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NANOSCALE SELF-ASSEMBLY FOR DELIVERY OF THERAPEUTICS AND IMAGING AGENTS

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Abstract

Self-assemblies are complex structures spontaneously organized from simpler subcomponents, primarily through noncovalent interactions. These structures are being exploited as delivery vehicles of therapeutic and imaging agents. They have two unique advantages in comparison to other vehicles: 1) they are able to assume complex structures that are difficult to attain by chemical synthesis, and 2) the dissociation of self-assembled structures can be triggered by external stimuli, which can serve as a mechanism of payload release. In this review, we discuss two naturally occurring (proteins and viral capsids) and five engineered self-assemblies (vesicles, micelles, proteins, hydrogels, and inclusion complexes) that are under development for delivery of drugs and imaging agents. For each class of self-assembled supramolecular structures, we examine its structural and physicochemical properties, and potential applications within the context of assembly, loading, and payload release.

Keywords

Self-assembly; Vesicle; Micelle; Protein; Hydrogel; Inclusion complex

INTRODUCTION

Self-assembly is a process where components interact with each other and form ordered structures or patterns without external guidance. In this review, the term “self-assembly” is also used to identify structures resulting from self-assembling processes. Self-assembly occurs on many different length scales: nanoscale, mesoscopic, and macroscopic, depending upon the size of the components (150). This review focuses on nano-mesoscale self-assembled systems of molecular components that have been developed for delivering therapeutic or diagnostic agents.

Nanoscale carriers can be useful for the delivery of diagnostic and therapeutic agents by: 1) improving their solubility, if the agents have poorly aqueous solubility; 2) improving their biodistribution and pharmacokinetics; 3) providing a platform for the attachment of targeting moieties so that these agents can be directed to specific tissues and cells; 4) enabling macromolecular agents to cross membrane barriers; and 5) enabling combination therapies or theranostics by co-delivering combinations of drugs and imaging agents (41,42,85).

Self-assembled nanoscale carriers have two attributes that make them useful for the delivery of imaging agents and drugs. First, molecular self-assembly offers a simple “bottom-up” method to fabricate nanoscale carriers with a specified shape and size from molecular components. In many instances, the molecular components can be readily produced in large quantity by chemical recombinant synthesis, such that bottom-up fabrication of the nanoscale constructs in solution by self-assembly is an attractive route for their cost-effective manufacturing. Second, self-assembly can provide a mechanism to release a payload by dilution or in response to an external trigger. This feature of self-assembly is particularly important for drug delivery, as most payloads need to be released to reach their physiological targets.

BIOMOLECULAR SELF-ASSEMBLY IN NATURE

Because life is dynamic, self-assembled structures play an essential role in life. Many biological events (e.g., molecular recognition, the cell cycle, tissue remodeling during development) rely on structures that are temporally dynamic (105,146). These structures need to assemble or disassemble based on environmental changes and physiological need. For example, in eukaryotes, the completion of the cell cycle requires the nuclear membrane to disassemble and re-form (105). Self-assemblies meet these requirements because they are built through reversible processes, yet they support defined structures and stable functions. Noncovalent bonding is the key to self-assembly, as it allows structures to be formed, while facilitating the rapid disassembly into subcomponents once the structures are no longer required.

Nanoscale biomolecular self-assemblies can be categorized into two groups: intramolecular and intermolecular assemblies. Intramolecular assemblies such as proteins are three-dimensional structures folded from single linear precursors. Intermolecular assemblies such as the cell membrane and viral capsids require the organization of many molecules. Both of these groups of self-assembled structures have been exploited as delivery vehicles (Fig. 1), and are hence discussed in this review.

Proteins play two distinct roles in drug delivery: as payloads and carriers. As a therapeutic payload, proteins can be encapsulated within a self-assembled structure, conjugated to a nanoscale carrier, or used directly without any attachment or encapsulation in a self-assembled structure (94). Proteins can also be used as carriers of other drugs and imaging agents (46,74,141). For example, human serum albumin (HSA) has been used as a vehicle for the delivery of drugs (Table 1) because of its extraordinarily long half-life of ~19 days in humans. It has been used in a variety of formulations that have made it to clinical trials including a methotrexate–HSA conjugate (58), a noncovalently associated paclitaxel–HSA nanoparticle called Abraxane (75), and a glucagon-like peptide–HSA fusion protein (71).

Naturally occurring, intermolecular self-assemblies have also been used as carriers. The most common examples include bacteriophage and viral capsids such as cowpea chlorotic mottle virus (CCMV) (36,95), cowpea mosaic virus (CPMV) (81,97), hibiscus chlorotic ringspot virus (HCRSV) (121), phage M13 (12), and phage Q (138). Among these, CCMV is the most thoroughly characterized virus for the delivery of drugs and imaging agents. This virus is composed of an icosahedral capsid consisting of 180 identical 20-kDa protein units. The capsid undergoes reversible pH-dependent swelling, resulting in the formation of 60 separate 20 Å pores that provide a convenient mechanism for the loading and release of drugs and other agents (136). This loading mechanism has been verified through the encapsulation of the imaging contrast agent Gd^{3+} and a 9-kDa anionic polymer (poly-anetholesulphonic acid) (36,95).

Although the chemical components, sizes, and shapes of these biological self-assemblies are diverse, they share a number of principles (18,31) that can be applied to the design of synthetic self-assembled structures:

1. Biological self-assemblies are generally the product of concerted, multiple weak forces such as the hydrophobic effect, hydrogen bonding, Van der Waals interactions, and electrostatics (31). On the other hand, while these weak non-covalent bonds are critical for self-assembly, it remains a challenge for biomolecular engineers to simultaneously optimize more than two of these interactions in engineered self-assembled systems.
2. There is a defined hierarchy in biological self-assemblies (18). In the case of viral capsids, polypeptides assemble into proteins, which in turn organize into the capsid shell. This hierarchical organization permits the use of simple, chemically synthesized building blocks to construct assemblies of great complexity.
3. Biological self-assemblies demonstrate self-healing behavior. Following disruption, the self-assembled structure is able to return to its ordered state, provided that it is the most thermodynamically stable state (18).
4. Biological self-assembling processes are dynamic (18). These dynamic self-assemblies provide organisms with a capacity to respond to environmental cues or to exhibit complex behaviors.

