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Engineered protein and protein-polysaccharide cages for drug delivery and therapeutic applications

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Isha Ghosh[1], Ujjwal Sahoo[2], Souvik Basak[2]

[1]Birla Institute of Technology, Mesra, Ranchi, India

[2]Dr. B.C. Roy College of Pharmacy & Allied Health Sciences, Durgapur, India

13.1 Introduction

Biopolymers are polymers obtained from biological sources including plant, animal, and marine sources. For the last few decades, applications of biopolymers have gained momentum in drug delivery systems due to their nontoxicity, high compatibility, biodegradability, easy availability, and low cost. Biopolymers are extensively used in the field of medicine and biology due to their several environmental benefits. They have potential applications as drug carriers, lubricants, cosmetics, adsorbents, textiles, packaging materials, tissue scaffolds, thickeners, and grafting materials (Bal & Swain, 2020; Brütsch, Stringer, Kuster, Windhab, & Fischer, 2019; Kaity et al., 2013; Nerkar, Mahajan, Ige, & Solanki, 2016).

Although a great deal of reports have been made earlier on biopolymers (Hassan, Bai, & Dou, 2019; Kawalkar, 2015; Singh, 2011), proteins and specially designed polysaccharides as biopolymeric carrier have attracted attention in the last few years owing to their structural and functional compatibilities to human cells, differential ionization over various physiological conditions, and improved cellular uptake (U.S. Congress, Office of Technology Assessment, 1993). These biopolymers are also biodegradable and thus environment-friendly, which have helped people overcome nondegradability issues of synthetic polymers across the globe (Tokiwa, Calabia, Ugwu, & Aiba, 2009).

Q2 Keeping with the tremendous onslaught on biopolymer-based research and to highlight its recent advancement in biomedical applications, we review the recent dynamics of the same in this study focusing on a special biopolymer, that is, protein. Although a great deal of studies have been undertaken to use protein as biopolymeric carrier in theranostics and drug delivery, the recent exploration on “protein cages” (PCs) has emancipated newer horizons on protein Q3 applications in biomedical fraternity. Due to magnificent improvement of properties such as stability, guest encapsulation, cellular uptake, compartmentalization of internal cavities, metal coordination, and last but not the least, scope of engineering as per requirement and target have made it a promising carrier of choice to biomedical scientists. Q4 Furthermore, the stepping up of PCs by complexation with polysaccharides have evolved further newer dimensions of drug delivery or drug–gene cocktail targeting. In this chapter, we summarize the updated approaches in this domain Q5 involving synopsis on various types of PCs, purposes on exploitation of PCs, a set of engineering approaches to manipulate the cages to augment their properties, targeting and improved release of cargoes from such cages, improving stabilities of such delivery systems and other theranostics related to engineered PCs. In addition, fabrication of protein-polysaccharide cages (PPCs), various therapeutic applications of the same, and particularly the value-added properties Q6 of PPCs over other drug delivery systems are another prima face of this chapter.

13.2 Proteins

Proteins are the most diverse copolymer made up of up to 20 different amino acids, which act as building blocks. Protein has a three-dimensional (3D) structure, and amino acids are linked by amide bonds (U.S. Congress, Office of Technology Assessment, 1993) to form a polymeric chain. Proteins are the unit of life, and it is present abundantly in all living cells. Proteins can coordinate or control inter- and intracellular communication and also take place in cellular

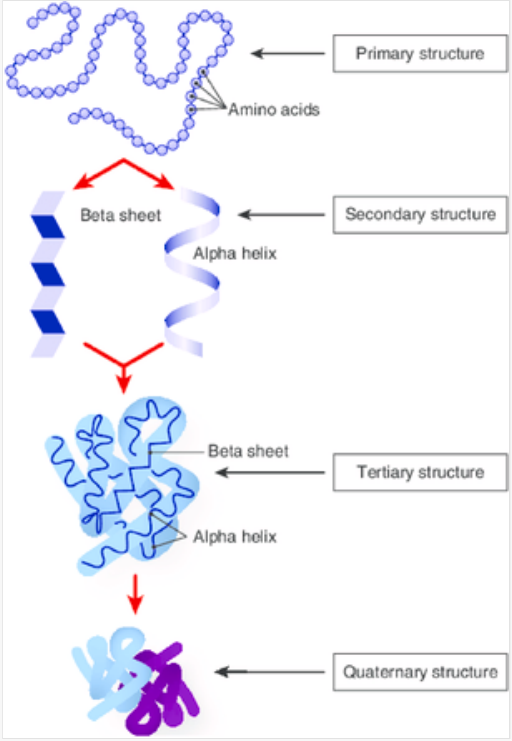
functions. Proteins can produce enzymes, hormones, immunoglobulins, cell walls, and many other pure or hybrid biomolecules in living body. Proteins can have 50 or more amino acids linked by amide bonds that can lead them to be high molecular-weight natural polymers (Savale, 2016). Protein plays a vital role as enzyme to carry out metabolic reactions, as hormones to transfer chemical information, as receptors to bind other molecules, as antibodies in immune system, as structural unit of tissue and bones, and many other functions as well (Littlechild, 2013; McLachlan, 1972). Proteins have four different structures (Protein structure, 1941) as primary, secondary, tertiary, and quaternary structures. Primary structure has a linear sequence of amino acids. The length of primary structure mainly varies from protein to protein. It may be of short sized like insulin (51 amino acids) as well as it may be of large sized like apolipoprotein B-100 (4536 amino acids) (McLachlan, 1972). Secondary structure is the secondary folding of primary structure.

The secondary structure is mainly of two types, namely, α-helix and β-sheet. α-Helix is the most common spiral structure found in protein. It was first proposed by Pauling and Corey in the year 1951 (Littlechild, 2013; McLachlan, 1972). The α-helix is a right-handed coiled strand (Protein structure, 1941). This helix is stabilized by a number of hydrogen bondings, which is present between one strand's peptidic −H atom and another strand's amino −N atom or between one strand's −H atom and another strand's peptidic −C=O group, the oxygen atom being the hydrogen bond acceptor (Littlechild, 2013). Each turn of this helix contains 3.6 amino acids, and this structure is the most stable structure due to extensive H-bonding. β-Sheet is the second type of structure, also proposed by Pauling and Corey (McLachlan, 1972). It exists as completely stretched structure. It is composed of mainly two or more segments of completely extended peptide chains. The antiparallel sheet is more stable than the parallel one because it has more close packing than the parallel one but the parallel one has high steric hindrance that makes the same less tightly packed, which means it is less stable.

Tertiary structure is the 3D arrangement of protein structure. It mainly contains the hydrophilic groups on the surface of protein molecule and hydrophobic chains are present in interior sides. This structure is stabilized by several interactions such as cross-linking, hydrophobic interaction, disulfide bonds, and van der Waals interaction (McLachlan, 1972).

The quaternary structure of the proteins has the most closely packed structure. Some proteins consist of two or more polypeptides, which may be identical or nonidentical. Such proteins have quaternary structures. The individual polypeptide chains are called protomers. For example, the hemoglobin proteins contain this structure (Lukin et al., 2003). Fig. 13.1 summarizes primary, secondary, tertiary, and quaternary structures of proteins.

Figure 13.1



Primary, secondary, tertiary, and quaternary protein structures (Haque, Tamilsena, & Adhikari, 2016).
Source: Reprinted with permission from Haque, A., Timilsena, Y. P., & Adhikari, B. (2016). Food proteins, structure, and function. In Reference module in food science. Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.03057-2.

Proteins have been studied as carriers for therapeutic molecules involving drugs, other peptides, DNAs, and RNAs for the last few decades because of their biocompatibility, improved pharmacokinetics, reduced toxicity, and target-specific delivery (Bruno, Miller, & Lim, 2013). Depending on the origin, proteins can be divided into three major classes, namely, plant protein (gliadin, zein, soy protein, legumin), animal protein (albumin, gelatin, keratin, collagen, elastin-like polymer, casein, whey, silk, fibrin), and virus protein (viral capsid) (MaHam, Tang, Wu, Wang, & Lin, 2009), and these proteins can be used to prepare several number of drug delivery systems (Table 13.1) (Jao, Xue, Medina, & Hu, 2017; MaHam et al., 2009) fibrin. Amongst the myriads of drug delivery systems, the proteins are used to fabricate delivery systems such as nanoparticles, microspheres, needles, films, and hydrogels.

Table 13.1

The table layout displayed in this section is not how it will appear in the final version. The representation below is solely purposed for providing corrections to the table. To preview the actual presentation of the table, please view the Proof.

Q48 Proteins used in several drug delivery systems with their properties.

Proteins	Properties of protein relevant for drug delivery applications	Drug delivery systems
Animal proteins		
Albumin	Present abundantly in plasma protein. Biocompatible.	Nanoparticles (Elzoghby, Samy, & Elgindy, 2012)
Gelatin	Has low toxicity. Biocompatible and biodegradable. Have many –COOH groups for cross-linking. Used as ingredient in formulation.	Microsphere (Vandelli et al., 2004), hydrogel (Ozeki, Ishi, Hirano, & Tabata, 2001)
Keratin	Very viable, cost-effective, biodegradable, and very tunable.	Sponge (Tachibana, Kaneko, Tanabe, & Yamauchi, 2005), film (Tanabe, Okitsu, Tachibana, & Yamauchi, 2002)
Collagen	Versatile protein and biocompatible. Contains three peptide	Microparticles (Rössler, Kreuter, & Scherer,

	chains. Contains –COOH and $-NH_2$ groups for cross-linking.	1995) hydrogel (Yuan et al., 2014)
Elastin	Natural elastin is insoluble. Most stable protein of our body. Biodegradable.	Nanoparticles, hydrogel (Annabi et al., 2009), film (Da Costa et al., 2015)
Casein	Hydrophobic drug incorporation is easy. Nontoxic and biodegradable. No sulfide bond present.	Microparticles (Vino, Lokesh, Vijayaragavan, Jayaraman, & Ghosh, 2011)
Silk protein (fibroin and sericin)	Best for long-term drug release. High tensile strength and elasticity. Biocompatible.	Microneedle (Tsioris et al., 2012)
Whey protein	Best for hydrophilic drug loading, pH-independent. Forms gel and emulsion.	Hydrogel (Zand-Rajabi & Madadlou, 2016), microsphere (Heelan & Corrigan, 1998)
Plant proteins		
Gliadin	Derived from wheat. Best for protecting drug from breaking in stomach. Biocompatible.	Nanoparticles (Duclairoir, Nakache, Marchais, & Orecchioni, 1998)
Zein	Has antibacterial activity. Best for hydrophobic drug delivery. Protects Ivermectin from photodegradation.	Microsphere (Hurtado-López & Murdan, 2005), nanofibers (Huang et al., 2013)
Soy protein	Easy to load hydrophilic drug. Water-soluble and biodegradable. Less costly.	Film (Brandenburg, Weller, & Testin, 1993)
Virus proteins		
Virus capsid	Viral diversity gives different size and shape. There are three features such as interior, exterior, and interface for drug loading.	Protein cage (Belletti et al., 2017)
CCMV	Chemical modification can be done. Biodegradable and stable at acidic pH. Has high cell penetration rate.	Protein cage, vaccines (Lee & Wang, 2006)
CPMV	Chemical and genetic modifications can be done. Stable at a wide range of pH (3–9). Thermostable. Has enhanced cellular uptake.	Protein cage, vaccines, antiviral and antitumor applications (Lee & Wang, 2006)
TMV	Nanotube-like formation. High aspect ratio is advantageous for drug delivery, high biodistribution, and tumor targeting. Also devoid of major immune response.	Protein cage, injectable biologics, vaccines (Smith, Fitzmaurice, Turpen, & Palmer, 2009)

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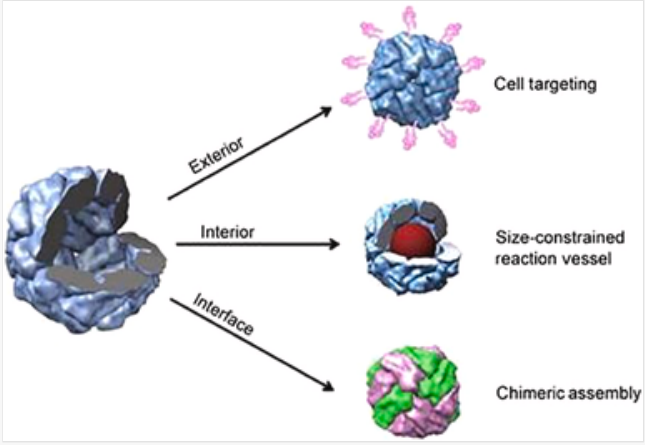
CCMV, Cowpea chlorotic mottle virus; *CPMV*, cowpea mosaic virus; *TMV*, tobacco mosaic virus.

