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Journal

Materials Chemistry Frontiers, 5(18)

ISSN

2052-1537

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Publication Date

2021-09-21

DOI

10.1039/d1qm00717c

Peer reviewed



HHS Public Access

Author manuscript

Mater Chem Front. Author manuscript; available in PMC 2024 February 09.

Published in final edited form as:

Mater Chem Front. 2021 September 21; 5(18): 6672–6692. doi:10.1039/d1qm00717c.

USE OF ARTIFICIAL CELLS AS DRUG CARRIERS

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Abstract

Cells are the fundamental functional units of biological systems and mimicking their size, function and complexity is a primary goal in the development of new therapeutic strategies. Recent advances in chemistry, synthetic biology and material science have enabled the development of cell membrane-based drug delivery systems (DDSs), often referred to as “artificial cells” or protocells. Artificial cells can be made by removing functions from natural systems in a top-down manner, or assembly from synthetic, organic or inorganic materials, through a bottom-up approach where simple units are integrated to form more complex structures. This review covers the latest advances in the development of artificial cells as DDSs, highlighting how their designs have been inspired by natural cells or cell membranes. Advancement of artificial cell technologies has led to a set of drug carriers with effective and controlled release of a variety of therapeutics for a range of diseases, and with increasing complexity they will have a greater impact on therapeutic designs.

Keywords

Artificial cells; liposomes; drug delivery; therapeutic drug delivery

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1. Introduction

Cells are the fundamental functional units of living systems with assemblages of internal structures organized to support the specialized functions of growth, division, communication, and molecular transport [1–3]. Human-made cell models that simulate one or more of the complex cellular components and perform selected cellular functions can be referred to as “artificial cells”, “protocells” or “minimal cells” (Figure 1). The first artificial cell, constructed in 1957 by Chang, consisted of a vesicle made of lipids found in the cell membrane and embedded proteins [4]. With advances in materials science and a greater understanding of cellular components and their function, the science of developing artificial cells has advanced considerably. In particular the use of these cellular mimics for directed delivery and controlled release of therapies has burgeoned with advances in molecular biology, proteomics, nanotechnology, biotechnology, and polymer chemistry [5–7]. We focus our discussion on the concept of building simple functional units that recapitulate living cells, protocells, that can respond to and deliver targeted therapies.

Artificial cells have been described in many different ways in various studies, alternatively having been called “synthetic cells,” “mimicry cells,” or “protocells” [8, 9]. We will use here the term “artificial cell”, which is defined as an entity with two main features: 1) comprised of biomolecules (lipids, enzymes, membrane channels, structural proteins, etc.) that enable the entity to perform some features of living, biological cells, such as evolution, self-replication, and metabolism; and 2) produced in different structural forms using elements (polymers, combined lipid-polymer complexes, nanoparticles) that can mimic one, or more, structural or functional properties of natural cells such as transport, energy production or adaptation. In these synthetic systems, surface properties, dimensions, morphologies, and chemical responses can be modified to more closely match characteristics of natural cells, providing a simplified experimental context in which to better understand cellular functions including cell-cell interactions, and communication across distances that can inform the design of tools for biologically controlled drug delivery, which is the focus of this review [10]. These tools can be designed from a top-down or a bottom-up approach and each has an impact various features of artificial cell systems. The composition and design of artificial cells, when used as therapeutic drug delivery systems (DDS), can vary, and commonly used techniques for construction include liposomes (protocells), polymersomes, dendrimersomes, and microfluidic-based droplets. This is an emerging field with numerous opportunities as well as challenges and we discuss these in the context of existing techniques with a view toward future innovations.