Biological self-assemblies, the accumulated products of evolution, provide inspiration for man-made self-assembled structures, which are discussed next in this review.

ENGINEERED SELF-ASSEMBLY ON THE NANOSCALE

In the past decade, there has been a rapid increase in the number of materials designed to deliver diagnostic or therapeutic agents. Some of these materials self-assemble into nanostructures that are used for delivery (Table 2). Based on their structural characteristics and the forces that drive self-assembly, these nanoscale self-assemblies can be categorized into five groups: vesicles, micelles, proteins, hydrogels, and inclusion complexes (Fig. 2). The first two groups are intermolecular self-assemblies of polarized amphiphiles—molecules in which the hydrophobic and hydrophilic segments are oriented linearly along the length of the molecule. Proteins, on the other hand, are intramolecular assemblies of polypeptide chains that fold into a defined three-dimensional structure. Most polypeptides are not polarized amphiphiles; however, folded proteins or individual protein domains typically have a mosaic surface where hydrophobic patches are embedded in a sufficiently polar background to enable their folding into a structure that is well solvated and hence soluble in water. Proteins that have tertiary structures can further exhibit hierarchical self-assembly, wherein folded subunits can self-assemble into multisubunit protein complexes. Hydrogels can self-assemble from polarized amphiphiles or from hierarchical self-assembled complexes (proteins, nanofibers, polysaccharides, etc.). Inclusion complexes are a distinct group of self-assembled carriers, because they are the product of intermolecular interactions between complementary chemical moieties. Inclusion complexes can be used either to link payloads to the carrier or to provide specific structural attachments (128).

Vesicles

Vesicles are hollow spheres with a bilayer membrane that are formed by the association of amphiphilic molecules (10,60,73) (Fig. 2). An amphiphilic molecule consists of two covalently linked blocks: a water-soluble (hydrophilic) block and a water-insoluble (hydrophobic) block. With the appropriate balance between these blocks, these amphiphiles can spontaneously self-assemble into tightly packed bilayers. Hydrophobic blocks from each

layer associate with each other to form a tightly packed, hydrophobic inner core that is sandwiched between two hydrophilic blocks, which are in contact with the aqueous environment. Depending on the chemical identity of the amphiphilic molecule, vesicles can be classified as liposomes or polymersomes. Liposomes are composed of primarily lipid monomers, whereas polymersomes are assembled synthetic block copolymers, or polymer–polypeptide hybrids.

Liposomes

Other than proteins, liposomes remain the most widely used nanoscale drug delivery vehicles in the clinic (10,127). Commonly used drug-loaded liposomes are generally 50–200 nm in diameter, but liposomes can range from about 30 nm to several microns in diameter. Liposomes can have as few as a single lipid bilayer (unilamellar) or can contain multiple bilayers (multilamellar). The bilayer characteristics and sizes of liposomes define four basic subgroups: multilamellar vesicles (MLV), giant unilamellar vesicles (GUV), large unilamellar vesicles (LUV), and small unilamellar vesicles (SUV) (Fig. 2). MLVs are prepared by hydrating dried lipids at a temperature above their characteristic melting point (131). GUVs can be generated by a slow gentle hydration of a very thin lipid film (120). MLVs or GUVs can be converted to SUVs through ultrasonication, which promotes the formation of liposomes with a minimum stable radius of curvature (64). Alternatively, polydisperse MLVs can be converted to LUVs by high-pressure homogenization and extrusion through narrow pores (24). In addition to these approaches, there are many methods to control the liposome diameter, encapsulation degree, and lamellar nature, as a function of the liposome cargo and end use.

Liposomes offer many of the common benefits of nanoscale carriers. In addition, liposomes can enhance cellular uptake of their payload by fusion of their lipid bilayer with the cell membrane, thereby releasing their payload directly into the cytosol (39). Several liposome-based delivery systems have been approved by the FDA for cancer treatment. Doxil, for example, is a liposomal formulation of the cancer chemotherapeutic doxorubicin. Doxorubicin is a potent chemotherapeutic that is used to treat breast and esophageal carcinomas, Hodgkin's and non-Hodgkin's lymphomas; however, it suffers from severe cardiotoxicity and poor local bioavailability in tumors (133). Doxil—a liposomal formulation of doxorubicin—improves the pharmacokinetics and biodistribution of the drug, thereby alleviating its side effects (110). Doxil has been approved by the FDA to treat Kaposi's sarcoma and ovarian cancer (16). Liposomes have also been exploited to deliver agents for the treatment of eye disorders and infectious diseases (49), facilitate the transport of drugs across the blood/brain barrier (BBB) (127), and deliver magnetic resonance imaging (MRI) contrast agents such as chemical exchange saturation transfer (CEST) agents (132) and radionuclides for single photon emission computed tomography (SPECT) and positron emission tomography (PET) (152).

Although liposomes have seen some clinical success, much remains to be done to further enhance their clinical utility. Avenues to optimize this class of nanoscale carriers include: 1) optimization of their formulation and fabrication procedures to improve their stability and reduce drug leakage [one approach to do so incorporates 3% (v/v) Tween 80 in the formulation and uses sucrose as a lyoprotectant during the freeze-drying fabrication procedure] (157); 2) invention of new formulations—such as stealth liposomes—to improve the in vivo pharmacokinetics and biocompatibility of liposomes (73,113); 3) modification of liposome surfaces with targeting ligands or antibodies to realize active targeting (112,153,154); and 5) expansion of the application of liposomes to deliver biomolecular therapeutics, such as proteins and oligonucleotides (26,156). All of these research directions are crucial to allow more liposome-formulated drugs and imaging agents to move into the market. As liposomes have a history of success and continue to generate a significant

amount of interest, it is reasonable to believe that liposomes will account for a significant fraction of self-assembled vehicles used for the clinical delivery of diagnostic and therapeutic agents in the foreseeable future.