13.3 Protein cages: engineering and therapeutic applications

Though proteins are one the most versatile biopolymers as mentioned earlier, which is an important property for a carrier of drug molecules, the shortcomings of protein-based drug delivery systems lie in the fact that the free proteins are not stable inside the body. The instability of proteins is largely attributed to the fact that the proteins are vulnerable to enzymatic degradation, agglutination, opsonization, and phagocytic uptake. Effective drug delivery mainly depends on high specificity, less toxicity, cell uptake, high treatment competence, systemic clearance, and controlled drug release at target site (Bruno et al., 2013; Molino & Wang, 2014; Roudi, Saraygord-Afshari, & Hemmaty, 2017). Over the years, the nano-scale delivery system involving protein nanocages is gaining importance because of its properties such as high stability, target specificity, suitable pharmacokinetics, efficient cell permeability, stimulus-responsive character, and a wide range of flexibility, which meet all the requirements of an ideal drug delivery system (Bhaskar & Lim, 2017).

PCs have been explored as a potential drug delivery system, which has caged structure with monodisperse size distribution made up of assembly of corresponding protein subunits (MaHam et al., 2009; Molino & Wang, 2014; Roudi et al., 2017). The cage acts as a career of drug molecules or storage of iron or metal ions or as host of reactions such as biomineralization reaction (Gomez, Arnaiz, Cacioppo, Arcudi, & Prato, 2018). The PCs (Fig. 13.2) have three different interfaces, exterior, interior, and intersubunit, which can be modified by chemical or genetic engineering to get desired response at the target site. These modifications are reported to control surface charge, drug encapsulation, ligand expression, drug release, and target specificity of proteins (Bhaskar & Lim, 2017; Molino & Wang, 2014). The surface modification of PCs, in turn, depends on the surface chemistry of the cage, its diameter, and surface charge of the same (Sahandi Zangabad et al., 2017).

Figure 13.2



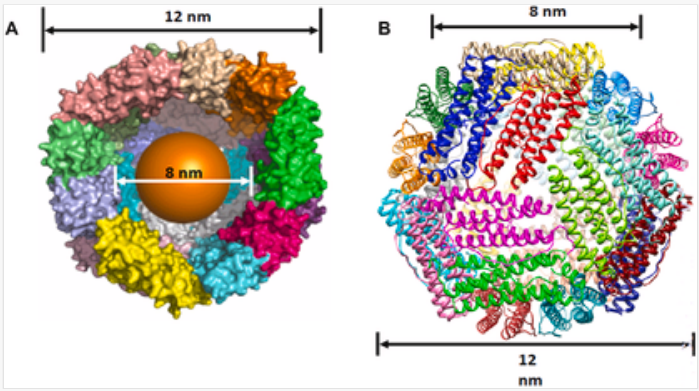
External, internal, and intersubunit modification of protein cages.
Source: Reprinted with permission from Bhaskar, S., & Lim, S. (2017). Engineering protein nanocages as carriers for biomedical applications. NPG Asia Materials, 9(4), 1–18. https://doi.org/10.1038/am.2016.128.

13.3.1 Natural protein cages/scaffolds

13.3.1.1 Ferritin

It is one of the major nonviral proteins, first isolated from horse's spleen in the year 1937 (Roudi et al., 2017). Later, it has been found in almost all organisms except yeast. Ferritin is reported to balance the iron level at target site by storing and releasing iron on physiological demand. Ferritins are ubiquitous protein that belongs to a family of iron storage proteins and consists of 24 subunits that are assembled to form a hollow sphere (Crichton & Declercq, 2010; Korpi, Anaya-Plaza, Välimäki, & Kostiainen, 2020; Palombarini, Fabio, Boffi, Macone, & Bonamore, 2020; Roudi et al., 2017). It has an outer diameter of 12 nm and an inner cavity diameter of 8 nm (Fig. 13.3). It consists of both H chain and L chain, which form an inner aqueous cavity by self-assembling both the chains at specific pattern. Notably, any modification in its exterior surface does not change the properties of interior cavity (Roudi et al., 2017). Ferritins devoid of iron cores are known as *apoferritin* (Dopamine-conjugated, 2019).

Figure 13.3



Structure of ferritin nanocages. (A) Ferritin representing 24 subunits assembled to form hollow spherical protein with 12 nm diameter exterior and 8 nm diameter interior cavity with an encapsulated mineral. (B) Ribbon diagram representation of ferritin nanostructure.
Q45 *Source: From Watt, R., Petrucci, O., & Smith, T. (2013). Ferritin as a model for developing 3rd generation nano architecture organic/inorganic hybrid photo catalysts for energy conversion. Catalysis Science & Technology, 3, 3103–3110.*

Ferritins are thermodynamically stable and can tolerate temperature up to 85°C and pH up to 8.5–9 without any disruption in their quaternary structure. It is also biodegradable and nonimmunogenic in nature and has electrophoretic mobility, so it is widely used in medicinal field as drug delivery careers (Roudi et al., 2017). The globular ferritin cage stores the iron in nontoxic and insoluble form, but delivers iron to cells with augmented bioavailability by converting it

to its soluble form. Subsequently, the iron remains as ferric oxyhydroxide crystal within the cell (Bhaskar & Lim, 2017), which regulates the uptake and release of drug molecules from the caged protein within the cell.

The formation of protein nanocages depends on the self-assembly of the proteins by protein–protein interaction. However, in the presence of specific stimuli such as pH, ionic concentration, and metal abundance, this assembly gets disjoined, subsequently releasing the molecular cargo from the cage (Bhaskar & Lim, 2017; Zhang & Orner, 2011).

13.3.1.2 Heat shock protein

Under stressful conditions (high temperature, inflammation, hypoxia, infection), heat shock proteins (HSPs) can protect the damaged proteins by maintaining their proper 3D structure through their chaperone-like activity. HSPs are classified depending on their molecular weight, for example, HSP100, HSP90, HSP70, HSP60, HSP27, and small HSPs (Kim, Kim, & Kim, 1998; Roudi et al., 2017; Sahandi Zangabad et al., 2017). The chaperone-like activity of HSPs forms stable complexes with the client protein, where the client molecule gets bound to the internal cavity of the former (Sahandi Zangabad et al., 2017).

Q9 Kim et al. elucidated the structure of sHSP and showed that it consists of 24 subunits, which form spherical nanoparticles with an inner diameter of 6.5 nm and an outer diameter of 12 nm. It has pores of about 3 nm, which is larger than other nanocage pores. So solute particle exchange is quite easy between exterior bulk solvent and cavity. It contains 147 amino acids without any cysteine residue, but can be modified to have cysteine residue with will conjugate with thiol group at specific site (Kim et al., 1998; Sahandi Zangabad et al., 2017).

Q10

13.3.1.3 Vaults

It was first reported in the year of 1986, having mass of 13 MDa, which is found in all eukaryotic cells having several functions such as mRNA localization, nuclear pore assembly, and nuclear cytoplasmic transport. Vaults are mainly ribonucleoproteins containing many copies of RNA and also several copies of three protein residues. Vaults make
Q11 barrel-shaped nanocages by assembling itselves. It forms a hollow cage-like structure of 72.5 nm length and 41 nm width. Vaults can be irreversibly dissociated into halves at pH 3–4 (Goldsmith, Yu, Rome, & Monbouquette, 2007; Lee, Lee, & Kim, 2016). Vaults consist of several highly conserved proteins with major vault proteins (MVPs). This MVP comprises up to 70% of the overall mass of vaults. Vaults consist of 78–96 MVPs, which are arranged from C- to N-terminus from cap to waist. It forms a protein coat covering the internal cavity. The N- terminus is present in the interior side of vault. Though MVPs are covalently bonded, it has a dynamic structure, which opens and closes; this phenomenon is called breathing. This phenomenon helps to insert drug molecule, proteins, or other molecules into the inner cavity (Bhaskar & Lim, 2017). Earlier studies have shown that vaults are stable at a wide range of conditions such as from pH 4 to 8 and temperature from 10°C to 90°C. In addition, special properties of vaults such as structural stability and nonimmunogenicity make them appropriate for drug delivery or immunogenic protein delivery at specific sites (Lee et al., 2016).

13.3.1.4 E2 protein

It is derived from pyruvate dehydrogenase multienzyme complex. It has 24 subunits in *Escherichia coli* and 60
Q12 subunits in *Geobacillus stearothermophilus*. This protein forms a hollow cage-like structure, which can carry the drug molecule. It forms a cubic core or icosahedrons with 12 openings. Pyruvate dehydrogenase multienzyme consists of pyruvate decarboxylase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3). In the presence of E2 enzyme, E1 and E3 do not get disassociated and E2 forms the structural core of the cage. The protein obtained from thermophilic bacteria *G. stearothermophilus* has high stability in extreme conditions. Modification of external surface of this protein with ligands does not affect the core structure (Bhaskar & Lim, 2017). Sometimes intersubunit modification is needed to prepare stimuli-sensitive system.

Peng and Lim (2011) investigated protein–protein interaction at intratrimer and intertrimer interfaces of E2 PC from *Bacillus stearothermophilus* and introduced histidine residue to prepare pH-sensitive PCs. By using this strategy encapsulated drug release from the carrier system can be controlled and can also be stimuli-dependent. For example, tumor cells are more acidic than normal cells. Hence this strategy would help target tumor cells using the stimuli-sensitive PCs.