2. Artificial Cell Technologies and Fabrication: From Simple Single Vesicle Structures to Complex Integrated Systems.

Cells are independent entities capable of a wide variety of interactions leading to a network of cellular centers in which controlled biochemical reactions take place within cells and are communicated throughout the network [11, 12]. An understanding of cellular dynamics and the complex structures that support integrated functions is necessary to develop artificial cells. Selecting key functions and integrating them into simple structures

enables development of advanced delivery tools with broad applications in medicine. When utilizing artificial cells as bio-responsive carriers, a communication element connecting the external and internal environment mediates the sensing and responsiveness of the carrier to signals in the environment [13]. Artificial cells can encapsulate chemicals and biologics with therapeutic functions, and sets of diverse, programmed stimuli can be used to control the timing and location of their release [3, 14]. Thus, synthesis should allow for the encapsulation of biomolecules without denaturation or loss of functionality, and, for clinical applications, carriers must be biocompatible and biodegradable [15, 16]. The opportunity for fine tuning therapeutic delivery of the right amount to the right place at the right time is the benefit of artificial cells as therapeutic drug carriers. Natural cells do this every moment of every day in our bodies, and we can learn from processes how to better deliver therapeutics to where they are needed, such that we can reduce off-target, or side-effects, in the treatment of disease or restoration of function.

Both top-down and bottom-up approaches have been used to synthesize nano- and micro-sized drug carriers [8]. Carriers prepared using the top-down approach require fewer purification steps and may be easier to scale up than those using the bottom-up approach; however, carriers prepared using the top-down approach exhibit broad size distributions while those obtained by the bottom-up approach can be more easily controlled to maintain tight size distributions [17]. Understanding these synthetic approaches and their products is critical to the design and use of advanced bio-responsive drug carriers.

2.1. Top-down approach for the development of artificial cells

Top-down construction of artificial cells is intended to simplify the complexity of natural cells, using these cells as the starting material (Figure 2) [17]. Specifically, this approach consists of removing all cellular components that are not essential for survival of the cell [18], and can include adding back in desired functions. Methods and technologies, for stripping or replacing cellular components have been developed as part of the study of cell biology, and these approaches can be used to re-engineer cells. This top-down approach is less complicated than building a cell from scratch (i.e., from the bottom-up) [19]. Synthetic eukaryotic cells have yet to be successfully created, and therefore the top-down approach is necessary to engineer these cells, but simpler biological systems have been completely engineered. De novo synthesis of a virus that infects human cells was first reported in 2002, and there have been numerous publications using similar approaches since that initial report [20]. To create an artificial infectious poliovirus, Cello *et al.* synthesized full-length poliovirus cDNA which was then transcribed into infectious viral RNA with T7 RNA polymerase and used to create a poliovirus similar to natural viruses. This study showed the possibility of synthesizing more complex living organisms by chemical/biochemical methods without using a genetic template [20]. Subsequently, Venter *et al.* designed a computer-based genome sequence, synthesized the corresponding cDNA, stitched into the yeast genome and then transplanted the new genome into *Mycoplasma capricolum* recipient cells. These studies marked a technological turning point, raising the possibility of creating artificial cells and controlling organisms at the molecular level [21, 22]. The complexity of even these so-called simple systems, necessitated the use of existing cellular structures to propagate synthetic genomes in these examples of top-down designed artificial biological

entities. Building from the bottom-up is a more challenging enterprise given that each component, pathway and structure would need to be engineered.

2.2. A Bottom-Up Approach to Build an Artificial Cell

Recently, simplified features of living cells have been reproduced in artificial systems using nanometer- or micrometer-sized compartments [23]. In the bottom-up approach, artificial cells consist of membrane-like carriers of biomolecules that are minimally necessary to re-create specific cellular functions, generally quite simple functions like binding and membrane fusion. The bottom-up approach can yield specific bioactivities without the need to consider and manage the complex interactions among the millions of other components, processes and structures that are present in natural cells [24], but the complexity is greatly reduced. The bottom-up approach can be used to develop transfer vehicles a variety of molecular cargos including nucleic acids [25], small molecules therapeutics [26], components of vaccines [27], and enzymes [28]. In the most complex configurations, multiple components can be integrated to comprise specific biological processes.

While this approach can be used to fabricate biomimetic systems, that contain nucleic acids or molecular pathways, a semipermeable outer membrane is required to enable interactions with cells or transfer of reactions into the interior of the cell where the exchange of biomolecules and ionic species occurs [19]. Currently, lipid vesicles such as liposomes, polymersomes, and dendrimersomes are used for bottom-up construction of artificial cells [6, 29, 30], and will be discussed in detail in the following sections. The preparation of water-in-water emulsion droplets using microfluidic technologies is one approach for bottom-up construction of artificial cells which provides the advantages of simple and rapid preparation, size control, and relatively small volumes. As such, microfluidic-based droplets are widely used as DDSs [31].