Polymersomes

Polymersomes are vesicles composed of amphiphilic block copolymers. While polymersomes and liposomes form similar structures, polymersome bilayers are more stable and less fluid than those of liposomes. These differences stem from the thickness and adjustable permeability of the bilayers: polymersome bilayers are 8–21 nm thick whereas liposomes are only 3–5 nm (7,15). The thicker polymersome bilayers are the result of the large molecular weight amphiphilic blocks used to assemble polymersomes (2–20 kDa). In contrast, the molecular weight of lipids comprising a bilayer are less than 1 kDa (7,11). The physical properties of polymersomes are very similar to viral capsids, as both structures are robust, stable, and have low permeability (35). Because polymersomes are composed of synthetic block copolymers, the rich diversity of synthetic polymer chemistry can be harnessed to design polymersomes whose assembly and disassembly can be triggered by external stimuli. The most common method used to drive polymersome formation is to dissolve the copolymer in one solvent, then add a second solvent drop-wise, which selectively dissolves one block of the copolymer into the first solvent (158). Other methods include the direct rehydration of polymer thin films, the use of water–oil–water double emulsions (98), and electroformation, which utilizes an alternating current between submerged electrodes to generate polymersomes from polymer thin films (33). Recently, microfluidic technology has also been used to prepare polymersomes with a narrow size distribution and high loading efficiency (137,139).

Precise control over the relative molecular weights of the hydrophilic and hydrophobic blocks has provided insights into the structural determinants of polymer self-assembly. By synthesizing PEG-*b*-PLA copolymers with varying molecular weight ratios between the two blocks, Discher's group discovered that the hydrophilic ratio, f —defined as the ratio of the molecular weight of the hydrophilic block to the total molecular weight—is predictive of the structure of the assembly. Amphiphiles with an f value greater than 50% form micelles; f values between 40% and 50% generate worm-like micelles termed filomicelles; f values between 25% and 40% lead to polymersome formation; and at an f value below 25%, amphiphiles form inverted microstructures (3,35). It was subsequently shown that polymersomes with larger f values are able to more rapidly disrupt cellular membranes. The membrane disruption rate constant, k_{lysis} , is believed to scale directly with the molecular weight of the hydrophobic block ($k_{\text{lysis}} \sim \text{MW}_{\text{hydro}}^{1.5}$) (2,35). As many self-assembled delivery vehicles consist of linear amphiphiles, the knowledge and insights gained from polymersome research will be useful for the design of other self-assembling block copolymers.

Polymersomes are useful for drug delivery because drug payloads can be encapsulated within the hydrophilic, aqueous core of a polymersome or within the inner, hydrophobic layer of the polymersome membrane depending on the drug's hydrophobicity. Hydrophilic agents are generally loaded through direct encapsulation during polymersome rehydration (89), water–oil–water double emulsions (53), remote loading with pH (28), gradients of ionic precipitants (2), or inkjets (137). Hydrophobic agents, on the other hand, are generally loaded through via the forces that drive the assembly of the structure (hydrophobic effect, hydrogen bonding, etc.) (51) or by the use of water–oil–water double emulsions (53). Polymersomes have been exploited to deliver a diverse range of diagnostic and therapeutic agents, such as porphyrin-based near-infrared fluorophores (50,51), Gd-labeled dendrimers (27), doxorubicin (1,2), docetaxel (145), paclitaxel (83), siRNA (72), plasmid DNA (65),

insulin (107), and zidovudine (93). These polymersome-based delivery strategies have been explored to treat cancer, diabetes, and infectious diseases (1,87,93,107).

Control over the timing and mechanism of release are critical issues in the design of polymersomes. One common strategy is to release the payload by destabilizing the membrane. To this end, polymersomes have been designed to disassociate in response to an external, physiologically relevant stimulus. Stimuli that have been explored include: pH changes (19,38,45), temperature (84,118), light (123), in vivo hydrolysis (1), oxidation (32,107), reduction (25), and concentration changes of small molecules, such as plasma glucose (107). A detailed discussion of each of these triggers is beyond the scope of this review, and readers are instead referred to comprehensive reviews of this topic by Li et al. (82) and Meng et al. (98). Another evolving research frontier in polymersome research involves the incorporation of targeting moieties on the polymersome surface to target uptake of drugs or imaging agents to specific cells or tissues (23).

Another frontier, which to date has received less attention, is the expansion of the building blocks beyond synthetic copolymers and polymer-peptide hybrids to polymersomes that are composed purely from peptidic building blocks (45). Polypeptides are an attractive choice for the design of polymersomes because they can be genetically encoded. This enables the design of intrinsically biodegradable building blocks that they can be recombinantly synthesized with exquisite control over the molecular weight and size distribution. These attributes are essential for drug delivery, where size, polydispersity, and clearance of nanoscale carriers controls their in vivo localization and ultimate disposition.