13.3.1.5 Virus-based particles

Most of the viruses consist of several protein subunits, which form the protein coat and in which they store viral DNA or RNA. They infect the living cells and replicate their DNA into host cells. Viruses have the innate ability to protect their genome which they transfer to the specific cell and also they can get self-assembled and form stable virus-like particles (VLPs). Thus it is highly used as functional drug carrier system. VLPs do not have pathogenicity. They can be of different shapes and sizes. The size can vary from 10 nm to 1 μm, and they can be rod-shaped, spherical-shaped, or isodecahedron. VLPs can also be chemically modified or genetically engineered at their exterior, interior, and intersubunit sites. The target ligands can also be attached to the exterior of the VLPs by chemical modifications (Bhaskar & Lim, 2017).

Q13 Cowpea mosaic virus (CPMV) is the first nanocage that is shown to be self-assembled and can act as a functional drug carrier. It is a plant virus, which is stable under a wide pH range, temperature, and in different solvents. Multiple reactive sites can be prepared by genetic modifications to which targeting moieties can be attached (Bhaskar & Lim, 2017).

There are several other viruses that can be used as protein nanocages such as cowpea chlorotic mottle virus (CCMV), which contains 180 protein subunits that get assembled into an icosahedral structure, bacteriophage MS2, Qβ bacteriophage, and others (Lee et al., 2016).

The external surface of these PCs can be modified to increase systemic circulation time, cell permeability, local accumulation, and cell targeting. Several modifying molecules such as antibodies, peptides, nucleotides (DNA, RNA, and DNA aptamers), polymethacrylate, and polymers (Bhaskar & Lim, 2017) are used to manipulate VLP cages for specific applications.

13.3.1.6 Other natural scaffolds

Some species of bacteria forms nano and micro-sized structure that contains protein complexes. Those are called bacterial microcompartments. Carboxysomes were found to be the first bacterial nanocages to show these kinds of compartmentalized functions. It has icosahedral structure with a cross section of 100–150 nm. It has also a protein coat containing 6–10 proteins. They help in autotrophic CO_2 fixation via RuBisCO and carbonic anhydrase enzymes. This bacteria can retain its self-assembling property in the absence of RuBisCO enzyme (Bhaskar & Lim, 2017).

13.3.2 Engineered protein cages

Reportedly, engineering of PCs has been performed either experimentally through modifying its specific subunits (intermeric or intrameric, external or internal) by covalent cross-linking, ionic interactions, and hybridization with various target-specific ligands, or through de novo designing novel self-assembling peptide/protein nanocages with desired geometry in 3D space. The engineered architecture of protein is achieved by strategic doping with tunable covalent bridging, noncovalent interactions, van der Waals forces, electrostatic attraction, π–π interactions, metal–protein coordinations, metal–ligand coordinations, and other relevant forces. Exploitation of chemistry of amino acids, its protonation states, tertiary structure conformation, vicinity or perturbation of one protein loop through another's field,
Q14 spanning of domain over another, proxemics and kinesics of intra-domain residues in dynamic state, entropy-guided thermodynamic stability all are involved in achieving symmetric or higher order protein structure, ultimately converting to cage-like conformation. A synopsis of such approaches has been summarized in this review.

13.3.2.1 Engineered bioinspired protein cages

This type of engineered nanocages is basically a synthetic protein nanocage where the final PC is either used as a biosimilar protein multimer or strategically used as a cargo-boat to discharge molecular payload at specific target. For example, amyloidogenesis yields a highly symmetric fibroidal protein nanocage, which was first achieved by designing the charged and symmetric polymerization of proteins de novo, followed by benchtop assembling of protein fibrils involving domain swapping and symmetric oligomerization (Bhaskar & Lim, 2017). The leucine zipper motif, in this sort, was also created by dimerization of coiled protein structures. Also, de novo–guided self-assembling protein nanocages were designed for hydrogelating self-assembling nanocages, smart pH-responsive protein nanostructures, and collagen mimetic protein ultrastructures utilizing the amphiphilic nature of proteins. Furthermore, protonation states in respective pH media and subsequent end-to-end interspersing of proteins have been coutilized to yield higher order protein structures (Banwell et al., 2009; Burkhard, Stetefeld, Strelkov, & Burkhard, 2001; Pandya et al., 2000; Papapostolou et al., 2007).

Q15 To further advancement of this de novo designed protein nanocages, Rosetta Design software has been used to design the assembly of protein subunits into oligomeric macrostructures having definite geometries. Using system Q16 thermodynamics paradigm of attaining lowest energy conformation, pentameric protein nanocage using axes of symmetry, tetrahedron geometry–based protein "nanohedras," and octahedral geometry–based fused protein oligomer whose outer diameter of 225 Å and inner porosity of 100 Å signify its huge capacity to accommodate guest molecules, have also been fabricated successfully (Lai, Cascio, & Yeates, 2012; Lai et al., 2014; Padilla, Colovos, & Yeates, 2001; Woolfson, 2017).

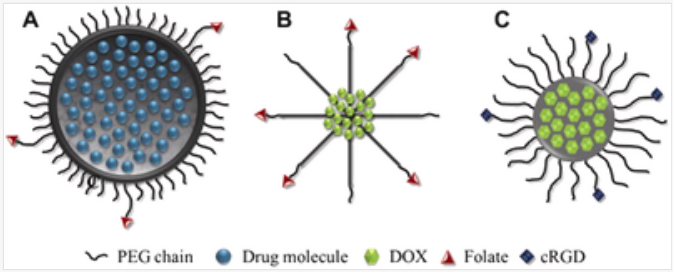
13.3.2.2 Engineered protein cages based on hybridization

Sometimes, PCs are conjugated with other molecules such as carbohydrates, synthetic or semisynthetic polymers, lipids, and relevant epitopes to prepare hybrid cages. Virus PCs such as CPMV, tobacco mosaic virus, and adenovirus can also be hybridized with synthetic polymer (Raja et al., 2003). Polymerizable vinyl groups can be grafted on protein pockets, which leads to polymerization in the presence of cross-linker and monomers. It forms a thin degradable or nondegradable polymeric coat on the protein. Smart polymers can also be grafted on the nanocage to get stimuli-sensitive properties. They exhibit response only in the presence of target stimuli such as ionic strength, heat, light, and pH subsequently delivering molecular cargos in that specific microenvironment (Bhaskar & Lim, 2017).

13.3.2.3 Engineered protein cages with exterior modifications

Several receptor-specific ligands such as antibody, folate, and transferrin can be attached to PCs to target the very receptors expressed in several tissues such as cancer cells, tumor cells, inflammatory site, blood cells, asthmatic lungs, infected organs, and many others. These ligands undergo ligand-receptor recognition, resulting in active targeting of the protein nanocages. Folate receptors are overexpressed in tumor cells of ovarian carcinoma, uterine sarcoma, and other tumors. Hence folate can be attached to nanocages as receptor ligand and targeted to folate receptors where it gets internalized by cells and the drug gets released from the carrier (Sahandi Zangabad et al., 2017; Steichen, Caldorera-Moore, & Peppas, 2013), for example, doxorubicin (Yoo & Park, 2004). Fig. 13.4 shows the schematic of external Q17 modifications, due to folic acid, PEG (Polyethylene Glycol) chain, and other ligands.

Figure 13.4



Q46 Schematic representation of external modifications of protein cages. Targeted particles: (A) Example of a folate receptor–targeted particle. Liposome functionalized with PEG tethers to impart STEALTH characteristics and folate for tumor targeting. (B) Folate-conjugated PLGA-PGA polymeric micelle loaded with encapsulated DOX and (C) cRGD-functionalized PCL-PEG polymeric micelle containing encapsulated DOX. *cRGD*, Doxorubicin-containing PEG polymeric micelle; *DOX*, doxorubicin.
Source: Reprinted with permission from Palombarini, F., Fabio, E. D., Boffi, A., Macone, A., & Bonamore, A. (2020). Ferritin nanocages for protein delivery to tumor cells. Molecules (Basel, Switzerland), 25(4).

13.3.2.4 Engineered protein cages with interior modifications

The inner cavity of the PCs can be functionalized or modified by genetic or chemical alterations to increase binding affinity of the drug, to increase encapsulation efficiency, or to modulate release of the drug molecules. Protein drugs, DNA, RNA, and imaging agents can be encapsulated easily in the interior cavity of the protein nanocages.

As one of the most strategic approaches, introducing cysteine residue in the interior surface of the protein manipulates the inner cavity significantly. This strategy is particularly useful in three major cases. First, for efficient loading of drug through drug-cysteine –S–S– disulfide linkage; second, for reductive opening of cavity by converting –S–S– linkage into –SH group and strategic release of the molecule in such environment; and third, compartmentalization of protein

nanocage into several microcompartments through forming multiple disulfide cross-bridges within the same cavity (Bhaskar & Lim, 2017).

The interior cavity of HSPs can also be modified by phenylalanine residue, lipid substances, and polymers. Abedin, Liepold, Suci, Young, and Douglas (2009) increased the thermal stability of HSP nanocage by synthesizing cross-linked branched polymeric network at genetically inserted cysteine residues, present in the inner cavity of the HSP. This synthesis of cross-linked polymeric network at the interior of the nanocages was created by sequential coupling of monomeric units using click chemistry. Using click chemistry the authors introduced functionalizable pendant groups, that is, free amines into the branched polymeric network (Abedin et al., 2009). Subsequently, these sites have been tuned as the area for covalent addition of the drug molecule. The authors acknowledged that these branched polymers stabilize the PC and covalently cross-link the protein subunits.

Vault can also be chemically modified to make it active for imaging and efficient for drug delivery (Benner et al., 2017). Benner et al. modified vault lysine and cysteine residues using three reactions, namely Micheal addition, nucleophilic substitution, and disulfide exchange reaction, and made vault more selective and efficient.

Q18 Interestingly, gold has been acknowledged to create engineered nanocage by adding it strategically with self-assembling protein monomers. In 2019 Malay et al. reported the addition of gold (I) triphenylphosphine into protein subunits to synthesize 11-mer protein nanocage with specific geometry. The authors demonstrated that the addition of gold led to the formation of micro-compartmentalized supra-assembly of protein nanocages, especially due to cross-linking of sulfur of cysteine with gold (Au) to yield –S–Au–S– coupling. The resulting geometry of the nanocage was of Archimedean snub cube type, and the opening or closure of this cube was also acknowledged to be regulated by strategic addition and depletion of gold in the medium (Malay et al.).

13.3.2.5 Engineered protein cages with improved biocompatibility and biodegradability

An ideal nanocage, which is used as drug carrier, must be significantly biodegradable in nature. The carrier must be highly biocompatible, which means the carrier should interact with body without showing toxicity, immunogenicity, thrombogenicity, and carcinogenic response (Naahidi et al., 2013). It must hold the guest molecule for a longer period of time in systemic circulation and must not show any systemic toxicity. So the half-life of the carrier is an important factor to show therapeutic effect in a sustained manner. In addition, it should not cause any harm to the nature and must be biodegradable. It must possess a proper systemic clearance after its function in the body without exhibiting any accumulation effect (Bhaskar & Lim, 2017).

