake has been observed after PEGylation of their capsid surfaces.[151,185] In the latter study, it was shown that the capsid could be simultaneously conjugated to a fluorescent dye. Thus, even though PEGylation might be required for successful *in vivo* applications in the future, this does not exclude other surface modification for, for instance, targeting purposes.

Conclusions

Nature has provided us with a wide variety of protein nanocages, all with different dimensions, shapes and physical properties. These particles have the unique advantage of structural uniformity, in addition to the ease of functionalization and production on large scale. Protein containers are especially useful for drug delivery purposes due to favourable properties such as water solubility, high uptake efficiency and biocompatibility. Many different modification strategies aimed at targeting specific tissue and delivering the required drug have been developed (Table 1). Genetic tailoring has allowed the introduction of functional handles in protein nanocages and site-selective conjugation of i.a. targeting ligands, therapeutics and polymers. Cysteine, lysine and glutamic and aspartic acid have evidently been mostly used in functionalization of protein nanocages for drug delivery applications. These functionalization strategies have proven to be robust, applicable to many types of proteins and versatile regarding the type of desired cargo. Also, high loading efficiencies can be achieved using these types of modification. Moreover the introduction of non-natural amino acids is a very convenient method for orthogonal functionalization of protein nanocages at specific locations or with specific cargo densities.
Although initial *in vivo* studies show promising results regarding the toxicity, immunogenicity and biodistribution, these properties have to be fully evaluated. Additionally, too little studies have focussed on drug release properties, encapsulation efficiencies and targeting abilities *in vivo* yet. These type of experiments are crucial in order to provide safety guidelines for the application of protein nanoparticles as therapeutics in clinical trials and eventually as approved therapeutic systems. Most nanocarriers that have reached the clinical trial phase up to now are liposomes, which have also been the most widely studied and successfully developed nanocarriers.[180] But also other types of nanoparticles have been approved or have been used in clinical trials, such as polymeric micelles, antibody-drug conjugates, polymer-protein conjugates and protein-drug conjugates.[195-197] However, to our best of knowledge, no protein-based nanocage has been tested in clinical trials, apart from several plant-made vaccine technologies.[198]

Acknowledgements

The Ministry of Education, Culture and Science (Gravity program 024.001.035) is acknowledged for financial support.

Notes and references

[a] Institute of Molecules and Materials, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands.

† No discrimination has been made between direct functionalization and functionalization via a linker (only the initial type of modification is described) and between genetically introduced residues and endogenous residues. Also, exterior and interior modifications are not separated in this overview.

Table 1 Overview of cargo attachment strategies described in this review.†

Residue	Type of modification	Nanocontainer	Cargo
AHA & HPG	Click chemistry	MS2	Oligonucleotide[118], PEG[118], Protein[118]
		Qβ	Fluorophore[128], Oligonucleotide[118], PEG[118], Protein[118,128]
Cysteine	S-S bond formation	CCMV	Peptide[79]
		JC virus	β-cyclodextrin[178], Fluorophore[178]
	Thiol-(iodo/bromo)acetamide	CCMV	Photosensitizer[81]
		CPMV	Fluorophore[84,99], Stilbene[84,86]
		P22	Fluorophore[162]
	Thiol-maleimide	BMV	Fluorophore[143], PEG[143], Peptide[143], Protein[143], Therapeutic[143]
		CCMV	Fluorophore[79,187]
		CPMV	Biotin[93,94], Fluorophore[39,93,94,95], Oligonucleotide[88], PEG[93], Peptide[93,95], Protein[89]
		HFn	β-cyclodextrin[63], PEG[60]
		HK97	Fluorophore[149], Protein[149]
		LiDps	Antibody[67], Fluorophore[67]
		MjHsp	Fluorophore[68,70,74,76,77], Therapeutic[69,77]
		MS2	Fluorophore[119,122], Photosensitizer[123], Therapeutic[127]
		P22	Biotin[160], Fluorophore[156,159]
		PfFn	Fluorophore[64]
		SV40	Protein[176]
		TMV	Fluorophore[138]
Glutamic & Aspartic acid	Carboxylic acid-carbodiimide	CPMV	Fluorophore[99]
		M13	Therapeutics[164,165]
		TMV	Fluorophore[139]