Micelles

Micelles are structures consisting of a compact hydrophobic core surrounded by a hydrophilic corona. Micelles can assume spherical, disk-like, or worm-like geometries, with sizes ranging from 5 to 100 nm in diameter (8). Micelles can be formed from lipids, surfactants, or block copolymers, although lipids and surfactants generally have a critical micelle concentration (CMC) greater than that of polymer micelles by several orders, and are hence less useful for the delivery of drugs and imaging agents than polymer micelles. Polymeric micelles are assembled from amphiphiles with sufficiently high hydrophilic ratios ($f > 40\%$). This rule-of-thumb applies to polymeric micelles, which are primarily stabilized by steric repulsion between flexible hydrophilic blocks (159). Within the range of polymeric micellar structures, the amphiphiles that form worm-like micelles have a slightly lower f value ($40\% < f < 50\%$) than those of spherical micelles ($f > 50\%$) (35). It has been reported that some disassembly pathways of polymersomes include a transition from vesicular structures to loose micellar structures. In this case, micelles and polymersomes coexist in solution (35).

Micelles are useful for the delivery of therapeutic and diagnostic agents, especially small hydrophobic molecules, by physical encapsulation in their hydrophobic core (92). Examples of these agents include: paclitaxel (44), doxorubicin (92, 148), camptothecin (14), methotrexate (160), Gd^{3+} (106), and superparamagnetic iron oxide (4). Micelles have also been used to deliver hydrophilic macromolecules such as DNA (103,104,117). One strategy to deliver charged, hydrophilic molecules like DNA is to use micelles that are composed of a hydrophobic core with a polycationic outer shell. The anionic DNA binds to the cationic shell (109). A second, orthogonal strategy uses copolymers with a neutral hydrophilic block and a polycationic block to condense the DNA within the core of a nanostructure. Polylysine, polyethyleneimine (PEI), and polyphosphoramidate (PPA) have been used as the polycationic block while PEG is frequently used as the hydrophilic block (103,104). Before the addition of DNA, the copolymers lack amphiphilicity and remain in monomer form. After mixing, the positively and negatively charged polymers interact to form polyplex,

which leaves the PEG hydrophilic block on the corona of the particle. Thus, the condensation of oppositely charged polyelectrolytes constitutes an alternative approach to assemble polyplex micelles (57,104).

We recently developed a third strategy, attachment-triggered self-assembly, to assemble micelles from a recombinant peptide polymer by attaching multiple copies of the small hydrophobic anticancer drug, doxorubicin, to the C-terminus of a polypeptide. This genetically encoded chimeric polypeptide consisted of two segments: a long, 61-kDa hydrophilic block followed by a short, 1.9-kDa hydrophobic polypeptide that provided multiple Cys residues as drug attachment sites. The conjugation of doxorubicin to the cysteine-rich segment imparted the hydrophobicity necessary to drive the formation of the chimeric polypeptide into micelles (92). In contrast to the physical encapsulation of hydrophobic drug molecules within the core of a micelle, this approach covalently links drugs to Cys residues that are sequestered on one end of the polypeptide via a pH-sensitive bond, and releases the drug by cleavage of the linker in low pH compartments within the cells.

Similar to many other nanoscale self-assembled delivery systems, the incorporation of targeting moieties as well as optimization of the size and drug release mechanisms of micelles have been extensively discussed in a review by Branco et al. (21), so that we restrict our discussion herein on the stability of micelles, a topic that is equally important to the success or failure of micelle drug delivery, but that is less discussed in the literature. The stability of micelles has a thermodynamic and kinetic component. Thermodynamic stability is characterized by the critical micelle concentration (CMC) and/or the critical micelle temperature (CMT), a concentration and temperature above which amphiphiles are thermodynamically driven to assemble into micellar structures. The CMC of a micelle is determined by the interplay of many factors, including the composition and length of the hydrophobic segment relative to the hydrophilic segment (6). For example, changing the hydrophobic core of a PEG-PS micelle (MW ratio: 10,400: 3700 Da) from polystyrene (PS) to polylactic acid (PLA) increases its CMC from 1 to 35 mg/L (55,151). The kinetic stability of micelles depends on several factors as well, including the hydrophobic content of the core, the surface charge of the corona, and the lengths of both the hydrophilic and hydrophobic blocks (6,8). While kinetic and thermodynamic stability are interrelated, it has been shown that micelles can remain kinetically stable for extended periods of time below the CMC (69).

One advantage of using micelles for drug delivery stems from the ability to modulate the properties of the micelle by controlling the polymer composition and length. The *in vivo* stability of polymer micelles has been manipulated by altering the MW (148), composition and size of the hydrophobic core (86), the hydrophilic/hydrophobic ratio of the block copolymer, and the surface charge (69) of the micelle (102). In this regard, peptide polymers are attractive for the formation of micelles as the surface charge, amphiphilicity, and size—all factors that influence micellar stability—can be readily tuned at the genetic level. For example, Dreher et al. tuned the stability of a series of micelles of genetically engineered peptide block copolymers by precisely and systematically varying their hydrophilic to hydrophobic ratios and MWs (37). Because of the ease with which these molecular parameters can be precisely engineered into peptide polymers at their genetic level, we believe that genetically encoded polypeptide micelles are likely to play an increasing role in the design of the next generation of nanoscale carriers of drug and imaging agents.

Hydrogels

Polymer hydrogels are three-dimensional, continuous networks of their molecular constituents that are surrounded by and entrap water (115). The nature of the interactions

that support the network defines the subcategories of chemical and physical gels (114). Chemical gels are assembled through covalent bonds whereas physical gels are held together through molecular entanglement and/or non-covalent molecular interactions between the polymer chains. This review focuses exclusively on physically cross-linked gels as they meet the definition for self-assembled complexes.

Due to the fact that hydrogels entrap large volumes of water, they are useful vehicles to deliver hydrophilic molecules. Hydrogels have been used to deliver a number of small hydrophilic molecules, such as lidocaine (122) and pilocarpine (101). However, an increasingly popular strategy is to use hydrogels to deliver macromolecules such as peptides and proteins. Interleukin-2 (IL-2) (149), insulin (13), epidermal growth factor (EGF) (129), bone macrophage protein-2 (BMP-2) (62), and basic fibroblast growth factor (bFGF) (61) are a few examples of macromolecular therapeutics that have been delivered by hydrogels. The high water content of hydrogels allows proteins to retain their native conformation. The interactions supporting hydrogel self-assembly can also respond to environmental changes such as temperature, pH, ionic strength, physical stress, and solutes. Disrupting these interactions can allow the release of therapeutic agent from the hydrogel to be modulated (115).