13.3.2.6 Engineered protein cages for improved release

The delivery of the active molecule is mainly controlled by chemical immobilization, noncovalent and covalent interactions with the carrier molecule (Bhaskar & Lim, 2017). The inner cavity of the PC has reactive sites to which drug molecule get attached by nonspecific interactions depending on their affinity. To the reactive side chains of amino acids such as amines, carboxyls, sulfhydryls, and hydroxyls and to the nonside chains, the drug molecules get attached by posttranslational attachment and click chemistry, respectively. The release of the drug from the carrier depends on the conjugation chemistry in between the drug molecules, drug–carrier interaction, and cellular microenvironment. The
Q19 pores present in PCs take an important part on drug release by diffusion. Gated pores or pores of stimuli-sensitive nanocages open at certain pH, ionic concentration, and heat shock, and subsequently the drug gets released. This mechanism helps to release the drugs at target site. Sometimes repulsive forces in the microenvironment may come to interplay, finally triggering the disassembly of the protein subunits at specific target site, resulting in release of the payloads (Bhaskar & Lim, 2017).

13.3.2.7 Engineered protein cages for improved targeting

Targeting of nanoparticles such as protein nanocages to a cancer cell becomes very effective because of the enhanced
Q20 permeation and retention (EPR) effect. Due to this effect, the nano-sized drugs get accumulated in cancer tissues than other organs, and the half-life of the drug gets increased because their size exceeds the renal excretion threshold (Brown et al., 2010). However, nonspecific accumulation of PCs due to EPR effect, called passive targeting, is not found as effective as its active counterpart. Hence active targeting has become the first choice amongst scientists in protein-based drug delivery system over the last few decades. In cancer cell targeting, natural ligands are used to target the receptors expressed in cancerous cells. Other ligands such as antibody, peptides (Kang, Park, Shin, Park, & Kang,

2012), DNA aptamers (Stephanopoulos, Tong, Hsiao, & Francis, 2010), and nonnatural proteins are also reported to be used for biological active targeting. The targeting ability of a PC mainly depends on the ligand density present on the exterior surface of the cage (Bhaskar & Lim, 2017) which in turn helps in innervations in specific tissues with plausible permeability (Sahandi Zangabad et al., 2017) previously reported as challenging and difficult to achieve.

Q21

Passive targeting

This targeting is dependent on the size, nature, shape, and surface characteristics of the PC and pathological condition of the target site. It is reported that on reducing the size or diameter of the protein nanocage, the circulation half-life of the same gets increased, together with enhanced accumulation of the cages in the target tissue. However, target tissue variation and protein nanocage interaction with macromolecules and phagocytes in circulation system may lead to alteration of the results.

Nanocage shape is another important factor for effective targeting. Rod-shaped and spherical-shaped particles larger than 100 nm are revealed to have more cell uptake efficiency than cylindrical and cubic-shaped cages. For the nanocages with size lesser than 100 nm, spherical-shaped particles showed the highest cell uptake (Albanese, Tang, & Chan, 2012).

Surface charge has also been an essential factor of targeting. Positively charged particles showed more efficient cellular uptake than the negatively charged or neutrally charged particles. Slightly negatively charged particles showed cell uptake by electrostatic attractions (Sahandi Zangabad et al., 2017).

In the conventional method for passive targeting, sometimes nanocages are coated with hydrophilic polymers such as PEG, which is called PEGylation, to increase residence time in systemic circulation (Suk, Xu, Kim, Hanes, & Ensign, 2016). However, lower cellular uptake, less circulation half-life, and early clearance are the limitations of these nanocages. So coating techniques such as glycans shielding, polyketal hybridization, and many others have been employed to solve these problems (Sahandi Zangabad et al., 2017).

Active targeting

Active targeting with peptides, antibodies, and receptor ligands is more efficient than passive targeting due to its lesser side effects in normal cells and increased therapeutic activity only at targeted sites. It has two major approaches. First, naturally occurring nanoparticle structure is used for targeting; and second, targeting ligands are attached to the surface of the nanocage (Sahandi Zangabad et al., 2017).

Peptide labeling on the exterior surface of the protein nanocages has been a popular target to improve active targeting and subsequent cellular uptake. Virus and bacteriophage are also reported to be used to attach peptides on their exterior surface for target specificity (Karimi et al., 2016). For cell-specific delivery, various peptides involving positively charged peptides (TAT peptide) or cell penetrating peptides (penetratins) have also been used (Sahandi Zangabad et al., 2017).

DNA and RNA-based aptamers, which are single-stranded oligonucleotides, have several advantages such as easily modifiable structure, high binding affinity, target specificity, and high cell uptake. These aptamers can be used as target ligands for nanocages for efficient targeted drug delivery. For example, MS2 bacteriophage capsid is modified with DNA aptamer through oxidative coupling and made specific for Jurkat cell (Sahandi Zangabad et al., 2017; Stephanopoulos et al., 2010).

13.3.2.8 Drug loading in protein nanocage contributions of engineering

As described earlier, several functionalizations and modifications of nanocages increase encapsulation efficiency, target specificity, and cellular uptake. The therapeutic efficacy of the drug delivery system depends on the chemistry and technique of drug loading, which eventually leads to drug release at desired conditions. Two major strategies of drug loading have been reported for protein nanocages: drug loading by noncovalent attachment and drug loading by covalent attachment.

Drug loading by noncovalent attachment

In this approach, drugs are entrapped inside the cage cavity, which depends on the affinity of the cage toward drug molecule and also on the genetic fusion. In the presence of stimuli such as pH, ionic concentration, heat shock, and osmotic shock, the structure of nanocages changes, that is, the pores get open and it also leads to disassembly/reassembly of protein units that help in encapsulation of drug as well as release of drug (Lee et al., 2016).

In ferritin nanocages, iron ions enter into the cage mainly by channels of 10 Å present on the cage (Munro & Linder, 1978). It is presumed that when the iron ions enter into the nanocages through channels, they form ferrihydrites of 2 nm and cover the entrance channels. Molecules that are larger than the iron ions and channels get internalized by ferritin (Lee et al., 2016). Zhen et al. modified ferritin nanocages with RGD peptide which will target tumors efficiently by RGD–integrin $\alpha_w\beta_3$ interaction. Doxorubicin was first complexed with Cu (II), and then it was loaded in RGD-modified ferritin nanocages with more efficiency than without precomplexion. It showed longer circulation half-life and less cardiotoxicity than free doxorubicin (Kilic et al., 2013).

Viral nucleic acids such as DNA and RNA can be inserted into the nanocages (VLP) by electrostatic interaction between the negative charges present on the nucleic acids and the positive charges of the nanocages (VLP). This natural characteristic is used for the insertion of negatively charged molecules into the VLPs. Sometimes receptor-specific ligands such as folic acids are attached to the carriers for tumor targeting. Zeng et al. (2013) loaded doxorubicin in folic acid–tagged cucumber mosaic virus (CMV) and obtained highly encapsulated doxorubicin -loaded proteasome with improved cellular uptake. In this engineering the drug is usually complexed by noncovalent interaction with negatively charged CMV RNA and targeted toward ovarian epithelial adenocarcinoma–expressed OVCAR-3 cells. Folic acid has been attached to this system via *N*-ethyl-*N′*-(3-dimethylamino propyl)-carbodiimide hydrochloride (EDC) coupling reaction (Naahidi et al., 2013).

In another approach, drugs have been attached to PCs through genetic fusion. Choi, Choi, Jeon, Kim, and Ahn (2011) formed a chimeric capsid protein for siRNA delivery, which showed pH- dependent dissociation. O'Neil, Reichhardt, Johnson, Prevelige, and Douglas (2011) genetically modified bacteriophage P22 and encapsulated fluorescent protein into it.

Drug loading through covalent attachment

Covalent attachment is another strategy of drug loading into the PC. Insertion of nonnative amino acids such as azidohomoalanine and homopropargylglycine can lead drugs to be attached by covalent attachment. Furthermore, controlled release of the drugs could be achieved when drugs are linked with the cage by covalent interactions.

Ren, Kratz, and Wang (2011) mutated the aspartic acid of E2 to cysteine and formed an engineered cage to load fluorescent dye and doxorubicin conjugate. The dye was coupled to the internal cavity through the maleimide linker that ultimately led to the formation of drug-maleimide-thiol triplet, thiol group anchoring to mutated cysteine. 6-Maleimidocaproyl hydrazone was used to coload doxorubicin, and the system was reported to exhibit pH-dependent drug release basically at pH 5.

Sometimes drugs can be attached covalently at the exterior side of the cage. Aljabali, Shukla, Lomonossoff, Steinmetz, and Evans (2013) activated the carboxylates present on the exterior surface of the CPMV to which 80 doxorubicin molecules covalently attached. Doxorubicin was covalently attached to the carboxylates through EDC (1-Ethyl-3-[3-dimethylami- nopropyl]-carbodiimide hydrochloride)/NHS (N-Hydroxy succinimide)-mediated coupling, which showed more activity toward HeLa cells than solitary doxorubicin.

Genetic alterations of cysteine or other residues inside the cage have become an emerging strategy to introduce drug–carrier bonding inside PC. Kang et al. (2010) genetically introduced cysteine residue at interior surface of bacteriophage P22 protein. It was modified for site-specific attachment of biotin linkers via which large tetrameric protein streptavidin was finally bound inside the cage.

13.3.3 Therapeutic applications of protein cages

A predominant application of PCs has been in delivery of drugs through various covalent and noncovalent engineering strategies. A synopsis of these strategies has already been discussed in the "Drug loading by noncovalent attachment" and "Drug loading through covalent attachment" sections. We suggest to refer to those sections for a judicial snapshot

to those approaches. Furthermore, there are some more approaches related to therapeutic applications of protein nanocages that are summarized in the following sections.

13.3.3.1 As drug carriers

Nanocages are genetically or chemically modified to increase encapsulation efficiency, long circulation half-life, and target specificity, and can also be modified to carry drugs, genes, nucleic acids, contrasting agents, and proteins. The interior of the PCs such as E2 and HSP, and virus nanocages can be genetically engineered, and cysteine residue can be incorporated into the cage for the selective attachment of the antitumor drug molecule through pH-sensitive linker, which will release the drug molecules at specific pH (Bhaskar & Lim, 2017). Sometimes receptor ligands such as folic acids can be attached to the exterior of the cages for target specificity. It has been shown that antitumor drugs encapsulated in the PC exhibit cytotoxic effect on carcinogenic cells. A detailed summary of these target-specific drug, protein, or gene delivery approaches through engineered PCs have been presented in the "Passive targeting" and "Active targeting" sections.

Some VLPs such as CCMV and CPMV act as a carrier of exogenous DNAs and RNAs. Choi et al. engineered HBV (Hepatitis B virus) capsid protein for siRNA delivery. RGD peptides are mostly used on the exterior of the capsid, which guides the loaded cage to target specific cells (Choi et al., 2011).

Q24 Protein-based drugs or therapeutics can also be delivered by PCs because of their larger cavity. VLPs can deliver proteins as a part of Gag proteins or can also deliver through the surface of VLPs (Kaczmarczyk, Sitaraman, Young, Hughes, & Chatterjee, 2011).