2.2.1. Liposomes—Many of the artificial cells developed as therapeutic drug carriers have been based on liposomes and comprise a number of widely used drugs including doxil—encapsulated doxorubicin [32–34]. Liposomes are spherical, supramolecular assemblies of amphiphilic molecules, including phospholipids, steroids, and polymeric materials, that constitute an aqueous core enclosed by a lipid bilayer (Figure 3A) [35]. Although, these synthetic structures do not inherently have growth, replication, communication, or other dynamic properties of natural cells, they model the lipid bilayers of living systems and have contributed significantly to the concept of artificial cells.

Several different liposome preparation methods are available, including thin-film hydration or the Bangham method, solvent injection, reverse-phase evaporation, sonication, and membrane extrusion [36]. Liposomes can range from 20 nm to 10 μ m in diameter. They can be classified accordingly as small (<50 nm), large (50–500 nm) or giant (10–100 μ m) liposomes, depending on their size and lamellarity [37, 38]. Lamellarity, defined as the number of bilayers surrounding the liposome, and these multivesicular vesicles can encapsulate smaller vesicles. . Giant liposomes are widely used as artificial cells because their size approaches that of the eukaryotic cells, making them easily observable by light microscopy and single-level vesicle analysis methods [39]. The hydration method, which is

also called the swelling method and electroformation method are the traditional methods for preparing giant liposomes and these methods are widely used as they can easily produce giant liposomes in sufficient quantities for experiments. However, in these methods, it is difficult to control monodisperse liposome size, encapsulation concentration, and asymmetry of lipid membrane [40, 41]. Researchers have also investigated methodologies for vesicle formation using microfluidics technology, aiming to create artificial cells, achieve a high encapsulation efficiency of vesicles and produce asymmetrical lipid bilayers [42]. In addition to the ability to formulate appropriate structures as *in vivo* drug carriers with sizes comparable to natural cells, liposomes also have the advantage of colloidal (particle) stability, [43]. Both physical stability, which is a very significant parameter for the storage and transport of a colloidal system, and chemical stability, a key parameter for predicting drug metabolism, efficacy, and toxicity, are significant for artificial cells. At the same time, it is essential that liposomes used for DDSs can be produced with precise size distributions to enable homogeneous and continuous drug release [44]. The size of particles used for drug delivery is an important consideration relative to being retained within the vasculature and targeting the endothelial cells or extravasating into the tissues, approximating the size of a cell serves to retain the entity in the blood vessel.

Liposomes can encapsulate both hydrophilic and hydrophobic drugs due to their unique core/shell structure [45]. The liposomal core can be loaded with drugs, vaccines, immunoadjuvants, or antigens through passive or active means. Passive loading involves encapsulation of target agents before or during liposome formation, while active loading takes place after liposome formation (Figure 3B) [46, 47]. Liposomes can be designed to release their cargo based on cell surface interactions, local changes in temperature or pH, or by external application of specific frequencies of light, ultrasound or magnetic fields [14].

One of the most important problems in studies on liposomes is that they collapse easily in response to environmental changes and mechanical forces due to their low bending modulus. The fragility of liposomes causes uncontrolled leakage of retained compounds and thus, complicates their use in biomedical applications and artificial cell experiments. Cell membrane and cytoskeleton are essential components that determine the shape of the living cell. Cell membrane defines the autonomy of the cell by separating the cytoplasm from the extracellular environment. Cell membranes are tolerant to environmental changes and mechanical forces. The stability of the cell membrane results from the cytoskeleton situated beneath the membrane. The cytoskeleton is a filamentous network in the cytoplasm and mechanically supports the cell membrane. The membrane is supported by actin, which forms a thin cortical shell. Based on this basic knowledge, Tsai *et al.* have proposed a new, facile, quick, and reproducible method to fabricate giant liposomes filled with an active actin-myosin cytoskeleton to improve mechanical strength of liposomes [48]. In another study Kurokawa *et al.* have constructed an artificial cytoskeleton using DNA. The DNA cytoskeleton significantly enhances mechanical stability and hence confers tolerance to osmotic shock to liposomes like the cytoskeleton in living cells. They noted that due to its biocompatibility and ease of implementing design changes, the DNA cytoskeleton could become an important stabilizer of liposomes [49].