Hydrogels are not restricted to synthetic polymers, as they can also be created from polypeptides and proteins (21,40). In particular, genetically engineered peptide polymers are especially attractive for the formation of hydrogels because their composition, sequence, and sites for physical cross-linking can be precisely designed on their genetic level (20,48). Peptide polymers and proteins are also intrinsically biodegradable, which facilitates their clearance from the body. In summary, these features of polypeptides and proteins make them appealing materials to formulate hydrogels.

Hydrogels are frequently used in cell delivery for tissue regeneration. One example of such a delivery system is the peptide-based rigid hydrogel, α -hyperin, developed by Schneider and Pochan (125). This 20-amino acid sequence consists of two β -sheet-forming segments of alternating lysine and valine residues flanking a tetrapeptide with a propensity to form type II turns. Under basic conditions, the peptide adopts a β -hairpin secondary structure with a hydrophobic and a hydrophilic face, which is able to self-assemble into rigid hydrogels. Schneider and Pochan have shown that these hydrogels are compatible with many mammalian cell types, including fibroblasts, mesenchymal stem cells, chondrocytes, and hepatocytes (56,70,76). Another appealing feature of this hydrogel is that under mechanical shear, the cell-hydrogel construct shear-thins and adopts a low viscosity. Upon dissipation of the mechanical force, the network reforms and regains its original mechanical properties and this process does not affect the growth of the trapped cells. This reversible shear-thinning feature allows the hydrogel and associated cells to be delivered via injection, which is convenient for clinical applications (56).

Proteins

Proteins differ from intermolecular self-assemblies in that their structural integrity depends upon their primary sequence and environment, but not on their concentration. In contrast, micelles and vesicles dissociate when the concentration drops below their critical aggregation concentration (CAC). Although the dissociation of intermolecular self-assemblies can provide a mechanism for release of drugs that are encapsulated within these structures, it may also complicate their pharmacokinetics and biodistribution. In contrast, the structures of proteins are not concentration-dependent, and this difference offers a distinct advantage in the use of proteins as delivery vehicles because their structural integrity is not jeopardized during the inevitable dilution that occurs when they are administered in vivo.

Advancements in genetic engineering have led to the development of many new proteins, some of which have been used as delivery vehicles. Monoclonal antibodies (mAb) and their derivatives are one example that have long been used clinically to deliver radionuclides for radioimmunotherapy and scintigraphy (9,116) because mAbs enable radionuclides to be targeted to cells that express the corresponding antigen. However, the clinical application of MAb has been hampered by several issues: 1) undesired immunogenicity generated by xenogeneic mAbs (91); 2) inadequate accumulation or penetration of mAbs inside diseased tissues because of their large size or their high affinity to antigens proximal to the vascular space; and 3) undesired accumulation of the mAbs in healthy organs, such as the kidney (100). These issues are gradually being resolved with new advances in genetic engineering, such as antibody humanization and the generation of smaller, but highly potent antibody fragments (47,99,134,135,143).

One major prospect to reduce the immune response against mAbs is to harvest them from humanized mice that are able to generate human mAbs against human antigens (90). It has been reported that these human mAbs elicit immune responses in cancer patients less frequently than xenogeneic mAbs. The lowered immunogenicity also imparts a longer plasma half-life to these mAbs, thereby increasing their efficacy. In addition, these humanized mAbs maintain comparable affinities to human antigens as xenogeneic mAbs (124). Another approach to generate human mAbs against human antigens is the use of phage display (96). As of 2008, these two approaches have led to a total of 61 mAbs in clinical development (91). Two of these mAbs, adalimumab, and panitumumab, have been approved by the FDA for the treatment of inflammatory diseases and colorectal cancer, respectively (43,100,124). Although largely developed as therapeutics, these human mAbs are also useful for the delivery of small molecule therapeutic and imaging agents.

Two strategies have been pursued to increase the overall accumulation and penetration distance of mAbs in target tissues: 1) using smaller fragments of mAbs rather than intact mAbs to retain the specificity of the full antibody while reducing its molecular weight, and 2) using covalent and non-covalent linkages to assemble multivalent antigen-binding units, which significantly improves the binding affinity between these mAb derivatives and their antigens (155). These mAb fragments often include only the regions of the antibodies that are essential for antigen recognition. The tailoring of mAbs helps to avoid immune responses toward their constant (Fc) domain. These antibody fragments include nanobodies (47), domain-deleted mAbs (99), hypervariable domain region peptides (134), and variable domain (Fv) fragments (135). On the other hand, multivalent antigen-binding units are generally assembled from these smaller mAb fragments to simultaneously provide lower immunogenicity than the parent, intact mAb and a higher binding affinity. Examples of these multivalent structures include dia-, tria-, and tetra-bodies (143).

It is also interesting to note that mAbs have been conjugated to albumin and to synthetic polymers to increase their plasma half-life (30,81). However, while this strategy succeeds in increasing the plasma half-life through reduction in renal clearance, it also decreases the penetration distance of the conjugate from plasma into tissue. Thus, controlled release of a therapeutic payload from mAbs and their derivatives is essential to balance the need for long systematic circulation, good tissue penetration, and high affinity binding to the target receptor. This balance remains a major challenge that needs to be solved to extract the full potential of mAbs and their derivatives for the precise, targeted delivery of drugs to their cellular targets in diseased tissues.