Q25 As other notable applications, enzymes such as caspase-8, TRAIL (tumor necrosis factor–related apoptosis-inducing ligand), Interferon-γ can be loaded onto avian sarcoma leukosis VLP; interleukin-2, clopidgrel, and siRNA can be entrapped into hepatitis VLP; miRNA, siRNA, and paclitaxel have been caged inside MS2 bacteriophage VLP; endothelial growth factor, porphyrin, and clopidogrel have been encapsulated with Qβ VLP; trastuzumab, duanomycin, cisplatin, and AP-1 peptide have been loaded inside ferritin nanocages (Molino & Wang, 2014).

13.3.3.2 As imaging agents

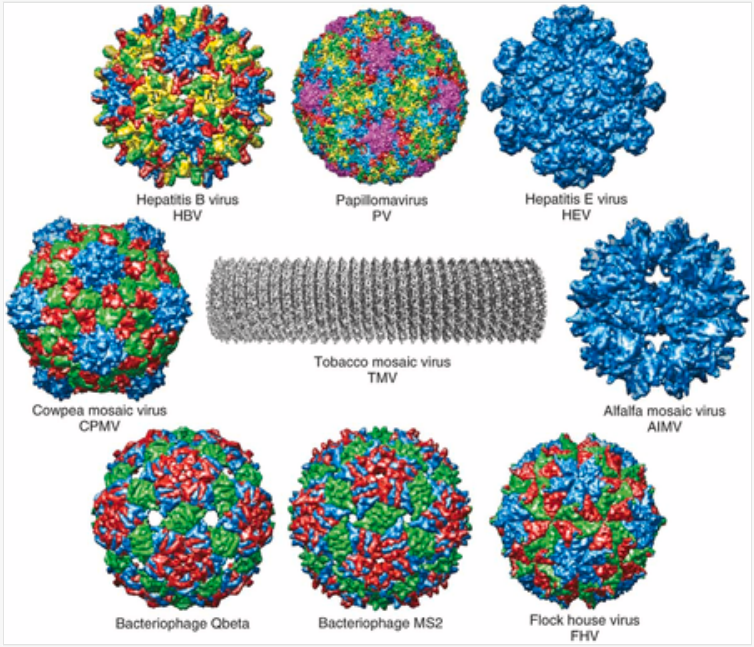
For in vitro or in vivo imaging the most essential thing is to get high concentration of imaging agent on a specific area. Protein nanocages are reported to encapsulate and carry the contrasting agents, which is required for OCT (Optical Coherence Tomography), magnetic resonance imaging (MRI), and near-infrared fluorescence. Ferritin is reportedly one of the best PCs for carrying contrasting agents. It stores the iron ions as ferric oxyhydroxide particles, which is preferable as contrasting agent. So, these ferritin nanocages can be used for effective delivery of contrasting agents for MRI (Bhaskar & Lim, 2017; Ruggiero, Alberti, Bitonto, & Crich, 2019). PCs are engineered and modified to increase the encapsulation efficiency and loading capacity of fluorophores. The contrasting agent is generally attached at the specific positions of the PCs by chemical reaction. In another approach, gadolinium (Gd) with HPDO3A formed gadoteridol contrasting image complex (Gd-HPDO3A) for MRI, which was encapsulated in PC through pH-mediated assembly and disassembly reaction. The PC was further loaded with curcumin and used as therapeutic cum magnetic
Q26 imaging machinery especially targeted for liver diseases (Belletti et al., 2017). The presence of dye molecule is acknowledged to prevent the contrasting agents from aggregating with each other and thus decreases the quenching of fluorophores (Bhaskar & Lim, 2017).

13.3.3.3 As vaccines

In 1976 Edward Jenner produced the first vaccine for small pox, which was virus-based. Since then several vaccines came into the market to prevent hepatitis A, rubella, measles, mumps, influenza, and chicken pox. The live attenuated vaccines are difficult to administer and also risky that they can revert back to their pathogenic form, which can be fatal to the subject. The advanced approach in the field of vaccine includes immunization with some of the portions of pathogen which shows strong immune response (Bhaskar & Lim, 2017). It is termed as subunit vaccine. Here, by the innate characteristic of viral capsid subunits, they get self-assembled and form VLPs that mimic the virus particles and improve the efficacy of the vaccine. VLPs consist of capsid proteins that show immune response but do not contain genetic material for replication or immunogenicity. VLPs and nanoparticles are used as vaccines and can also be used as base to attach foreign epitopes which shows strong immune responses. These particles thus increase the efficacy of vaccines used for specific diseases. HBV and HPV (Human papilloma virus) vaccines with two nonenveloped VLPs

are now commercialized for clinical use. Furthermore, VLP-based vaccines against coronavirus, chikungunya, Ebola virus, Marburg virus, hepatitis C virus, and filovirus are in pipeline and under investigation before being destined for clinical trials. Fig. 13.5 shows some virus capsids that are used as VLPs' vaccine (Plummer & Manchester, 2011).

Figure 13.5



Example of virus capsids that are used as virus-like particles.
Source: From Plummer, E., & Manchester, M. (2011). Viral nanoparticles and virus-like particles: Platforms for contemporary vaccine design. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 3(2), 174–196. https://doi.org/10.1002/wnan.119.

Vaccines against respiratory infections such as influenza and coronaviruses are being extensively explored. In the development of coronavirus subunit vaccine, only the small antigenic fragments of virus, which may not involve any infectious portions of viruses, are included. It has been shown that the S protein of the coronavirus is the major antigen, which induces neutralizing antibodies and thus blocks viruses to bind to respective receptors inhibiting the infections. So S protein and its fragments are the major targets to prepare subunit vaccine against coronavirus (Jiang, Du, & Shi, 2020; Wang, Shang, Jiang, & Du, 2020).

13.4 Protein-polysaccharide cages: engineering and therapeutic applications

Q27 Engineering PCs with various cross-linking agents, polymers, or metallic gold ~~have~~has been discussed in this chapter, which primarily aims to accommodate guest molecules with better efficiency, elicit smarter responses against stimuli such as pH, temperature, or other environmental factors, and finally have controlled release of the guest in biological fluid. PPC or nanohybrid of protein-polysaccharide is another extension to that because polysaccharide is an excellent complexation material owing to their polyhydric alcoholic groups and ionization capacities (Jones & Mcclements, 2011
Q28). Various proteins such as albumin, lactoferrin, α-lactalbumin, β-lactoglobulin, casein, lactoferrin, and lysozyme often result in complexation with polysaccharides such as chitosan, carrageenan, pectin, chondroitin sulfate, and alginates (Schmitt & Turgeon, 2011). The successful protein-polysaccharide complexation or cage formation depends on successful choice of binding partners among each other, which further depends on their chemistry, overlaying functional groups, steric effects, backbone of both polymers, their hydrophilicities, and various bonding interactions.
Q29 Various drug deliveries involving curcumin, resveratrol, folic acid, methotrexate, vitamin D3, rutin have been succesfully achieved through protein cage based drug delivery systems (Ha, Jeon, Kim, & Han, 2016; Luo, Pan, & Zhong, 2015; Teng, Luo, & Wang, 2013; Yang, Peng, Wang, & Liu., 2010; Yin, Deng, Xu, Huang, & Yao, 2012). Not only this, but also various biomacromolecules such as enzymes (e.g., β-lactamase), recombinant DNA, and quantum dots have been successfully loaded into PPCs and efficiently administered in vivo to improve stability, targeting, and release profile together with reducing their toxicity (Li et al., 2015; Member et al., 2016; Zorzi et al., 2011). The PPC, based on their chemistry, mode of formation, and tunable engineering techniques, could be majorly divided into four

major types of nanocomplexes or nanocages: electrostatic precipitation complexes, chemical reaction–mediated complexes, electrospun nanohybrid–mediated complexes, and posttranslational modification (PTM)-aided protein-polysaccharide block copolymer complexes.

13.4.1 Electrostatic precipitation complexes/cages

The electrostatic precipitation protein-polysaccharide complexes/cages are formed by two principal methods, single-step complexation and core–shell complexation.

13.4.1.1 Single-step complexation

The electrostatic precipitation single-step complexes are formed when oppositely charged protein and carbohydrates interact with each other and undergo associative separation out of the phases. This method relies on setting a reaction between positively charged polysaccharide and negatively charged albumin, where the medium pH is adjusted tactfully to induce such charges on both agents. For example, doxorubicin-loaded chitosan-albumin complex (Varshosaz, Hassanzadeh, Sadeghi, Khan, & Rostami, 2013) or aceclofenac-loaded chitosan-albumin nanogel (Jana, Manna, Kumar, & Kumar, 2014) has been manufactured by exploiting this strategy. Herein, medium pH was set at 5.4, which was higher than isoelectric point of albumin (pI 4.7) and lower than that of chitosan (pI 6.5). Thus at this pH, albumin was negatively charged, whereas the net charge on chitosan was positive. Hence they were subjected to electrostatic interaction, following the formation of associative precipitation complex. Similarly, careful selection of protein-polysaccharide binding partners and thus choosing the medium pH to induce opposite charges on both polymers have led to the formation of a large variety of electrostatic precipitation complex, where medium pH, heating, rate of aeration, and sonication have been finely tuned to incur spectrum of engineering regarding their size, shape, charge, hydrophilicity, and cavity formation. The thus formed electrostatic precipitation complexes with drug delivery applications are narrated in Table 13.2.

Table 13.2

The table layout displayed in this section is not how it will appear in the final version. The representation below is solely purposed for providing corrections to the table. To preview the actual presentation of the table, please view the Proof.

Q50 Protein-polysaccharide nanocages formed by electrostatic precipitation with therapeutic outcomes.

Protein-polysaccharide cage	Drug	Key outcomes
Albumin-CMC	DTX	Enhanced antitumor activity was demonstrated via an active targeting mechanism
Q51 Albumin-chitosan	DOX	Significant cytotoxicity enhancement in HepG2 cells for retinoic acid–targeted nanohybrids in comparison to nontargeted ones
Albumin-chitosan, alginate, and dextran	Insulin	Enhanced stability of insulin, release modulation according to pH, and enhanced intestinal permeability
Q52 BSA-chitosan	GEM (Gemcitabine)	Higher cytotoxicity in A549 and H460 lung adenocarcinoma cell lines in comparison to sole albumin nanoparticles
BSA-chitosan	psiRNA-hH1GFPzeo	Higher biocompatibility in comparison to chitosan alone
BSA-i-carrageenan	Curcumin	Enhanced the stability and the antioxidant activity of curcumin
BSA-i-carrageenan	EGCG	Enhanced stability and antioxidant activity of EGCG
BSA-alginate	TRAIL and DOX	Synergistic cytotoxic activity was achieved even in the DOX-resistant L929 cell line
BSA-gum acacia	–	Successful fabrication of nanohybrids with a size of 108 nm
BSA-chitosan-dextran	DOX	Significant prolongation in the survival of hepatoma (H22)-bearing mice in comparison to free DOX
Egg albumin-chitosan	Aceclofenac	Enhanced skin permeation and higher antiinflammatory activity in comparison to