2.2.2. Polymersomes—Polymersomes are attractive tools for bottom-up construction of artificial cells as DDSs as they have tunable membrane properties and good stability making them excellent tools to deliver a wide variety of compounds [18]. Polymersomes are synthetic analogs of liposomes being comprised of diblock or triblock copolymers that self-assemble into polymer vesicles. Compared to other polymeric carriers, polymersomes can encapsulate and deliver relatively large macromolecular payloads. Regulation of size, structure, shape, surface activity, and environmental responsiveness of polymersome-based carriers are highlighted in several recent reviews [51–53]. Polymersomes are generally more stable and less permeable than liposomes, but these properties can be modulated by the types and ratios of block co-polymers that are selected for their fabrication and the method by which they are prepared. Their properties are also determined by the size of the vesicle and the conditions under which it is stored.

Polymersomes can be produced by solvent switch methods [54], film rehydration [55, 56], solid (or bulk) rehydration [57], and electroformation [24, 58]. Formed by the self-assembly of amphiphilic molecules, polymersomes can be fabricated from several polymers, including poly(L-glutamic acid) (PGlu), poly(ϵ -caprolactone) (PCL), polystyrene (PS), poly(N-isopropyl acrylamide) (PNIPAM), poly(acrylic acid) (PAA) and poly(ethylene glycol) (PEG)/poly(ethylene oxide) (PEO). PEG is used frequently in the fabrication because it confers long circulation times in blood, has low immunogenicity, and has a long track record of use in human therapeutics [59]. Polymersomes are robust versions of liposomes and are well-suited for the development of multicompartimentalized systems that mimic cells and encapsulate cellular processes.

2.2.3. Dendrimersomes—Dendrimersomes also have improved stability and versatility over liposomes and are being developed as drug delivery vehicles (Figure 4) [60]. Like their macromolecular analogs polymersomes, dendrimersomes can be used to simultaneously encapsulate both hydrophilic and hydrophobic compounds. Dendrimersomes are assembled by simple injection of a solution containing an amphiphilic Janus dendrimer, repetitively branched macromolecules with hydrophilic and hydrophobic components, into an appropriately buffered, aqueous solution [61]. Distinct from liposomes and polymersomes, dendrimersomes have superior uniformity of size, ease of formation, and high stability. Dendrimer building blocks, which are highly branched macromolecules, enable further chemical functionalization [62]. Dendrimersomes have excellent mechanical stability compared to liposomes and polymersomes while maintaining a flexibility close to that of natural cellular membrane structures. [63].

Onion-like dendrimersomes, so-called because they have multiple superficial layers (Figure 4), have enabled development of DDSs with controlled release of encapsulated agents over time as the layers can dissolve over long periods [37]. They can consist of as many as 20 bilayers, and long-term dosing, or sequential delivery, is conceivable by enclosing the same or different therapeutic molecules within each layer [62, 64]. Despite these advantages, the mechanism by which onion-like dendrimersomes form still remains poorly understood [65], and their fabrication is a challenging and time-consuming process that limits their wide-spread use.

2.2.4. Droplet-Based Artificial Cells—The methods discussed above (Sections 2.2.1–2.2.3) for the generation of artificial cell DDSs depend primarily on traditional chemical synthesis methods (bulk synthesis methods). However, these methods are critically limited by a lack of precision, resulting in highly non-uniform size distributions of drug carriers that consequently increase the variability of drug release profiles [66]. Thus, development of methods capable of handling smaller volumes in order to provide greater control and reproducibility in the preparation of these carriers have been investigated to create systems that better recapitulate the function of living cells. Microfluidic biofabrication and biosynthesis have emerged in the fields of chemistry [67], medicine [68], biotechnology [69] and pharmacology [70], and microfluidic-based manufacturing would enable greater functionality in the development of artificial cells as droplets [71, 72]. Advanced microfabrication techniques have led to the development of devices with precisely sized microfluidic channels, which translates to a high degree of control over the formation of droplets. Changes in particle size alter the other properties like shape, surface area and porosity, which then can affect bulk properties, product performance, processability and stability. In the fabrication of any cell mimetic, monitoring particle size is paramount to the function, stability and nature of the entity and monitoring its size throughout the entire development and manufacturing process is critical.