	NHS activation, NHS-amine	CCMV	Fluorophore[79]
		CPMV	Therapeutic[110], Viologen[99]
		M13	Fluorophore[167]
		Rotavirus	Therapeutic[153]
		TYMV	Fluorophore[154,155], Peptide[155]
Lysine	Amine-isothiocyanate	CPMV	Fluorophore[83]
	Amine-succinimidyl ester	CCMV	Biotin[80,81,83], Digoxigenin[80], Fluorophore[79]
		CMV	Folic acid[147]
		CPMV	Biotin[83,93], Carbohydrate[83,85,101,102,103,104], Fluorophore[83,92,97,100,101,104,106,107,109,151,188,193,194], Folic acid[107], Oligonucleotide[88], PEG[86,87,92,93,101,106,151,193], Peptide[91,92,101,108,109], Protein[89,101], Photosensitizer[90], Stilbene[86], Therapeutic[110]
		HCRSV	Folic acid[148]
		HK97	Fluorophore[149]
		λ	Fluorophore[142], Protein[142]
		M13	Antimicrobial[166], Fluorophore[167], Folic acid[168]
		MjHsp	Antibody[70], Fluorophore[68,70,74,76,77], Peptide[76]
		MS2	Biotin[126], PEG[126], Peptide[114,115], Protein[113,116]
		PfFn	Biotin[64]
		PVX	Biotin[150], Fluorophore[150,151], PEG[150,151]
		Qβ	Carbohydrate[102,103,132], Fluorophore[130,131,133,136,189], Peptide[134], Protein[130], Photosensitizer[90,132,133]
		RCNMV	Peptide[152]
		Rotavirus	Carbohydrate[153]
		TMV	Fluorophore[137]
		TYMV	Fluorophore[154]
pAF	Oxidative coupling	MS2	Oligonucleotides[122,123], PEG[124], Peptides[121,124]
Tryptophan	Oxidative coupling	MS2	PEG[117], Peptide[117]
Tyrosine	Diazonium-coupling	M13	Biotin[167], Fluorophore[167]
		MS2	Fluorophore[126]
		TMV	Biotin[139], Fluorophore[140], PEG[139,140], Peptide[140], Phosphate[140,141]
	Oxidative coupling	CPMV	Fluorophore[105]
		MS2	PEG[117], Peptide[117]
Loop region	Genetic	CPMV	Peptide[109]
		Murine polyomavirus	Protein[169]
		P22	Peptide[156]
		PfFn	Peptide[65]
		SV40	Peptide[175]
C/N-terminus	Genetic	CCMV	Peptide[82]
		HBV	Protein[146]
		HFn	Peptides[52,56,58-60], Protein[62]
		MjHsp	Peptide[70,74,77,78]
		Murine polyomavirus	Protein[171]
		P22	Peptide[159]
		Qβ	Protein[131]

1. D. J. Brayden, *Drug Discovery Today*, 2003, **8**, 976-978.
2. F. Alexis, E. M. Pridgen, R. Langer and O. C. Farokhzad, *Handb. Exp. Pharmacol.*, 2010, DOI: 10.1007/978-3-642-00477-3_2, 55-86.
3. H. E. van Kan-Davelaar, J. C. M. van Hest, J. J. L. M. Cornelissen and M. S. T. Koay, *British Journal of Pharmacology*, DOI: 10.1111/bph.12662.
4. R. J. Watts and M. S. Dennis, *Curr. Opin. Chem. Biol.*, 2013, **17**, 393-399.
5. M. X. Sliwkowski and I. Mellman, *Science*, 2013, **341**, 1192-1198.
6. J. M. Montenegro, V. Grazu, A. Sukhanova, S. Agarwal, J. M. de la Fuente, I. Nabiev, A. Greiner and W. J. Parak, *Adv Drug Deliv Rev*, 2013, **65**, 677-688.
7. J. A. Zasadzinski, B. Wong, N. Forbes, G. Braun and G. Wu, *Current Opinion in Colloid & Interface Science*, 2011, **16**, 203-214.
8. R. Chandrawati and F. Caruso, *Langmuir*, 2012, **28**, 13798-13807.
9. S. Kumar and J. K. Randhawa, *Mater. Sci. Eng., C*, 2013, **33**, 1842-1852.
10. R. P. Brinkhuis, F. P. J. T. Rutjes and J. C. M. van Hest, *Polymer Chemistry*, 2011, **2**, 1449.
11. M. Elsabahy and K. L. Wooley, *Chem. Soc. Rev.*, 2012, **41**, 2545-2561.
12. N. Kamaly, Z. Xiao, P. M. Valencia, A. F. Radovic-Moreno and O. C. Farokhzad, *Chem. Soc. Rev.*, 2012, **41**, 2971-3010.
13. A. Kumar, X. Zhang and X. J. Liang, *Biotechnol. Adv.*, 2013, **31**, 593-606.
14. X. Wang, M. Q. Shao, S. Zhang and X. L. Liu, *J. Nanopart. Res.*, 2013, **15**, 1892-1907.
15. A. Valizadeh, H. Mikaeili, M. Samiei, S. M. Farkhani, N. Zarghami, M. Kouhi, A. Akbarzadeh and S. Davaran, *Nanoscale research letters*, 2012, **7**, 480-493.
16. C. E. Probst, P. Zrazhevskiy, V. Bagalkot and X. Gao, *Advanced drug delivery reviews*, 2013, **65**, 703-718.
17. P. M. Harrison, F. A. Fischbach, T. G. Hoy and G. H. Haggis, *Nature*, 1967, **216**, 1188-1190.
18. D. M. Lawson, P. J. Artymiuk, S. J. Yewdall, J. M. Smith, J. C. Livingstone, A. Treffry, A. Luzzago, S. Levi, P. Arosio, G. Cesareni, C. D. Thomas, W. V. Shaw and P. M. Harrison, *Nature*, 1991, **349**, 541-544.
19. P. M. Harrison and P. Arosio, *Biochim. Biophys. Acta*, 1996, **1275**, 161-203.
20. N. D. Chasteen and P. M. Harrison, *Journal of structural biology*, 1999, **126**, 182-194.
21. M. Uchida, S. Kang, C. Reichhardt, K. Harlen and T. Douglas, *Biochim. Biophys. Acta*, 2010, **1800**, 834-845.
22. K. K. Kim, R. Kim and S. H. Kim, *Nature*, 1998, **394**, 595-599.
23. R. Kim, K. K. Kim, H. Yokota and S. H. Kim, *PNAS*, 1998, **95**, 9129-9133.
24. K. K. Kim, H. Yokota, S. Santoso, D. Lerner, R. Kim and S. H. Kim, *Journal of structural biology*, 1998, **121**, 76-80.