		the marketed gel
Ovalbumin-chitosan	–	Enhanced nanohybrid stability upon long-term storage with pH responsiveness
Gelatin-chitosan	CPs	Higher apoptotic activity was achieved against THP-1 leukemic cells in comparison to free CPs
Gelatin-chitosan	Fluorescein	The intravenous administration was found optimum for achieving higher tissue concentrations rather than the intraperitoneal one
Gelatin-chitosan	5-FU and HYL	Release modulation of 5-FU and HYL to COLO-205 and HT-29 colon cancer cells
Gelatin-CMC	Alpha-amylase	Efficient carrier systems for the immobilization of alpha-amylase
Gelatin-alginate	Curcumin	Enhanced cytotoxic activity against MCF-7 cell line
Gelatin-gum Arabic	Jasmine oil	Stability enhancement against humid heat
Cationized gelatin-dextran or CS	pEGFP	Enhanced plasmid ocular delivery
Cationized gelatin-dextran or CS	pMUC5AC	Enhanced MUC5AC protein expression in conjunctival cells
α-La or β-Lg-chitosan	–	Successful fabrication of the nanohybrid system with potential applications for drug, food, cosmetic, or nutraceutical delivery
β-Lg-alginate	Quercetin	Enhanced the stability of quercetin threefold and modulated its release profile
β-Lg-pectin	DHA	Transparent nanohybrid systems with stability for DHA against oxidation
β-Lg-pectin	–	Cosolvents as glycerol and sorbitol could modulate nanohybrid properties formed by heat treatment
β-Lg-pectin	Pt(II) complex	Enhanced cytotoxicity against the colon cancer HCT116 cell line
β-Lg-pectin or carrageenan		β-Lg-pectin nanohybrids were more stable than their β-Lg-carrageenan counterparts against pH changes
β-Lg-KC or β-Lg-sodium alginate		Enhanced stability against aggregation
β-Lg-LMP	Oxali-palladium	A pH-sensitive targeted colon delivery of oxali-palladium was achieved
β-Lg-gum arabic or CMC		Enhanced stability against aggregation
WPI-inulin	Resveratrol	Intrinsic prebiotic activity with efficient encapsulation of resveratrol
WPI-alginate		Successful fabrication of the nanohybrid system with higher hydrophobic patches exposed for binding lipophilic drugs
Soy protein-soy polysaccharide	Curcumin	The nanohybrids enhanced the thermal stability of curcumin and modulated its release
Soy protein-soy polysaccharide	Folic acid	Enhanced folic acid protection and delivery in food and beverages
Soy protein-CMCS	Vitamin D3	Enhanced vitamin D3 loading, encapsulation efficiency, and release profile
Soy protein-KC	Quercetagetin	Improved light stability and the antioxidant properties of quercetagetin
Lf-pectin	–	Higher nanosystem thermal stability in comparison to Lf alone
Lf-pectin, carrageenan, or alginate	–	Improved stability at pH range 5–8
Lf-alginate or i-carrageenan	–	Alginate and i-carrageenan endowed Lf nanoparticles with higher stability against gastric digestion
Lf-pectin, i-carrageenan, or alginate	–	Enhanced stability against gastric digestion was demonstrated especially with i-carrageenan

Lysozyme-CS		CS endowed lysozyme with stability against degradation and biocompatibility
Lysozyme-CMC	5-FU	A more sustained drug release was achieved in simulated gastric fluid than in intestinal fluid
Lysozyme-CMC	MTX and QDs	Concomitant cancer imaging and therapy "theranostic nanohybrids" were developed
Lysozyme-CMC	–	The higher the substitution degree on CMC, the more is the negative charge and the stronger is the electrostatic interaction
Lysozyme-alginate	β-Lactamase	Prevention of the irreversible destruction of the enzyme with stimuli-responsive release
Casein-pectin	Vitamins	Successful incorporation of both hydrophilic and hydrophobic vitamins
Casein-pectin	Rutin	Enhanced stability in gastric conditions while releasing rutin in intestinal conditions
Casein-chitosan	–	Successful fabrication of stable soluble nanohybrid systems in the pH range 4.8–6.0
Protamine-HA	GFP-ODN	Enhanced cellular uptake and transfection efficiency of the GFP-ODNs
Protamine-HA	miR-34a	Enhanced delivery of miR-34a to breast tumor tissues
Protamine-heparin	Ferumoxytol	Enhanced cell labeling capability facilitating MRI monitoring
FSP-alginate		Successful fabrication of nontoxic stable nanohybrids
Zein-pectin		Successful fabrication of nanohybrids with potential emulsion stabilizing properties
Egg yolk LDL-pectin	Curcumin	Successful fabrication of nanohybrids with modulated curcumin release
Lactoferrin-chondroitin	DOX/ellagic acid	Superior internalization of the nanocomplex into lung cancer cells and hence enhanced antitumor efficacy in vitro and in vivo

BSA, Bovine serum albumin; *CMC*, carboxymethyl cellulose; *CMCS*, carboxymethyl chitosan; *CPs*, cocoa procyanidins; *CS*, chondroitin sulfate; *DHA*, docosahexaenoic acid; *DOX*, doxorubicin; *DTX*, docetaxel; *EGCG*, epigallocatechin-3-gallate; *FSP*, fish sarcoplasmic protein; *5-FU*, 5-fluorouracil; *GFP-ODN*, green fluorescent protein antisense oligodeoxynucleotide; *HA*, hyaluronic acid; *HYL*, hyaluronidase; *i-carrageenan*, iota-carrageenan; *KC*, κ-carrageenan; *LDL*, low-density lipoprotein; *Lf*, lactoferrin; *LMP*, low methoxyl pectin; *miR-34a*, a potent endogenous tumor suppressor microRNA; *MRI*, magnetic resonance imaging; *MTX*, methotrexate; *pEGFP*, plasmid for enhanced green fluorescent protein; *pMUC5AC*, plasmid for gel-forming mucin; *psiRNA-hH1GFPzeo*, vector for generating shRNA targeting GL3 luciferase; *Pt(II) complex*, bipyridine ethyl dithiocarbamate Pt(II) nitrate; *QDs*, quantum dots; *TRAIL*, tumor necrosis factor–related apoptosis-inducing ligand; *WPI*, whey protein isolate.

13.4.1.2 Core–shell complexation

Q30 This technique of PPC formation comprises two steps. In the first step the drug is loaded into gelatin or other protein nanocage by carefully adding ethanolic or isopropanolic solution of drug in gelatin buffer to facilitate coacervation. During coacervation, protein nanocage is formed and subsequently precipitated with the drug. In the second step, ~~T~~the~~n~~ ionized polysaccharide (dissolved in a buffer whose pH is strategically controlled for inducing required charge over the polymer) is coated over the protein nanocage. Both the electrostatically intercalated partners are further cross-linked with polylinkers such as tripolyphosphate (Tekade & Chougule, 2013) to induce stability and architecture of the PPC cavity. Gemcitabine, an anticancer drug, was successfully loaded into bovine serum albumin (BSA) shell–based nanocage, which was further wrapped with ionized chitosan and cross-linked with tripolyphosphate.

Q31 Lactoferrin (Lf), a popular globular glycoprotein having a molecular mass of 80 kDa, is often used to form core–shell complexes by heat denaturation of the same and precipitation with loaded drug to form core nanocage. Then the core cage is complexed with various carbohydrate polymers such as chitosan or carrageenan converted to their ionic form. Because lactoferrin has pI as high as 8.5, lowering medium pH anywhere below this value renders the protein to be electropositive. Now choosing a partner polysaccharide whose pI is below the medium pH is a key to this engineering. As subjecting the polysaccharide to that pH induces a net negative charge on it, electrostatic precipitation is favored between contra-ionic protein and polysaccharide, forming desired protein-polysaccharide complex or cage. A wide range of such complexes can be engineered by varying the medium pH below 8.5 and choosing various carbohydrates as per their pI (<medium pH).

Furthermore, another interesting engineering of protein-polysaccharide nanocage is to lay down alternative layers of protein and polysaccharides on previously drug-loaded nanocages. Gaber et al. (2018) reported that cationic lactoferrin (Lf) and anionic hyaluronate (HA) could be used to coload rapamycin and berberine with this technique of alternative wrapping. The thus formed complexes achieved better targeting and release against A549 lung cancer cells overexpressing CD44 receptors (Fang, Elkhodairy, & Elzoghby, 2018). Although the size, shape, and polymorphism of the protein-polysaccharide nanohybrid could be varied infinitesimally by changing the biopolymer ratio and the pH of the solution, one of the most influencing engineering techniques of forming such nanocomplex/cage has been the exploitation of ionic gelation. Addition of $CaCl_2$ has been reported to improve cross-linking between protein and polysaccharide such as between carboxymethyl chitosan and soya protein isolate (SPI), which has been otherwise impossible even by varying other parameters of the process (Teng et al., 2013). The Ca^{2+} ion bent bulky and conformationally rigid sugar groups by strong electrostatic interaction, increased the proximity between protein and polysaccharide and reinforced the bridge formation between the same. The authors reported that varying $CaCl_2$ and SPI concentration in the formulation (SPI act as buffer to eliminate the overdose of calcium), the size and stereochemistry of the PPC might be controlled.

13.4.2 Chemical reaction–mediated complexes/cages

The abundance of functional groups such as hydroxyl, carboxylic, or thiol groups over the proteins and the polyhydric alcohols over the carbohydrates provides opportunities to scientists for coupling the two reactive terminals of candidate polymers through chemical reaction. Often the addition of condensing agent or cross-linkers aids the reaction and facilitates the bonding. The main techniques of chemical conjugations have been discussed in the following sections.

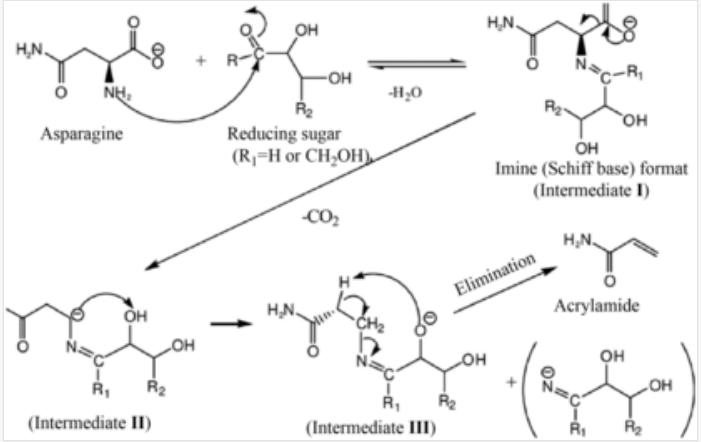
13.4.2.1 Chemical coupling

Chemical coupling is the technique of PPC formation where the protein and polysaccharide are interlinked in respective points by simple chemical reaction. This reaction often requires addition of some dehydrating or condensing agent in the medium. For example, the chemical coupling between alginic acid and BSA occurs by means of L-cysteine, which act as a cross-linker in this reaction. The cysteine first transfers its thiol (−SH) group into alginate by a reaction between −COOH groups of the latter and free amino terminals of the former. Later, a disulfide linkage (−S−S−) is introduced between cysteine and BSA by proportionate reaction between cysteine-alginate hybrid and the protein with the catalysis of β-mercaptoethanol. Drugs such as tamoxifen (Martínez, Muñiz, Iglesias, Teijón, & Blanco, 2012) and 5-fluorouracil (5-FU) have been successfully delivered through PPCs engineered by chemical coupling (Yi, Yang, & Pan, 1999).