Inclusion Complexes

An inclusion complex is a structure formed by host–guest chemistry. In an inclusion complex, one chemical compound, the “host,” forms a frame structure with a cavity that the

second chemical compound, the “guest,” can stably occupy. Cyclodextrins (CD) are the most extensively studied “hosts” for hydrophobic drug “guests” (Fig. 2). CDs comprise a family of cyclic oligosaccharides that are subcategorized into the α -, β -, γ -, δ -, and ϵ -CD groups based on whether the number of oligosaccharides are 6, 7, 8, 9, or 10, respectively (22). CDs have a bracelet shape with an outer diameter ranging from 1.5 to 2.0 nm, depending upon the number of oligosaccharides. The cavity of this bracelet-like structure is slightly hydrophobic and has a diameter that ranges between 0.4 and 0.8 nm (147). In aqueous solutions, CD structures are not in an energetically favored state if the cavities are occupied by water molecules. However, when water molecules are substituted by more hydrophobic molecules, CDs and the new “guest” molecules will form energetically stable inclusion complexes. Thus, CD-based self-assembled inclusion complexes are products of the noncovalent intermolecular interactions between CDs and their “guest” molecules, which can be substituted with drugs or imaging agents. CD-based carriers, therefore, are payload-stabilized self-assembled structures.

Although three types of CDs have been found in nature (α -, β -, and γ -, termed Schardinger’s sugars) (142), they are not ideal delivery vehicles due to their low aqueous solubility and shallow cavities. Chemically modified CDs were hence developed to overcome these drawbacks. The most frequent modifications to date are acetylation, hydroxypropylation, and sulfobutylation of the primary or secondary hydroxyl groups of α - and β -CDs (22). These modified CDs are mostly used for oral, transdermal, or ophthalmic delivery of hydrophobic therapeutics (88,131). More than 30 CD formulations of drugs are now on the market. In these formulations, CDs are used to increase the solubility, bioavailability, and stability of drugs (22).

It is worth noting that the preferred administration routes of CD-formulated drugs are different from other self-assembled nanoscale carriers, which are often developed and used for parenteral delivery. There are two main reasons why CDs are rarely used for parenteral delivery: 1) at high concentrations, CDs have been shown to cause toxicity to erythrocytes by forming tight complexes with membrane components, which destabilizes the membrane and drives cell lysis (66); and 2) fast renal clearance of CDs due to their small size (1.5–2 nm) (147). To address the first issue, modified CDs, such as 2-hydroxypropyl- β -CD (HP- β -CD) and sulfobutylether β -CD sodium salt (SBE β -CD), have been developed that display lower erythrocytic toxicity (22). To tackle the second issue, CDs have been used together with other nano-materials to build delivery vehicles that are large enough to avoid renal clearance, while maintaining the CD’s solubilizing function. For example, Davis et al. engineered a linear polymer decorated with multiple CDs. The linear polymers themselves are smaller than 10 nm in diameter and are below the renal cut-off size so that they can be cleared through the kidney. However, when a hydrophobic cancer chemotherapeutic, camptothecin (CPT), is conjugated to the linear polymers, the conjugates form nanoparticles, which are believed to be the result of inclusion complex formation between CPT and CDs on different strands. The nanoparticles are ~30–40 nm in diameter, which is larger than the renal cut-off size so that these particles are able to circulate in the bloodstream for an extended time. These nanoparticles disassociate once the CPTs are cleaved from the linear polymer and the resulting polymer molecules presumably clear through the kidney (29).

Another reported method to “enlarge” the size of CDs for delivery is to load preformed CD–drug inclusion complexes into the hydrophilic core of liposomes (63). With resolution of these issues that have limited the applications of CDs in parenteral delivery, it can be expected that CD-based inclusion complexes will be increasingly used for parenteral drug delivery, because of their established safety profile, high solubilizing capacity of hydrophobic drugs, and ease of manufacture (22).

GENERAL CONSIDERATIONS WHEN USING SELF-ASSEMBLY AS DELIVERY VEHICLES

The Mechanism of Self-Assembly

Almost all self-assembling materials used for delivery are amphiphilic in nature. Amphiphilicity, the spatial polarization of hydrophobic and hydrophilic segments, can be displayed by various types of molecules. For example, a linear molecule may have an uneven distribution of hydrophilic and hydrophobic residues along its length. Alternatively, hydrophobic moieties can be distributed throughout the chain such that formation of distinct secondary structures of molecules creates faces with amphiphilic properties, such as the secondary structure of α -hairpin peptides (130). Such amphiphilicity drives the formation of nano- and microscale self-assemblies (144). Hydrophobic interactions play a major role to transform amphiphilic nature of the subcomponents into self-assemblies. In addition to hydrophobic interactions, other noncovalent interactions such as electrostatics and hydrogen bonding can also shape the final structure of self-assemblies.

There are three features shared by all self-assembled structures, as pointed out by Whitesides and Grzybowski (150). First, the final structure and properties of self-assembled complexes are encoded by the properties—shape, polarity, charge, sequence, molecular weight, etc.—of individual components and by the solution environment. These components can be either individual amphiphilic molecules or elements of a molecule such as amino acid residues in a polypeptide. The physicochemical properties of these components determines their intra- and intermolecular interactions with solvent molecules, and can, when appropriately encoded, lead to self-assembly of the components into supramolecular structures. Thus, the ability to design components that contain the requisite physicochemical properties is the key to engineering self-assemblies with desired properties and functions. Second, the components of self-assembled structures must be dynamic, so that the assembled structures are able to sample a variety of energy states, and arrive at a local or global minimum in its free energy. Third, equilibration (reversibility) is critical for self-assembly, because self-assembly requires a dynamic exchange between the ordered (self-assembled) and disordered (unimer) states through equilibration. These three features are important factors to consider in designing self-assembled structures.