25. T. Douglas and M. Young, *Science*, 2006, **312**, 873-875.
26. E. V. Grgacic and D. A. Anderson, *Methods*, 2006, **40**, 60-65.
27. A. Ghasparian, T. Riedel, J. Koomullil, K. Moehle, C. Gorba, D. I. Svergun, A. W. Perriman, S. Mann, M. Tamborrini, G. Pluschke and J. A. Robinson, *Chembiochem : a European journal of chemical biology*, 2011, **12**, 100-109.
28. N. Kushnir, S. J. Streatfield and V. Yusibov, *Vaccine*, 2012, **31**, 58-83.
29. M. Manchester and P. Singh, *Advanced drug delivery reviews*, 2006, **58**, 1505-1522.
30. D. P. Cormode, P. A. Jarzyna, W. J. Mulder and Z. A. Fayad, *Advanced drug delivery reviews*, 2010, **62**, 329-338.
31. S. A. Bode, I. J. Minten, R. J. Nolte and J. J. Cornelissen, *Nanoscale*, 2011, **3**, 2376-2389.
32. A. L. Parker, S. A. Nicklin and A. H. Baker, *Current opinion in molecular therapeutics*, 2008, **10**, 439-448.
33. S. K. Campos and M. A. Barry, *Current gene therapy*, 2007, **7**, 189-204.
34. C. E. Flynn, S.-W. Lee, B. R. Peelle and A. M. Belcher, *Acta Mater.*, 2003, **51**, 5867-5880.
35. J. Rong, Z. Niu, L. A. Lee and Q. Wang, *Current Opinion in Colloid & Interface Science*, 2011, **16**, 441-450.
36. S. Y. Lee, J. S. Lim and M. T. Harris, *Biotechnol. Bioeng.*, 2012, **109**, 16-30.
37. J. A. Speir, S. Munshi, G. J. Wang, T. S. Baker and J. E. Johnson, *Structure*, 1995, **3**, 63-78.
38. T. W. Lin, Z. G. Chen, R. Usha, C. V. Stauffacher, J. B. Dai, T. Schmidt and J. E. Johnson, *Virology*, 1999, **265**, 20-34.
39. W. F. Ochoa, A. Chatterji, T. Lin and J. E. Johnson, *Chemistry & biology*, 2006, **13**, 771-778.
40. M. A. Canady, S. B. Larson, J. Day and A. McPherson, *Nature structural biology*, 1996, **3**, 771-781.
41. R. W. Lucas, S. B. Larson and A. McPherson, *J. Mol. Biol.*, 2002, **317**, 95-108.
42. T. J. Smith, E. Chase, T. Schmidt and K. L. Perry, *Journal of virology*, 2000, **74**, 7578-7586.
43. T. Stehle and S. C. Harrison, *Structure*, 1996, **4**, 183-194.
44. S. A. Wynne, R. A. Crowther and A. G. Leslie, *Molecular cell*, 1999, **3**, 771-780.
45. R. Golmohammadi, K. Valegard, K. Fridborg and L. Liljas, *J. Mol. Biol.*, 1993, **234**, 620-639.
46. R. Golmohammadi, K. Fridborg, M. Bundule, K. Valegard and L. Liljas, *Structure*, 1996, **4**, 543-554.
47. C. Helgstrand, W. R. Wikoff, R. L. Duda, R. W. Hendrix, J. E. Johnson and L. Liljas, *J. Mol. Biol.*, 2003, **334**, 885-899.
48. W. Earnshaw, S. Casjens and S. C. Harrison, *J. Mol. Biol.*, 1976, **104**, 387-410.
49. A. S. Khalil, J. M. Ferrer, R. R. Brau, S. T. Kottmann, C. J. Noren, M. J. Lang and A. M. Belcher, *PNAS*, 2007, **104**, 4892-4897.
50. C. Sachse, J. Z. Chen, P. D. Coureux, M. E. Stroupe, M. Fandrich and N. Grigorieff, *J. Mol. Biol.*, 2007, **371**, 812-835.
51. W. Shenton, T. Douglas, M. Young, G. Stubbs and S. Mann, *Adv. Mater.*, 1999, **11**, 253-256.
52. M. Uchida, M. L. Flenniken, M. Allen, D. A. Willits, B. E. Crowley, S. Brumfield, A. F. Willis, L. Jackiw, M. Jutila, M. J. Young and T. Douglas, *J. Am. Chem. Soc.*, 2006, **128**, 16626-16633.
53. W. Arap, R. Pasqualini and E. Ruoslahti, *Science*, 1998, **279**, 377-380.
54. T. V. Byzova, R. Rabbani, S. E. D'Souza and E. F. Plow, *Thromb Haemost*, 1998, **80**, 726-734.
55. A. S. Antonov, F. D. Kolodgie, D. H. Munn and R. G. Gerrity, *American Journal of Pathology*, 2004, **165**, 247-258.
56. M. Uchida, D. A. Willits, K. Muller, A. F. Willis, L. Jackiw, M. Jutila, M. J. Young, A. E. Porter and T. Douglas, *Adv. Mater.*, 2009, **21**, 458-462.
57. X. Lin, J. Xie, L. Zhu, S. Lee, G. Niu, Y. Ma, K. Kim and X. Chen, *Angew. Chem. Int. Ed.*, 2011, **50**, 1569-1572.
58. X. Lin, J. Xie, G. Niu, F. Zhang, H. Gao, M. Yang, Q. Quan, M. A. Aronova, G. Zhang, S. Lee, R. Leapman and X. Chen, *Nano Lett.*, 2011, **11**, 814-819.