Conventional microfluidic approaches for droplet production include T-junctions, co-flowing, and flow-focusing [73]. T-junction microfluidic devices have been used extensively since their first demonstration in the manufacture monodisperse water droplets in oil phase [74]. As shown in Figure 5A, this method employs microfluidic channels where two non-mixing liquids meet to produce droplets. The choice of the method depends on the materials used for droplets generation. For example, T-junction microfluidic devices fabricated by soft lithography methods are easily penetrated by organic solvents, leading to swelling of the channel walls. Thus, in this scenario, flow-focusing junction devices, which have two primary types as dripping and jetting (Figure 5B), that are fabricated of glass, are preferred [75]. In these devices, the dispersed phase is injected into a capillary centered in a larger diameter channel with a continuous phase flowing parallel so that co-flow occurs. Flow focusing is similar to co-flowing; however, droplet formation in flow focusing is achieved by passing the dispersed and continuous phase through a narrow opening, or orifice. The force applied by a continuous phase stream followed by a sudden decrease in the channel width facilitates droplet fragmentation and causes rapid and uniform droplet formation (Figure 5C) [75].

To date, uni- and multi-compartmental artificial cells have been fabricated using microfluidic techniques, including using natural biomolecules [76]. Multi-compartmentalization, a distinctive feature of living cells, can provide separation and protection of the intracellular matrix and protect biological processes from unwanted interference. This biological property has inspired the development of multi-compartment vesicles. Since Schmidt *et al.* first reported the microfluidic-based automated production of lipid-bilayer membranes in 2006 [77], microfluidic technologies have been used to produce artificial cell membranes that can mimic morphologies and functions found in living cells, including compartmentalized structures to mimic cellular organelles [78, 79]. Such basic

microfabrication techniques have provided researchers with facile methods for generating and studying lipid membranes and embedded proteins [80].

Generation of droplets with one or more mono- or hetero-compartments has been accomplished by employing the multi-emulsion method, which uses oil-water-oil (O/W/O) or water-oil-water (W/O/W) multi-emulsions [81]. However, uniformity of droplet size and fine control of the number of higher order droplets is better achieved through microfluidic methods. For example, Alexander *et al.* fabricated a hierarchical protocell model using interfacial self-assembly of copolymer molecules on biopolymer coacervate microdroplets by agitating the solutions with magnetic stir bars (Figure 6A). The copolymeric membranes of the developed artificial cells were naturally semipermeable, enabling incorporation of enzymatic cascades inside the membrane in response to stimuli outside of the membrane. This suggests that it may be possible to develop artificial cells that can participate in molecular communication with neighboring, non-synthetic cells and interact with varied responses depending on signals and environment [82].

In another study, Wang *et al.* connected three different microfluidic devices to create a system to generate emulsion structures in a highly controlled manner, where droplets generated in the first device can be encapsulated in secondary droplets successively generated in the second device (Figure 6B). These emulsion structures contained distinct compartments in which unique biomolecules can be encapsulated. Multi-component emulsions offer the possibility of creating microstructures that closely resemble the organization of natural cells, where organelle compartments are suspended inside the larger cell membrane. Furthermore, this technology enables the co-encapsulation of chemically incompatible molecules (e.g. hydrophobic and hydrophilic molecules), which can be useful in the fabrication of DDSs releasing synergistic compounds or the development of microreactors to carry out biochemical reactions in a manner similar to the vectoral metabolism of living cells [83].

Lu *et al.* produced compartmentalized microcapsules similar to those seen in eukaryotic cells, in terms of size and shape [84]. Typical setups for droplet microfluidics use immiscible aqueous and oily phases, which could be harmful to biological systems. However, Lu *et al.* used an inert gas (either air or nitrogen), instead of oil, in a flow-focusing, microfluidic device to generate biopolymer-containing aqueous droplets [84]. These droplets were then converted to capsules to exploit the electrostatic complexation of oppositely charged biopolymers, along with ionic cross-linking, upon contact with a reservoir solution. This approach provides control over the number and size of the inner compartments in multicompartment capsules and, most importantly, over the contents of each compartment [84].