Loading and Releasing Diagnostic and Therapeutic Payloads

Loading and releasing the payload are two key events in drug delivery, and these mechanisms are frequently interdependent. In contrast, while loading is the key for the design of self-assembled carriers of imaging agents, as in most cases these reagents do not need to be released from the carrier in vivo, but can perform their imaging function without the need for release. Drugs, in contrast, typically need to be released from their delivery vehicle to be effective. Methods to load diagnostic and therapeutic agents into self-assembled delivery vehicles can be categorized into three groups, based on the interactions between the delivery vehicles and the drugs or imaging agents: 1) physical encapsulation (such as that of aqueous payloads within liposomes); 2) encapsulation via secondary interactions (such as DNA condensation by ionic polymers or that of hydrophobic drugs within the lipid bilayer of liposomes); and 3) covalent conjugation. The loading efficiency of a drug or other agents into self-assembled carriers is quantified by two terms: drug loading (DL) or drug content, and entrapment efficiency (EE):

$$DL (\%) = \text{mass of loaded drug} / \text{mass of carrier} \times 100$$

$$EE (\%) = \text{mass of loaded drug} / \text{mass of total drug used in formulation} \times 100$$

Loading efficiency describes the overall performance and safety of a delivery system as it impacts the rate of release, the extent of drug release, and the amount of drug that can be injected. This is particularly true for encapsulated molecules. From a mechanistic standpoint, loading efficiency is dependent upon the type of carrier, the physicochemical properties of the drug, and cumulative interactions between the drug, the vehicle, and their environment.

Physical encapsulation is a common loading methodology used with vehicles such as liposomes, polymersomes, micelles, hydrogels, and inclusion complexes (Table 3) (34,54,59,77). The hydrophobicity of the molecule generally dictates the location of the encapsulated drug within these structures. Encapsulation can be performed during or following the self-assembly process. The most common methods of encapsulating a drug during the assembly process include: double emulsion, cold or hot homogenization, solvent evaporation, and dialysis (68). Encapsulation following vehicle formation is accomplished through remote loading, where agents are transported from the buffer into the encapsulation compartment of the vehicle by diffusing down pH or ion gradients (17). The release mechanisms include diffusion through the vehicle wall or bolus release of the payload following dissociation or degradation of the vehicles. Self-assembled carriers generally experience dissociation once the carrier concentration falls below their critical aggregation concentration (CAC), or in response to environmental triggers such as changes of pH, light, ion strength, and temperature that decrease the carrier stability.

Encapsulation via secondary interactions is mostly used to load charged payloads, particularly nucleic acids (Table 3) (103,104,109,117). In these cases, delivery vehicles usually have a segment or compartment that is oppositely charged to the payload and serves as a payload binding site (103,104,109). After the delivery vehicle is internalized by cells, the release of the payload occurs by counterion exchange, in which the payload is replaced by equivalently charged ions in cells (103). It is noteworthy that these delivery vehicles, if positively charged, can exploit the “proton sponge effect,” in which a buildup of osmotic pressure ruptures the endosome, thereby releasing the carrier–payload complex into the cytosol and ultimately allowing the cargo to reach their subcellular target, such as the nucleus (104).

Covalent conjugation is another loading method that can exploit environmentally sensitive tethers to regulate drug release (Table 3). The environmental cues or stimuli used in delivery include changes in microenvironmental triggers such as the change in extracellular pH between tumor tissue relative to healthy tissue, enzymatic activity, or an extrinsic trigger, such as light (92,148). Depending on the chemistry, conjugation can be performed before or following carrier assembly. As an example of the former case, conjugation of a hydrophobic molecule, doxorubicin to one end of a chimeric polypeptide triggered micelle self-assembly of the polypeptide (92). In contrast to conjugation-triggered self-assembly, the conjugation of radionuclides to antibodies for radioimmunotherapy is an example of postassembly conjugation of a drug to a self-assembled protein carrier (9).

Because self-assembly is thermodynamically reversible, dissociation of a self-assembled structure can occur by two mechanisms (Table 3). In the first—passive—mechanism, dilution can trigger dissociation of the self-assembled complex into its subcomponents; the dissociation of micelles at a concentration below their critical micelle concentration (CMC) is an example of this mechanism. A second mechanism is that of triggered release, in which

the self-assembled complex dissociates in response to a specific external cue such as a sharp change in pH or temperature. Lee et al., for instance, designed a PEG-*b*-poly(histidine) construct that formed stable micelles at physiological pH, but which rapidly became unstable when the histidine core became charged below pH 7 (79). From this perspective, in principle, self-assembled structures can be designed to display controlled disassembly after they reach the target organs, tissues, or cells to liberate their therapeutic payload; at the same time, the need for disassembly must be counterbalanced with the need to impart sufficient structural integrity to the carrier to ensure that it does not disassemble during its transit in systemic circulation on its way to its target organ or tissue.

FUTURE RESEARCH DIRECTIONS

There are many interesting and useful research directions in this field that are motivated by the following challenges: 1) biophysical characterization—the biophysical properties of self-assembled structures may differ significantly between well-defined in vitro conditions and the complex biological environment in organisms; 2) rational design of biocompatible materials—there is limited knowledge that predicts the safety of new architectures and biomaterials; and 3) discrepancy between the need for complexity of function and ease of manufacturing of self-assembled materials—with the development of multifunctional self-assemblies, the manufacturing scale-up of a uniform product will become prohibitive for some technologies.

Decades of evidence demonstrate that many nanoparticles have different in vitro and in vivo physicochemical properties. For example, protein films form on metallic nanoparticles in the presence of plasma proteins (108). This bio-fouling changes the particle size, surface area, electrostatic charge, and hydrophobicity (108). Therefore, nanoparticle characteristics (shape, structure, stability, affinity, rates of release, etc.) obtained in pure solutions may have limited relevance to physiochemical properties in an organism. To address this discrepancy, better methods are required to assess these characteristics in vivo. One development toward this end is the introduction of correlative microscopy (140). This microscopy method systematically combines fluorescence microscopy with electron microscopy to enable construction of an image with the benefit of fluorescent labels while retaining nanoscale resolution. Another interesting approach that was recently demonstrated by Schmidt and coworkers uses dynamic light scattering to quantify the aggregation of polymeric drug carriers in serum (119).