59. Z. Zhen, W. Tang, H. Chen, X. Lin, T. Todd, G. Wang, T. Cowger, X. Chen and J. Xie, *ACS Nano*, 2013, **7**, 4830-4837.
60. L. Vannucci, E. Falvo, M. Fornara, P. Di Micco, O. Benada, J. Krizan, J. Svoboda, K. Hulikova-Capkova, V. Morea, A. Boffi and P. Ceci, *International journal of nanomedicine*, 2012, **7**, 1489-1509.
61. N. Normanno, A. De Luca, C. Bianco, L. Strizzi, M. Mancino, M. R. Maiello, A. Carotenuto, G. De Feo, F. Caponigro and D. S. Salomon, *Gene*, 2006, **366**, 2-16.
62. X. Li, L. Qiu, P. Zhu, X. Tao, T. Imanaka, J. Zhao, Y. Huang, Y. Tu and X. Cao, *Small*, 2012, **8**, 2505-2514.
63. C. Kwon, Y. J. Kang, S. Jeon, S. Jung, S. Y. Hong and S. Kang, *Macromolecular bioscience*, 2012, **12**, 1452-1458.
64. Y. J. Kang, D. C. Park, H. H. Shin, J. Park and S. Kang, *Biomacromolecules*, 2012, **13**, 4057-4064.
65. H. J. Kang, Y. J. Kang, Y. M. Lee, H. H. Shin, S. J. Chung and S. Kang, *Biomaterials*, 2012, **33**, 5423-5430.
66. S. Kang, P. A. Suci, C. C. Broomell, K. Iwahori, M. Kobayashi, I. Yamashita, M. Young and T. Douglas, *Nano Lett.*, 2009, **9**, 2360-2366.
67. P. A. Suci, S. Kang, M. Young and T. Douglas, *J. Am. Chem. Soc.*, 2009, **131**, 9164-9165.
68. M. L. Flenniken, D. A. Willits, S. Brumfield, M. J. Young and T. Douglas, *Nano Lett.*, 2003, **3**, 1573-1576.

69. M. L. Flenniken, L. O. Liepold, B. E. Crowley, D. A. Willits, M. J. Young and T. Douglas, *Chem. Commun.*, 2005, 447-449.
70. M. L. Flenniken, D. A. Willits, A. L. Harmsen, L. O. Liepold, A. G. Harmsen, M. J. Young and T. Douglas, *Chemistry & biology*, 2006, **13**, 161-170.
71. S. H. Choi, I. C. Kwon, K. Y. Hwang, I. S. Kim and H. J. Ahn, *Biomacromolecules*, 2011, **12**, 3099-3106.
72. P. Laakkonen, K. Porkka, J. A. Hoffman and E. Ruoslahti, *Nature medicine*, 2002, **8**, 751-755.
73. V. Fogal, L. Zhang, S. Krajewski and E. Ruoslahti, *Cancer research*, 2008, **68**, 7210-7218.
74. M. Uchida, H. Kosuge, M. Terashima, D. A. Willits, L. O. Liepold, M. J. Young, M. V. McConnell and T. Douglas, *ACS Nano*, 2011, **5**, 2493-2502.
75. A. Lo, C. T. Lin and H. C. Wu, *Mol Cancer Ther*, 2008, **7**, 579-589.
76. R. Toita, M. Murata, S. Tabata, K. Abe, S. Narahara, J. S. Piao, J. H. Kang and M. Hashizume, *Bioconjugate Chem.*, 2012, **23**, 1494-1501.
77. R. Toita, M. Murata, K. Abe, S. Narahara, J. S. Piao, J. H. Kang and M. Hashizume, *Chem. Commun.*, 2013, **49**, 7442-7444.
78. M. Murata, S. Narahara, K. Umezaki, R. Toita, S. Tabata, J. S. Piao, K. Abe, J. H. Kang, K. Ohuchida, L. Cui and M. Hashizume, *International journal of nanomedicine*, 2012, **7**, 4353-4362.
79. E. Gillitzer, D. Willits, M. Young and T. Douglas, *Chem. Commun.*, 2002, 2390-2391.
80. E. Gillitzer, P. Suci, M. Young and T. Douglas, *Small*, 2006, **2**, 962-966.
81. P. A. Suci, Z. Varpness, E. Gillitzer, T. Douglas and M. Young, *Langmuir*, 2007, **23**, 12280-12286.
82. I. J. Minten, L. J. Hendriks, R. J. Nolte and J. J. Cornelissen, *J. Am. Chem. Soc.*, 2009, **131**, 17771-17773.
83. Q. Wang, E. Kaltgrad, T. Lin, J. E. Johnson and M. G. Finn, *Chemistry & biology*, 2002, **9**, 805-811.
84. Q. Wang, T. W. Lin, L. Tang, J. E. Johnson and M. G. Finn, *Angew. Chem. Int. Ed.*, 2002, **41**, 459-462.
85. K. S. Raja, Q. Wang and M. G. Finn, *Chembiochem : a European journal of chemical biology*, 2003, **4**, 1348-1351.
86. Q. Wang, K. S. Raja, K. D. Janda, T. W. Lin and M. G. Finn, *Bioconjugate Chem.*, 2003, **14**, 38-43.
87. K. S. Raja, Q. Wang, M. J. Gonzalez, M. Manchester, J. E. Johnson and M. G. Finn, *Biomacromolecules*, 2003, **4**, 472-476.