13.4.2.2 Maillard reaction

Maillard reaction is a reaction that utilizes the amino terminal situated at distant position from α-carbon in a suitable amino acid. Such a kind of amino terminal reacts with free carbonyl terminal of suitable carbohydrates to first form a Schiff's base (imine) intermediate. Later this intermediate undergoes decarboxylation and intramolecular Amadori type rearrangement to yield favored amino acid-carbohydrate cage-like complex. For example, $-NH_2$ terminal at ε-position of lysine residue participates in this reaction, although indole group of tryptophan, imidazole of histidine, and guanidine of arginine may also take part in this reaction (Hodge, 1953). Later asparagine has also been revealed to exhibit this reaction. A schematic ~~coupling~~diagram of this coupling reaction is shown in Fig. 13.6.

Figure 13.6



A schematic representation of Maillard reaction (Krishnakumar & Visvanathan, 2014).
Source: From Krishnakumar, T., & Visvanathan, R. (2014). Acrylamide in food products : A review. Journal of Food Processing & Technology, 5, 7.

The pivotal technology in this process depends on three steps: initial addition of 0.1 N NaOH with protein-polymer solution, drying it either by spray drying or lyophilization, and further allowing the reaction at 50°C–60°C with 78% relative humidity. SPI-maltodextrin (MD) nanocomplex encapsulating eugenol (Shah, Davidson, & Zhong, 2012) or Maillard casein-MD nanocage entrapping vitamin D has been formulated by this method (Markman & Livney, 2012). To further improve the stability of the system, thermal gelation or ionic cross-linking–based engineering has been used. In thermal gelation, first charge-guided ionic interaction takes place between protein and polysaccharide as described earlier, later precipitating the complex with heating at 80°C. This technique has been successfully exploited to fabricate trinomial biocage comprising chitosan-BSA-dextran. In this process, first the BSA forms core–shell, chitosan is partially entrapped in the core–shell of BSA by ionic interaction. Later the complex is extended by the interaction with dextran through Maillard reaction (Qi, Yao, He, Yu, & Huang, 2010). Similarly glutaraldehyde cross-linked ibuprofen-loaded BSA-dextran conjugate (Li & Yao, 2009), sodium tripolyphosphate cross-linked catechin-loaded BSA-chitosan nanocage, tea polyphenols-loaded gelatin-dextran nanoboat cross-linked with genipin are also reported (Zhou et al., 2012).

In this chemically coupled PPCs, drugs such as ibuprofen, doxorubicin, tamoxifen, 5-FU, β-carotene, curcumin, vitamin D, epigallocatechin-3-gallate, curcuminoids, catechin, eugenol, and tea polyphenols have been reported to be delivered via BSA-dextran, BSA-dextran, BSA-alginate, BSA-alginate, casein-dextran, casein-dextran, casein-dextran, Q35 casein-MD, α-La-dextran, ~~wheat~~whey protein isolate (WPI)-chitosan, WPI-MD, gelatin-dextran PPCs, respectively, predominantly for improving stability, pH-dependent release, improved cell uptake, and enhanced encapsulation efficiencies (Fang et al., 2018).

13.4.3 Electrospun nanohybrid–mediated complexes/cages

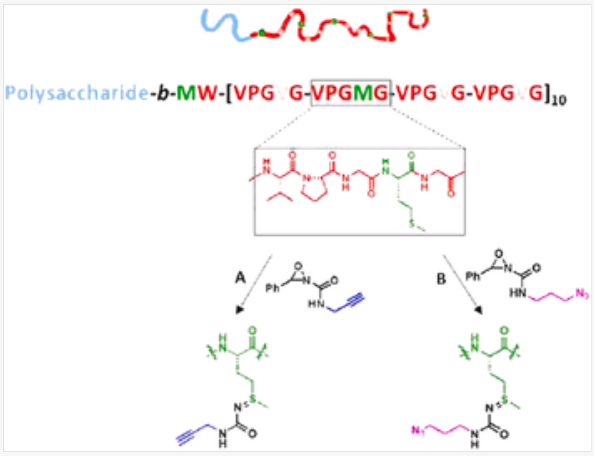
Electrospinning is a technology that accrues the combinatorial effects of hydrodynamic force of solution and high-voltage electric field applied on it. As a result, first a thin jet of solution forms, which spins down the lane when ejected as a jet of viscous stream. Explicitly, the viscous liquid containing the nanomaterial is ejected through a fine nozzle where a strong electric field is applied. Under this electric field, the liquid droplet forms a cone known as Taylor cone guided by its surface tension, and then falls down in the form of thin jets of nanometer dimension. Subsequently they are rapidly cooled to form nanofibers (Ashammakhi, Wimpenny, Nikkola, & Yang, 2009; Valizadeh & Farkhani, 2014). With this technology, egg albumin-cellulose acetate nanofiber, gelatin-microcrystalline chitosan, and zein-

microcrystalline nanofibers have been formed, which have been acknowledged for drug delivery (Wongsasulak, Patapeejumruswong, Weiss, & Supaphol, 2010; Zhuang, Cheng, Kang, & Xu, 2010).

13.4.4 Posttranslational modification–aided protein-polysaccharide block copolymer complexes/cages

PPCs could be engineered tactfully in a stimuli-responsive manner, the stimuli being any of pH, temperature, redox environment, soluble oxygen, and light. Xiao (2019) investigated this engineering with a special protein called elastin. The author recombinantly expressed elastin, posttranslationally modified it via chemical modifications, and lastly joined it with polysaccharide counterparts such as chitosan, hyaluronic acid, or dextran by "click chemistry" (Fig. 13.7). The author reported that elastin has an inverse transition temperature (Tt) below which it remains soluble and above which it forms an insoluble aggregate. This property could be further tuned by exploiting the presence of methionine residue in its reading frame and chemically modifying it with oxaziridine. This rendered the polysaccharide-b-elastin bioconjugate more susceptible to temperature change, as its T_t became finer and more sensitive to temperature change. The author acknowledged that this engineered protein-polysaccharide complex could successfully deliver drug in a temperature-responsive manner within specific microenvironment inside the body. The author also mentioned that strategic hybridization of the surface with CD44 receptors, which is highly expressed in a plethora of cancer cells, could render Q36 the device target-specific and operative in tumor microenvironments (116).

Figure 13.7



Q47 Chemoselective posttranslational modification of elastin-like protein hybridized with polysaccharide.
Source: Reprinted from Krishnakumar, T., & Visvanathan, R. (2014). Acrylamide in food products : A review. Journal of Food Processing & Technology, 5, 7 with creative common license.

Several generalized advantages of PPCs in drug delivery and theranostics applications are summarized in Table 13.3.

Table 13.3

The table layout displayed in this section is not how it will appear in the final version. The representation below is solely purposed for providing corrections to the table. To preview the actual presentation of the table, please view the Proof.

Q53 A summary of advantages of protein-polysaccharide cages obtained using techniques other than electrostatic precipitation (Fang et al., 2018).

Drug	Protein-polysaccharide complex	Major outcome
Docetaxel	Albumin-hyaluronic acid complex	Improved targeting toward SPARC and CD-44-expressed tumors.
Docetaxel	Albumin-CMC complex	Improved targeting toward SPARC-expressed tumors.
miRNA	miR-34a-loaded HA-protamine nanohybrid complexes	Improved cellular uptake in triple-negative breast cancer (MDA-MB-231) cells.

Rhodamine	Rhodamine-labeled dextran lysozyme nanogels	Improved biocompatibility.
Curcumin	Soy polysaccharide-soy protein nanohybrids	Slow and controlled release. At pH 4 or 7, 50%–60% drug released against ~90% from soy protein nanoparticles after 20 h.
Curcumin	i-Carrageenan-albumin, α-lactalbumin-dextran, soy polysaccharide-soy protein	Improved stability.
Vitamin D	Hydrophobic core of casein-maltodextrin nanohybrids	Improved stability.
EGCG	Matrix of dextran-casein	Improved stability, prevention of destruction of galloyl group.
DOX	Albumin-dextran	Reduced DOX toxicity.
Q54 DOX	Zein-chitosan hybrid nanofibers electrospun from TFA solution	Intrinsic antimicrobial activity.
Q55 CdTe QDs	Lysozyme-carboxymethyl cellulose complexes	Prolong fluorescence signal as imaging ~~agent~~tool from HepG2 and MCF-7 cancer cells.
Cy3-labeled plasmid DNA-loaded nanohybrids	Gelatin-based PPC	Enhanced ocular delivery and stability of DNA.

CMC, Carboxymethyl cellulose; *DOX*, doxorubicin; *EGCG*, epigallocatechin-3-gallate; *PPC*, protein-polysaccharide cage; *QDs*, quantum dots; *SPARC*, secreted protein, acidic, and rich in cysteine; *TFA*, trifluoroacetic acid.

13.5 Conclusion and future perspectives

Q37 Myriads of drug delivery systems are emerging every year in compliance with the need of the intricate physiological disorders present in mankind, to meet their criticalities, improving the treatment protocols, patient compatibility, reduce toxicity and pro-augmenting the therapeutic efficacy of earlier delivery regimens. As a subset of this, improving pharmacokinetics, prolonging the drug action, improving biocompatibility, target-specific delivery, and controlled release at the disease microcosmic environment have been in crying need of the medical communities over the last few decades. Protein and PPCs have been the two effective solutions to meet each of the demand in biomedical fraternities where engineering those carriers with genetic mutation, PTM, chemical functionalization, interspersing with smart or stimuli-responsive polymers, and tagging target-specific ligands have scaled these delivery systems to a new height to combat physiological disorders more efficiently. We hope this review will unravel the potential routes of accomplishing such delivery systems to investigate further manipulation of protein and protein-polysaccharide-based delivery systems for meeting such demands. Use of smart adjuvants and supramolecular protein complexes with synthetic and semisynthetic biopolymers, using metal activators, constructing protein-based dendrimers, recombinantly manufacturing protein-based vaccines, constructing protein-based polynomial drug delivery systems, exploiting toxin-antitoxin dynamics of natural or synthetic protein–based nanocages for healing infectious diseases, coupling PC with carbon nanomaterials for achieving better cellular penetration with delivery, fabricating protein-based capsosomes for multicompartment reactions in vivo are only a few of the immense possibilities of exploiting such protein or protein-polysaccharide-based drug delivery systems in future, which may take biomedical science to a nextgen platform in the 21st century's world.

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The corrections made in this section will be reviewed and approved by master copier.

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Further reading

The corrections made in this section will be reviewed and approved by master copier.

Manuscript A. Catalysis Science & Technology. 2013;(207890).