To move self-assemblies into clinical applications, their biocompatibility must be evaluated, which is typically assessed by pharmacokinetics, biodistribution, immunogenicity, acute toxicity, and chronic mutagenicity (5,126). These evaluation studies, however, seldom answer mechanistic questions surrounding the “why” of biocompatibility. Thus, few transferable principles in this regard can be generalized from the studies. For example, as a free polymer in solution, PEG is widely regarded as biocompatible (52,111); however, this knowledge has not been sufficient to predict the biocompatibility of PEG-modified self-assemblies (67,78). Therefore, there are great research opportunities in discovering and validating transferable principles that allow better prediction of biocompatibility of new biomaterials for drug delivery.

Finally, the incorporation of bioresponsive or targeting moieties into self-assembled carriers has followed a consistent trend toward complexity. While this trend may be unavoidable in building carriers with versatile functions, increasing complexity poses a challenge for manufacturing. The production of multifunctional nanoscale carriers often involves multiple steps, which may introduce polydispersity in size, shape, chain lengths, and the location and orientation of linkages between components. One promising strategy to efficiently produce

complex structures is to utilize genetic engineering and biosynthetic pathways to produce programmable and monodisperse materials such as polypeptides. The ease of polypeptide biosynthesis is not generally jeopardized by sequence complexity or the incorporation of multiple functionalities. Therefore, genetic engineering and biosynthesis may provide a strategy to overcome the trade-off between functional complexity and manufacturing scale-up of self-assembling materials.

CONCLUSIONS

Self-assembled nanoscale delivery vehicles can improve the bioavailability, biodistribution, and pharmacokinetics of payloads that can either be physically encapsulated or chemically conjugated to these carriers; these carriers, if appropriately designed, can also enable these payloads to cross physiological barriers that they would be unable to traverse if delivered by themselves. Self-assembled nanocarriers, have two unique attributes. First, because they are built from molecular or supramolecular components that come together with precise spatial definition, they can assume complex shapes and defined sizes in the nano-mesoscale length regime, which are difficult to attain by chemical synthesis. Second, because self-assembly is a dynamic, equilibrium process, self-assembled structures can be designed to disassemble “passively” by dilution or “actively” in response to an external cue to release their payload. These attributes make them enormously attractive for the in vivo delivery of drugs and imaging agents.

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Figure 1. Biomolecular self-assemblies and their typical delivery target, the human cell. Note: The sizes of objects are not drawn to scale.

Figure 2. Engineered self-assemblies and their typical delivery target, the human cell. Note: The sizes of the objects are not drawn to scale.

Table 1

Naturally Occurring Nanoscale Self-Assemblies

Type	Structural Characteristics	Advantages	Payload	Examples	Ref.
Protein	Intramolecular self-assembly	Biocompatible; Long plasma half-life; High in vivo bioavailability	Radionuclides; Small drug molecules	Albumin	74,141
Virus capsid	Intermolecular, hierarchical self-assembly	Inherent capacity for cell entry	Imaging contrast agents	CCMV	36,95

Table 2

Engineered Self-Assemblies Used as Delivery Vehicles

Type	Structural Characteristics	Variants	Advantages	Cargo	Examples	Ref.
Vesicles	Hollow, bilayer, spherical structure with a hydrophilic core, Intermolecular self-assembly	Liposome (lipid amphiphiles); Polymersome (polymer or polypeptide amphiphiles)	Relatively stable; Capable of delivering both hydrophobic and hydrophilic agents; Large compartment for payloads; Extensively researched	Small drug molecules; Imaging agents; RNA; DNA; Peptide	Doxil	110
Micelles	Single layer structure with a hydrophobic core and a hydrophilic corona; Intermolecular self-assembly	Sphere; Disk-like; Worm	Easy to prepare; Easy to adjust stability; Payloads can be conjugated to micelles	Small hydrophobic drugs; Imaging agents; DNA	Micelles formed by PEG- <i>b</i> -PPA copolymer with DNA	103
Hydrogels	Entangled three dimensional networks, less organized self-assembly		High capacity to deliver water-soluble macromolecules	Peptides; Proteins; Cells *	-Hyperin peptide hydrogel	56,76,125
Proteins	Intramolecular self-assembly with a hydrophilic surface and hydrophobic core		Biocompatible; Easy modification by genetic engineering	Radionuclides	Antibody fragment or derivative	47,99,134,135
Inclusion complexes	Host-guest chemistry; Payload-stabilized delivery vehicles		Biocompatible; Easy to manufacture	Small hydrophobic drugs	Cyclodextrins	22

* The hydrogels used for cell delivery are in micrometer range.

Table 3

Loading and Release Methods

Loading Mechanism	Specific Methods	Related Release Mechanisms	Examples of Self-Assembly	Ref.
Physical encapsulation	Double emulsion; Homogenization; Evaporation; Dialysis; Remote loading	Dissolution	Liposome	113
		Carrier dissociation upon environmental stimulations: changes of pH, temperature, or light	Polymersome	19,38,45,84,118
		Carrier dissociation after chemical reactions: hydrolysis, oxidation, or reduction	Polymersome	1,25,107
Encapsulation via secondary interactions	Electrostatic interactions/Condensation	Ion exchange, diffusion	DNA–polymer polyplex micelle	104
Covalent bonding	Chemical conjugation before or after self-assembling	Cleavage of covalent bonds upon environmental stimulation: changes of pH, temperature, or light	Chimeric polypeptide–doxorubicin micelle	92
		Enzymatic cleavage	Polymer–doxorubicin micelle	148