88. E. Strable, J. E. Johnson and M. G. Finn, *Nano Lett.*, 2004, **4**, 1385-1389.
89. A. Chatterji, W. Ochoa, L. Shamieh, S. P. Salakian, S. M. Wong, G. Clinton, P. Ghosh, T. Lin and J. E. Johnson, *Bioconjugate Chem.*, 2004, **15**, 807-813.
90. N. F. Steinmetz, V. Hong, E. D. Spoerke, P. Lu, K. Breitenkamp, M. G. Finn and M. Manchester, *J. Am. Chem. Soc.*, 2009, **131**, 17093-17095.
91. F. M. Brunel, J. D. Lewis, G. Destito, N. F. Steinmetz, M. Manchester, H. Stuhlmann and P. E. Dawson, *Nano Lett.*, 2010, **10**, 1093-1097.
92. N. F. Steinmetz, A. L. Ablack, J. L. Hickey, J. Ablack, B. Manocha, J. S. Mymryk, L. G. Luyt and J. D. Lewis, *Small*, 2011, **7**, 1664-1672.
93. A. M. Wen, S. Shukla, P. Saxena, A. A. Aljabali, I. Yildiz, S. Dey, J. E. Mealy, A. C. Yang, D. J. Evans, G. P. Lomonossoff and N. F. Steinmetz, *Biomacromolecules*, 2012, **13**, 3990-4001.
94. Q. Wang, T. Lin, J. E. Johnson and M. G. Finn, *Chemistry & biology*, 2002, **9**, 813-819.
95. A. Chatterji, L. L. Burns, S. S. Taylor, G. P. Lomonossoff, J. E. Johnson, T. Lin and C. Porta, *Intervirology*, 2002, **45**, 362-370.
96. N. F. Steinmetz, D. J. Evans and G. P. Lomonossoff, *Chembiochem : a European journal of chemical biology*, 2007, **8**, 1131-1136.
97. A. Chatterji, W. F. Ochoa, M. Paine, B. R. Ratna, J. E. Johnson and T. Lin, *Chemistry & biology*, 2004, **11**, 855-863.
98. A. Chatterji, W. F. Ochoa, T. Ueno, T. Lin and J. E. Johnson, *Nano Lett.*, 2005, **5**, 597-602.
99. N. F. Steinmetz, G. P. Lomonossoff and D. J. Evans, *Langmuir*, 2006, **22**, 3488-3490.
100. Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless and M. G. Finn, *J. Am. Chem. Soc.*, 2003, **125**, 3192-3193.
101. S. Sen Gupta, J. Kuzelka, P. Singh, W. G. Lewis, M. Manchester and M. G. Finn, *Bioconjugate Chem.*, 2005, **16**, 1572-1579.
102. E. Kaltgrad, M. K. O'Reilly, L. Liao, S. Han, J. C. Paulson and M. G. Finn, *J. Am. Chem. Soc.*, 2008, **130**, 4578-4579.
103. R. D. Astronomo, E. Kaltgrad, A. K. Udit, S. K. Wang, K. J. Doores, C. Y. Huang, R. Pantophlet, J. C. Paulson, C. H. Wong, M. G. Finn and D. R. Burton, *Chemistry & biology*, 2010, **17**, 357-370.
104. S. Sen Gupta, K. S. Raja, E. Kaltgrad, E. Strable and M. G. Finn, *Chem. Commun.*, 2005, 4315-4317.
105. S. Meunier, E. Strable and M. G. Finn, *Chemistry & biology*, 2004, **11**, 319-326.
106. J. D. Lewis, G. Destito, A. Zijlstra, M. J. Gonzalez, J. P. Quigley, M. Manchester and H. Stuhlmann, *Nature medicine*, 2006, **12**, 354-360.
107. G. Destito, R. Yeh, C. S. Rae, M. G. Finn and M. Manchester, *Chemistry & biology*, 2007, **14**, 1152-1162.
108. Z. Wu, K. Chen, I. Yildiz, A. Dirksen, R. Fischer, P. E. Dawson and N. F. Steinmetz, *Nanoscale*, 2012, **4**, 3567-3576.
109. M. L. Hovlid, N. F. Steinmetz, B. Laufer, J. L. Lau, J. Kuzelka, Q. Wang, T. Hyypia, G. R. Nemerow, H. Kessler, M. Manchester and M. G. Finn, *Nanoscale*, 2012, **4**, 3698-3705.
110. A. A. Aljabali, S. Shukla, G. P. Lomonossoff, N. F. Steinmetz and D. J. Evans, *Molecular pharmaceutics*, 2013, **10**, 3-10.
111. N. F. Steinmetz, C. F. Cho, A. Ablack, J. D. Lewis and M. Manchester, *Nanomedicine*, 2011, **6**, 351-364.
112. M. Wu, W. L. Brown and P. G. Stockley, *Bioconjugate Chem.*, 1995, **6**, 587-595.
113. W. L. Brown, R. A. Mastico, M. Wu, K. G. Heal, C. J. Adams, J. B. Murray, J. C. Simpson, J. M. Lord, A. W. Taylor-Robinson and P. G. Stockley, *Intervirology*, 2002, **45**, 371-380.