Bayoumi *et al.* synthesized an artificial cell using encapsulating the multi-compartments in the hydrogel to create vesicles. They incorporated aqueous droplets, stabilized in an oil/lipid bath, inside a hydrogel, which might serve as the basic unit for the bottom-up construction of a protocell, whose assembly could create a proto-tissue—i.e. a simple functional integrated system of interacting protocells [85]. Nuti *et al.* fabricated the multivesicular droplets derived cell structure for the study of compartmentalized biochemical reactions

and their method demonstrated the possibility to mimic artificial organelles with optimized reaction parameters (pH, ions, etc.) in each compartment [86].

Most recently, Elani *et al.* used microfluidics to encapsulate living cells in vesicle-based single droplets (Figure 6C), creating a hybrid cellular bionic system whose external vesicle architecture could provide protection for encapsulated, living cells from toxic environments [87]. Encapsulated cells can act as bioreactor modules that process encapsulated substrates, which are further processed by a synthetic enzymatic metabolism co-encapsulated in the vesicle. The combination of living cells and synthetic materials using this top-down approach may enable development of more complex artificial cells as a way to overcome the inability to recreate biochemical pathways and vectoral metabolism using a purely bottom-up approach [87]. Advantages and disadvantages of the different artificial cell constructs described above are listed in Table 1.

3. Artificial Cells as Advanced Drug Carriers

3.1. Cancer Therapeutics

Cancer is one of the leading causes of death worldwide [95] and, in the United States alone, causes one in every four deaths each year [96, 97]. Chemotherapies are a major treatment strategy that target the metabolic features of cancer cells such as increased cell division and DNA replication; however, despite being characteristics of cancer, these are necessary cellular functions and delivery with a lack of specificity leads to serious off-target effects. Toxicity to healthy cells has significant negative impacts on patient health and their quality of life, necessitating the development of safer, targeted chemotherapeutics [98, 99]. DDSs based on artificial cell technologies are being developed to address this need, as they can protect drug compounds within a self-assembled core while presenting biomolecules on the surface designed to target the drug payload to tumor cells [100]. Furthermore, artificial cell-based drug carriers can be designed with unique molecular signatures, as well as drugs, for the dual purpose of therapy and diagnostics, a concept referred to as theranostics [23, 101].

The use of advanced nanotechnologies to create artificial cell-based drug carriers has enabled targeting cancer cells through: (1) primary (targeted delivery to specific organs), (2) secondary (targeted delivery to cancer cells), or (3) tertiary (targeting the uptake of DDS into specific cellular compartments) means [102]. Targeting strategies can be active, using attachment of specific ligands to the surface of a DDS that recognize and bind cancer cells, or passive, controlled by the circulation time of the DDS in the blood and its accumulation in tumor sites through leaky tumor vasculature [103–105]. With the goal of active targeting, DDSs can be modified with various surface ligands [106], most commonly antibodies or their fragments, peptides, and aptamers.

Artificial cells have the promise to function as new drug carriers with increased therapeutic efficacy and reduced risk of toxic side effects. Doxorubicin (DOX) is one of the most widely used and effective chemotherapies [107]. However, the positive charge of DOX confers a high degree of affinity for negatively charged molecules. For example, DOX binding to cardiolipin, found in the cardiac tissues, increases cardiotoxicity, which has been evidently

related to its lifetime cumulative dose [108]. Therefore, artificial cell carriers are being developed to provide controlled release of DOX and cardiotoxic effects that may occur. The use of carrier formulations has been demonstrated to reduce cardiotoxicity due to lower myocardial drug concentrations [109].