Q44

Abstract

Protein and protein-polysaccharide cages (PPCs) have emerged as a promising tool for drug delivery in the last few years. The primary criterion of selecting protein as a carrier is its biocompatibility, which ensures safe delivery of its cargo without eliciting adverse immunogenic response. Second, due to its properties such as unique sequences, secondary and tertiary structures, folding, clustering, domain-to-domain interactions, entangling between its chains with ionic, covalent, van der Waals, or hydrogen bonding interactions, and casting bridges between its multimers, protein leverages a unique display of cage-like structures that could be used as host to accommodate guest drug molecules with improved stability. Also, PPCs have evolved as unique nanohybrids that allow novel interactions with guest molecules and wrap them with firm interactions vis-à-vis binding forces. When attributed to such kind of payloads, protein cages (PCs) and PPC could be exploited to deliver small to macromolecular drug molecules, control their release inside body, and deliver them in either stimuli-responsive or target-specific manner. These cages may further be engineered by metal-guided intra or intermeric cross-linking, metal lacing, chemical-guided disulfide or amide bridge formation, ligand-guided epitope appending, or aptamer-tagged target furnishing. Such kind of engineering could be employed to shape the PC for improving cage stability, cavity architecture, target specificity, cellular uptake, and release property. In this chapter, we have presented all traditional PCs, their architectures, release properties, engineering approaches, and their biomedical applications. In addition, comparatively newer engineering approaches such as posttranslational modification of proteins to tune their in vivo properties have also been discussed. The perturbations of PPC in drug delivery and therapeutic applications have also been reviewed. It is noteworthy that various engineering approaches such as electrostatic complexation, chemical coupling, and electrospinning have been exploited in contemporary ages to engineer PPCs for improving their intracellular uptakes, loading capacity, in vivo drug

Q1

release as well as stability. A succinct and magnanimous highlight of all these manipulations has been a major chord in this chapter, especially focusing their applications in recent days of biomedical world.

Keywords: Protein cages; protein-polysaccharide cages; multimer; chemical coupling; electrospinning

Queries and Answers

Q1

Query: Please check the sentence “A succinct and magnanimous highlight of all…” for clarity, and correct if necessary.

Answer: It is okay.

Q2

Query: Please check the sentence “Keeping with the tremendous onslaught on…” for clarity, and correct if necessary.

Answer: It is okay

Q3

Query: Please check the phrase “has emancipated newer horizons on protein applications in biomedical fraternity” for clarity, and correct if necessary.

Answer: It is okay

Q4

Query: Please check the sentence “Furthermore, the stepping up of PCs by…” for clarity, and correct if necessary.

Answer: It is okay

Q5

Query: Please check the sentence “In this chapter, we summarize the updated approaches in this domain involving synopsis…” for clarity, and correct if necessary.

Answer: it is okay

Q6

Query: Please advise whether “another prima face” can be changed to “prime focus.”

Answer: ok. please change to "prime focus"

Q7

Query: Please check the phrase “take place in cellular functions” for clarity, and correct if necessary.

Answer: it is okay

Q8

Query: Please check the sentence “It may be of short sized like insulin…” for clarity, and correct if necessary.

Answer: it is okay

Q9

Query: Please clarify whether it is “sHSP” or “HSP” in the sentence “Kim et al. elucidated ….”

Answer: it is okay

Q10

Query: Please check the sentence “It contains 147 amino …” for clarity, and correct if necessary.

Answer: it is okay

Q11

Query: Please check the sentence “Vaults make barrel-shaped nanocages by…” for clarity, and correct if necessary.

Answer: it is okay

Q12

Query: Please note that both “Geobacillus stearothermophilus” and “Bacillus stearothermophilus” have been used in the text. Kindly advise which one has to be used consistently.

Answer: Please keep "Geobacillus stearothermophilus"

Q13

Query: Please confirm edits to the sentence “Cowpea mosaic virus (CPMV) …” and correct if necessary.

Answer: it is okay

Q14

Query: Please check the phrase “spanning of domain over another” for clarity, and correct if necessary.

Answer: it is okay

Q15

Query: Please check the phrase “To further advancement of this de novo designed protein nanocages” for clarity, and correct if necessary.

Answer: it is okay

Q16

Query: Please confirm edits to the sentence “Using system thermodynamics …” and correct if necessary.

Answer: it is okay

Q17

Query: Please provide expansion for PEG, RGD, NHS, HBV, HPV, and OCT at their first mention in the text.

Answer: Thanks. All full extentions are now given inside text except RGD. Since RGD is a standard peptide name, keeping as such is fine for it.

Q18

Query: Please confirm edits to the sentence “Interestingly, gold has …” and correct if necessary.

Answer: It is okay

Q19

Query: Please check the sentence “The pores present in …” for clarity, and correct if necessary.

Answer: It is okay

Q20

Query: Please check the sentence “Due to this effect, the …” for clarity, and correct if necessary.

Answer: It is okay

Q21

Query: Please check the phrase “which in turn helps in innervations in specific…” for clarity, and correct if necessary.

Answer: It is okay

Q22

Query: Please check the sentence “Doxorubicin was first complexed with…” for clarity, and correct if necessary.

Answer: It is okay

Q23

Query: Please check the phrase “judicial snapshot” for clarity, and correct if necessary.

Answer: It is okay

Q24

Query: Please confirm edits to the sentence “Protein-based drugs or therapeutics can…,” and correct if necessary.

Answer: It is okay

Q25

Query: Please check the sentence “As other notable applications, enzymes such as…” for clarity, and correct if necessary.

Answer: It is okay

Q26

Query: Please check the phrase “therapeutic cum magnetic imaging machinery” for clarity, and correct if necessary.

Answer: It is okay

Q27

Query: Please confirm edits to the sentence “Engineering PCs with various crosslinking agents…,” and correct if necessary.

Answer: corrected inside text (a few grammatical corrections)

Q28

Query: Please advise whether “Lf” can be used as abbreviation for “lactoferrin” throughout the text.

Answer: Yes, it can be used

Q29

Query: Please check the sentence “Various drug deliveries involving curcumin, Resveratrol…” seems to be incomplete.

Answer: Thanks for pointing it out. Now it is completed inside text after the word 'rutin'

Q30

Query: Please note that there is a mention of two steps in the sentence “This technique of PPC formation comprises…,” but only the first step is described here. Kindly check .

Answer: Thanks for the comment. Now it is edited and the second step is inserted (4th line of the paragraph under the head "Core-shell complexation"

Q31

Query: Please check the sentence “Then the core cage is complexed with various…” for clarity, and correct if necessary.

Answer: It is okay

Q32

Query: The reference given here is cited in the text but is missing from the reference list – please make the list complete or remove the reference from the text: Gaber et al. (2018); Xiao (2019).

Answer: Please add the following Reference of Gaber et al 2018.

Gaber, M., Mabrouk, M.T., Freag, M.S., KHiste, S.K., Fang, J.-Y., Elkhodairy, K.A., Elzoghby, A.O. (2018). Protein-polysaccharide nanohybrids: Hybridization techniques and drug delivery applications. European Journal of Pharmaceutics and Biopharmaceutics, 133, 42-62.

Please omit the Xiao 2019 ref if present.

Q33

Query: The abbreviation "HA" has been expanded inconsistently in the text as "hyaluronate" and "hyaluronic acid". Please check and correct as necessary.

Answer: Hyaluronate is just the ionic form of hyaluronic acid which is produced natively. So scientifically, both are ok.

Q34

Query: Please confirm edits to the sentence "A schematic coupling of this reaction…," and correct if necessary.

Answer: Thanks for the comment. Now it is corrected inside text

Q35

Query: The abbreviation "WPI" has been expanded inconsistently in the text as "wheat protein isolate" and "whey protein isolate". Please check and correct as necessary.

Answer: Thanks for the comment. Now it is corrected to whey protein isolate inside the text.

Q36

Query: Please note that reference (116) is cited in the text but is missing from the reference list – please make the list complete or remove the reference from the text.

Answer:

Please insert this reference as Ref 116.

Ye Xiao (2019). Synthesis and self-assembly of polysaccharide-b-elastin-like polypeptide bioconjugates. Polymers. Université de Bordeaux, 2019. English. ffNNT : 2019BORD0172

Q37

Query: Please check the sentence "Myriads of drug delivery systems are emerging…" for clarity, and correct if necessary.

Answer: It is okay.

Q38

Query: Please check the sentence "As a subset of this, improving pharmacokinetics…" for clarity, and correct if necessary.

Answer: It is okay.

Q39

Query: Please check the sentence “Protein and protein-polysaccharide cages…” for clarity and correct if necessary.

Answer: It is okay.

Q41

Query: Please provide the complete bibliographic details of Bruno et al. (2013), Choi et al. (2011), Malay A.D., Miyazaki, et al., “Manuscript A. Catalysis Science & Technology. 2013;(207890)”, Member et al. (2016), “Protein structure. Nature. 1941;147(3734):646.”.

Answer:

Please use the following references:

Bruno, B.J., Miller, G.D., Lim, C.S. (2013). Basics and recent advances in peptide and protein drug delivery. Therapeutic Delivery 4(11), 1443-1467

Choi K-m., Choi, S-H., Jeon, H., Kim, I-S, Ahn, H. J. (2013). Chimeric capsid protein as a nanocarrier for siRNA delivery: stability and cellular uptake of encapsulated siRNA. ACS Nano 5(11), 8690-8699

Malay, A.D.; Heddle, J.G.; Tomita, S.; Iwasaki, K.; Miyazaki, N.; Sumitomo, K.; Yanagi, H.; Yamashita, I.; Uraoka, Y. Gold Nanoparticle-Induced Formation of Artificial Protein Capsids. Nano Lett. 2012, 12, 2056–2059

Please delete eny other extraneous references

Q44

Query: Further reading section contains references that are not cited in manuscript text. Please cite the references or confirm if we could delete it? Further reading will be retained if the references are not cited or deleted.

Answer: You may delete extra references.

Q45

Query: Please check the details of the credit line of Figs. 13.3, 13.5, and 13.6 for correctness.

Answer: They are correct

Q46

Query: Please provide expansion for RGD, PEG, PCL, PLGA, and PGA in the caption of Fig. 13.4.

Answer: RGD: keep as such since it is the peptide name

PEG: Polyethylene Glycol

PCL: Polycaprolactone

PLGA: Poly Lactic-co-Glycolic Acid

PGA: Polyglycolic Acid

Q47

Query: Please provide the better quality image for Fig. 13.7.

Answer: Please excuse me as better quality image than this is not available

Q48

Query: Please confirm edits to Table 13.1, and correct if necessary.

Answer: It is okay

Q49

Query: Please check the phrase "Also devoid of major immune response" in Table 13.1 for clarity, and correct if necessary.

Answer: It is perfectly okay

Q50

Query: Please note that we have used Table 13.2 given in the manuscript and ignored the one given in xlsx format. Kindly check and confirm.

Answer: It is okay

Q51

Query: Please check the entry "Significant cytotoxicity enhancement in HepG2 cells…" in Table 13.2 for clarity, and correct if necessary.

Answer: It is okay

Q52

Query: Please provide expansion for GEM, α-La, and β-Lg in Table 13.2.

Answer:

Gem- Gemcitabine

alpha La-alpha lactalbumin

beta-Lg- beta lactoglobulin

Q53

Query: Please confirm edits to Table 13.3 caption, and correct if necessary.

Answer: Correct

Q54

Query: Multi-hyphens have been replaced with "DOX". Please check and correct if necessary.

Answer: It is okay

Q55

Query: Please check the entry "Prolong fluorescence signal as imaging agent…" in Table 13.3 for clarity, and correct if necessary.

Answer: imaging agent has been replaced by imaging tool