114. C. E. Ashley, E. C. Carnes, G. K. Phillips, P. N. Durfee, M. D. Buley, C. A. Lino, D. P. Padilla, B. Phillips, M. B. Carter, C. L. Willman, C. J. Brinker, C. Caldeira Jdo, B. Chackerian, W. Wharton and D. S. Peabody, *ACS Nano*, 2011, **5**, 5729-5745.
115. B. Wei, Y. Wei, K. Zhang, J. Wang, R. Xu, S. Zhan, G. Lin, W. Wang, M. Liu, L. Wang, R. Zhang and J. Li, *Biomedicine & Pharmacotherapy*, 2009, **63**, 313-318.
116. F. A. Galaway and P. G. Stockley, *Molecular pharmaceutics*, 2013, **10**, 59-68.
117. K. L. Seim, A. C. Obermeyer and M. B. Francis, *J. Am. Chem. Soc.*, 2011, **133**, 16970-16976.
118. K. G. Patel and J. R. Swartz, *Bioconjugate Chem.*, 2011, **22**, 376-387.
119. D. S. Peabody, *Journal of nanobiotechnology*, 2003, **1**, DOI: 10.1186/1477-3155-1181-1185.
120. F. Tian, M. L. Tsao and P. G. Schultz, *J. Am. Chem. Soc.*, 2004, **126**, 15962-15963.
121. Z. M. Carrico, D. W. Romanini, R. A. Mehl and M. B. Francis, *Chem. Commun.*, 2008, 1205-1207.
122. G. J. Tong, S. C. Hsiao, Z. M. Carrico and M. B. Francis, *J. Am. Chem. Soc.*, 2009, **131**, 11174-11178.
123. N. Stephanopoulos, G. J. Tong, S. C. Hsiao and M. B. Francis, *ACS Nano*, 2010, **4**, 6014-6020.
124. C. R. Behrens, J. M. Hooker, A. C. Obermeyer, D. W. Romanini, E. M. Katz and M. B. Francis, *J. Am. Chem. Soc.*, 2011, **133**, 16398-16401.
125. J. M. Hooker, E. W. Kovacs and M. B. Francis, *J. Am. Chem. Soc.*, 2004, **126**, 3718-3719.
126. E. W. Kovacs, J. M. Hooker, D. W. Romanini, P. G. Holder, K. E. Berry and M. B. Francis, *Bioconjugate Chem.*, 2007, **18**, 1140-1147.
127. W. Wu, S. C. Hsiao, Z. M. Carrico and M. B. Francis, *Angew. Chem. Int. Ed.*, 2009, **48**, 9493-9497.
128. E. Strable, D. E. Prasuhn, A. K. Udit, S. Brown, A. J. Link, J. T. Ngo, G. Lander, J. Quispe, C. S. Potter, B. Carragher, D. A. Tirrell and M. G. Finn, *Bioconjugate Chem.*, 2008, **19**, 866-875.
129. D. E. Prasuhn, P. Singh, E. Strable, S. Brown, M. Manchester and M. G. Finn, *J. Am. Chem. Soc.*, 2008, **130**, 1328-1334.
130. D. Banerjee, A. P. Liu, N. R. Voss, S. L. Schmid and M. G. Finn, *Chembiochem : a European journal of chemical biology*, 2010, **11**, 1273-1279.
131. J. K. Pokorski, M. L. Hovlid and M. G. Finn, *Chembiochem : a European journal of chemical biology*, 2011, **12**, 2441-2447.

132. J. K. Rhee, M. Baksh, C. Nycholat, J. C. Paulson, H. Kitagishi and M. G. Finn, *Biomacromolecules*, 2012, **13**, 2333-2338.
133. A. M. Wen, M. J. Ryan, A. C. Yang, K. Breitenkamp, J. K. Pokorski and N. F. Steinmetz, *Chem. Commun.*, 2012, **48**, 9044-9046.
134. A. K. Udit, C. Everett, A. J. Gale, J. Reiber Kyle, M. Ozkan and M. G. Finn, *Chembiochem : a European journal of chemical biology*, 2009, **10**, 503-510.
135. A. J. Gale, D. J. Elias, P. M. Averell, P. S. Teirstein, M. Buck, S. D. Brown, Z. Polonskaya, A. K. Udit and M. G. Finn, *Thrombosis research*, 2011, **128**, e9-13.
136. J. K. Pokorski, K. Breitenkamp, L. O. Liepold, S. Qazi and M. G. Finn, *J. Am. Chem. Soc.*, 2011, **133**, 9242-9245.
137. M. Demir and M. H. B. Stowell, *Nanotechnology*, 2002, **13**, 541-544.
138. H. Yi, S. Nisar, S. Y. Lee, M. A. Powers, W. E. Bentley, G. F. Payne, R. Ghodssi, G. W. Rubloff, M. T. Harris and J. N. Culver, *Nano Lett.*, 2005, **5**, 1931-1936.
139. T. L. Schlick, Z. Ding, E. W. Kovacs and M. B. Francis, *J. Am. Chem. Soc.*, 2005, **127**, 3718-3723.
140. M. A. Bruckman, G. Kaur, L. A. Lee, F. Xie, J. Sepulveda, R. Breitenkamp, X. Zhang, M. Joralemon, T. P. Russell, T. Emrick and Q. Wang, *Chembiochem : a European journal of chemical biology*, 2008, **9**, 519-523.
141. G. Kaur, C. Wang, J. Sun and Q. Wang, *Biomaterials*, 2010, **31**, 5813-5824.
142. K. J. Koudelka, S. Ippoliti, E. Medina, L. P. Shriver, S. A. Trauger, C. E. Catalano and M. Manchester, *Biomacromolecules*, 2013, **14**, 4169-4176.
143. I. Yildiz, I. Tsvetkova, A. M. Wen, S. Shukla, M. H. Masarapu, B. Dragnea and N. F. Steinmetz, *RSC Advances*, 2012, **2**, 3670.
144. T. Yamada, Y. Iwasaki, H. Tada, H. Iwabuki, M. K. Chuah, T. VandenDriessche, H. Fukuda, A. Kondo, M. Ueda, M. Seno, K. Tanizawa and S. Kuroda, *Nat. Biotechnol.*, 2003, **21**, 885-890.
145. T. Kasuya, T. Yamada, A. Uyeda, T. Matsuzaki, T. Okajima, K. Tatematsu, K. Tanizawa and S. Kuroda, *Journal of bioscience and bioengineering*, 2008, **106**, 99-102.
146. N. Kurata, T. Shishido, M. Muraoka, T. Tanaka, C. Ogino, H. Fukuda and A. Kondo, *J. Biochem.*, 2008, **144**, 701-707.
147. Q. Zeng, H. Wen, Q. Wen, X. Chen, Y. Wang, W. Xuan, J. Liang and S. Wan, *Biomaterials*, 2013, **34**, 4632-4642.
148. Y. Ren, S. M. Wong and L. Y. Lim, *Bioconjugate Chem.*, 2007, **18**, 836-843.
149. R. K. Huang, N. F. Steinmetz, C. Y. Fu, M. Manchester and J. E. Johnson, *Nanomedicine*, 2011, **6**, 55-68.
150. N. F. Steinmetz, M. E. Mertens, R. E. Taurog, J. E. Johnson, U. Commandeur, R. Fischer and M. Manchester, *Nano Lett.*, 2010, **10**, 305-312.
151. S. Shukla, A. L. Ablack, A. M. Wen, K. L. Lee, J. D. Lewis and N. F. Steinmetz, *Molecular pharmaceutics*, 2013, **10**, 33-42.
152. D. M. Lockney, R. N. Guenther, L. Loo, W. Overton, R. Antonelli, J. Clark, M. Hu, C. Luft, S. A. Lommel and S. Franzen, *Bioconjugate Chem.*, 2011, **22**, 67-73.
153. Q. Zhao, W. Chen, Y. Chen, L. Zhang, J. Zhang and Z. Zhang, *Bioconjugate Chem.*, 2011, **22**, 346-352.
154. H. N. Barnhill, R. Reuther, P. L. Ferguson, T. Dreher and Q. Wang, *Bioconjugate Chem.*, 2007, **18**, 852-859.
155. Q. Zeng, S. Saha, L. A. Lee, H. Barnhill, J. Oxsher, T. Dreher and Q. Wang, *Bioconjugate Chem.*, 2011, **22**, 58-66.
156. S. Kang, G. C. Lander, J. E. Johnson and P. E. Prevelige, *Chembiochem : a European journal of chemical biology*, 2008, **9**, 514-518.
157. K. N. Parent, C. T. Deedas, E. H. Egelman, S. R. Casjens, T. S. Baker and C. M. Teschke, *Biomaterials*, 2012, **33**, 5628-5637.
158. L. Tang, E. B. Gilcrease, S. R. Casjens and J. E. Johnson, *Structure*, 2006, **14**, 837-845.
159. A. Servid, P. Jordan, A. O'Neil, P. Prevelige and T. Douglas, *Biomacromolecules*, 2013, **14**, 2989-2995.
160. S. Kang, M. Uchida, A. O'Neil, R. Li, P. E. Prevelige and T. Douglas, *Biomacromolecules*, 2010, **11**, 2804-2809.
161. J. Lucon, S. Qazi, M. Uchida, G. J. Bedwell, B. LaFrance, P. E. Prevelige, Jr. and T. Douglas, *Nature chemistry*, 2012, **4**, 781-788.
162. M. Uchida, D. S. Morris, S. Kang, C. C. Jolley, J. Lucon, L. O. Liepold, B. LaFrance, P. E. Prevelige, Jr. and T. Douglas, *Langmuir*, 2012, **28**, 1998-2006.
163. L. Chen, A. J. Zurita, P. U. Ardelt, R. J. Giordano, W. Arap and R. Pasqualini, *Chemistry & biology*, 2004, **11**, 1081-1091.
164. H. Bar, I. Yacoby and I. Benhar, *BMC biotechnology*, 2008, **8**, DOI: 10.1186/1472-6750-1188-1137.
165. D. Ghosh, A. G. Kohli, F. Moser, D. Endy and A. M. Belcher, *ACS synthetic biology*, 2012, **1**, 576-582.
166. I. Yacoby, M. Shamis, H. Bar, D. Shabat and I. Benhar, *Antimicrob. Agents Chemother.*, 2006, **50**, 2087-2097.
167. K. Li, Y. Chen, S. Li, H. G. Nguyen, Z. Niu, S. You, C. M. Mello, X. Lu and Q. Wang, *Bioconjugate Chem.*, 2010, **21**, 1369-1377.
168. N. Suthiwangcharoen, T. Li, K. Li, P. Thompson, S. You and Q. Wang, *Nano Research*, 2011, **4**, 483-493.
169. U. Schmidt, R. Rudolph and G. Bohm, *Protein Eng.*, 2001, **14**, 769-774.
170. E. K. Koepf, H. M. Petrassi, G. Ratnaswamy, M. E. Huff, M. Sudol and J. W. Kelly, *Biochemistry*, 1999, **38**, 14338-14351.
171. U. Schmidt, C. Gunther, R. Rudolph and G. Bohm, *FASEB J.*, 2001, **15**, 1646-1648.
172. A. Abbing, U. K. Blaschke, S. Grein, M. Kretschmar, C. M. Stark, M. J. Thies, J. Walter, M. Weigand, D. C. Woith, J. Hess and C. O. Reiser, *J. Biol. Chem.*, 2004, **279**, 27410-27421.
173. E. Boura, D. Liebl, R. Spisek, J. Fric, M. Marek, J. Stokrova, V. Holan and J. Forstova, *FEBS Lett.*, 2005, **579**, 6549-6558.
174. T. Inoue, M. A. Kawano, R. U. Takahashi, H. Tsukamoto, T. Enomoto, T. Imai, K. Kataoka and H. Handa, *J. Biotechnol.*, 2008, **134**, 181-192.
175. R. U. Takahashi, S. N. Kanesashi, T. Inoue, T. Enomoto, M. A. Kawano, H. Tsukamoto, F. Takeshita, T. Imai, T. Ochiya, K. Kataoka, Y. Yamaguchi and H. Handa, *J. Biotechnol.*, 2008, **135**, 385-392.
176. Y. Kitai, H. Fukuda, T. Enomoto, Y. Asakawa, T. Suzuki, S. Inouye and H. Handa, *J. Biotechnol.*, 2011, **155**, 251-256.
177. N. Ohtake, K. Niikura, T. Suzuki, K. Nagakawa, S. Mikuni, Y. Matsuo, M. Kinjo, H. Sawa and K. Ijiro, *Chembiochem : a European journal of chemical biology*, 2010, **11**, 959-962.
178. K. Niikura, N. Sugimura, Y. Musashi, S. Mikuni, Y. Matsuo, S. Kobayashi, K. Nagakawa, S. Takahara, C. Takeuchi, H. Sawa, M. Kinjo and K. Ijiro, *Molecular bioSystems*, 2013, **9**, 501-507.
179. D. A. Muruve, *Human gene therapy*, 2004, **15**, 1157-1166.
180. T. M. Allen and P. R. Cullis, *Science*, 2004, **303**, 1818-1822.
181. S. D. Li and L. Huang, *Molecular pharmaceutics*, 2008, **5**, 496-504.
182. P. Singh, D. Prasuhn, R. M. Yeh, G. Destito, C. S. Rae, K. Osborn, M. G. Finn and M. Manchester, *J. Controlled Release*, 2007, **120**, 41-50.
183. T. J. Molenaar, I. Michon, S. A. de Haas, T. J. van Berkel, J. Kuiper and E. A. Biessen, *Virology*, 2002, **293**, 182-191.
184. J. M. Hooker, J. P. O'Neil, D. W. Romanini, S. E. Taylor and M. B. Francis, *Molecular Imaging and Biology*, 2008, **10**, 182-191.
185. M. E. Farkas, I. L. Aanei, C. R. Behrens, G. J. Tong, S. T. Murphy, J. P. O'Neil and M. B. Francis, *Molecular pharmaceutics*, 2013, **10**, 69-76.
186. M. Wu, J. Shi, D. Fan, Q. Zhou, F. Wang, Z. Niu and Y. Huang, *Biomacromolecules*, 2013, **14**, 4032-4037.
187. C. R. Kaiser, M. L. Flenniken, E. Gillitzer, A. L. Harmsen, A. G. Harmsen, M. A. Jutila, T. Douglas and M. J. Young, *International journal of nanomedicine*, 2007, **2**, 715-733.
188. C. S. Rae, I. W. Khor, Q. Wang, G. Destito, M. J. Gonzalez, P. Singh, D. M. Thomas, M. N. Estrada, E. Powell, M. G. Finn and M. Manchester, *Virology*, 2005, **343**, 224-235.
189. D. Gatto, C. Ruedl, B. Odermatt and M. F. Bachmann, *J Immunol*, 2004, **173**, 4308-4316.
190. M. F. Bachmann, H. Hengartner and R. M. Zinkernagel, *European journal of immunology*, 1995, **25**, 3445-3451.
191. C. R. O'Riordan, A. Lachapelle, C. Delgado, V. Parkes, S. C. Wadsworth, A. E. Smith and G. E. Francis, *Human gene therapy*, 1999, **10**, 1349-1358.
192. M. A. Croyle, N. Chirmule, Y. Zhang and J. M. Wilson, *Human gene therapy*, 2002, **13**, 1887-1900.

193. N. F. Steinmetz and M. Manchester, *Biomacromolecules*, 2009, **10**, 784-792.
194. H. S. Leong, N. F. Steinmetz, A. Ablack, G. Destito, A. Zijlstra, H. Stuhlmann, M. Manchester and J. D. Lewis, *Nature Protocols*, 2010, **5**, 1406-1417.
195. D. Peer, J. M. Karp, S. Hong, O. C. FaroKHzad, R. Margalit and R. Langer, *Nature Nanotechnology*, 2007, **2**, 751-760.
196. T. Lammers, W. E. Hennink and G. Storm, *British journal of cancer*, 2008, **99**, 392-397.
197. A. Z. Wang, R. Langer and O. C. Farokhzad, *Annual review of medicine*, 2012, **63**, 185-198.
198. D. D. Kirk and S. R. Webb, *Immunology and cell biology*, 2005, **83**, 248-256.