The first DOX-loaded, PEG-modified liposome on the market, Doxil[®], was approved by the US Food and Drug Administration (FDA) in 1995 for treatment of ovarian cancer and acquired immune deficiency syndrome-related Kaposi's sarcoma [110]. Doxil[®] is marketed as Caelyx[®] in Europe. In 2012, a PEGylated, DOX-loaded liposome DDS, known as Lipo-dox[®] or Lipodox, was approved as a generic alternative to Doxil[®] in response to high demand for the drug. A 2012 meta-study compared controlled clinical trials evaluating free DOX or liposomal DOX and found that better patient tolerance and less toxicity effects in patients receiving liposomal DOX [111]. In 2016, Smith *et al.* published a study to compare toxicity, objective response rate (ORR), and time to progression (TTP) of Doxil[®] with Lipo-dox[®] when used for treatment of recurrent ovarian cancer [112]. Results showed that while Lipo-dox[®] and Doxil[®] had similar toxicities, treatment with Doxil[®] significantly improved ORR and TTP compared to Lipo-dox[®] [112]. However, the authors cautioned that additional studies of pre-clinical comparative efficacy and pharmacokinetic/tumor drug distribution studies are underway. To date, a prospective, randomized clinical study has not been published comparing Doxil[®] with Lipo-dox[®] in any other tumor type. Myocet[®] is a non-pegylated, DOX carrier has been approved for treatment of metastatic breast cancer in Europe and Canada since 2000, but has not been approved in the US [113]. Currently, there are a many liposome-based DDSs in U.S. clinical trials which use a variety of lipid formulations to encapsulate chemotherapies, including DOX, daunorubicin (DNR), and paclitaxel (PTX).

Like DOX, DNR is an anthracycline antibiotic drug with anti-cancer activity, but with significant side effects such as cardiotoxicity, alopecia, nausea, and vomiting [114]. As with DOX, researchers have posited that the encapsulation of DNR in artificial cell-like DDSs could be a useful approach to reduce side effects [115]. Creutzig *et al.* developed liposomal DNR (L-DNR), which they found in a U.S. clinical trial (NCT00111345) had increased overall antileukemic activity, and decreased treatment-related mortality when compared to treatment with idarubicin, an anthracycline antileukemic drug for the treatment of pediatric acute myeloid leukemia [116].

PTX is used for the treatment of many solid tumors such as breast, ovarian, lung and prostate cancer, but with severe acute toxicity. Compared to other chemotherapeutics, PTX has poor water solubility, making it challenging to incorporate into nanocarriers [117]. Water solubility and physical stability of paclitaxel can; however, be significantly improved by the loading into PEGylated liposomes [118]. Zhang *et al.* prepared PTX and gold nanoparticles (GNPs)-encapsulated liposomes (PTX-PEG@GNPs Lips), to fabricate a hybrid DDS capable of controlled co-release of multiple therapeutic molecules [119]. In mice with xenografts of HepG2 human hepatocellular carcinoma cells, this hybrid DDS led to sustained release of PTX and efficient tumor inhibition.

Over the past three decades there have been attempts to create DDSs that respond to external stimuli, such as temperature, enabling payload release in an environmentally responsive manner [120, 121]. The first study of drug release from heat-sensitive liposomes was carried out by Yatvin *et al.* in 1978 [122]. In 2010, Yarmolenko *et al.* showed that DOX-loaded, thermosensitive liposomes and mild-hyperthermia combined therapy increased median tumor growth time in mice inoculated with five different cancer cell lines (FaDu, HCT116, PC3, SKOV-3, and 4T07), with the slower-growing cancers (SKOV-3, PC-3) having the highest rates complete regressions and longest tumor growth delays [123]. In 2016, Mao *et al.* developed a thermosensitive, injectable hydrogel containing paclitaxel-loaded liposomes (PTX-lip), which were evaluated for the treatment of pancreatic cancer in mice (Figure 7A). The PTX-lip-gel system was retained near tumor tissue and showed no considerable toxicity to surrounding healthy tissues [124]. To visualize thermal release of small molecules from liposomes, Mackanos *et al.* fabricated thermal labile liposomes containing the small molecule, luciferin, as an amphipathic drug mimetic and injected these into genetically engineered mice that constitutively expressed the enzyme luciferase in all cells. When the liposomes were thermally disrupted with laser irradiation they released luciferin, and the enzymatic reaction with luciferase released photons that could be visualized in living animals as a measure of local drug delivery [125].

Hossann *et al.* demonstrated that vesicle size has a significant impact on the release of drug, specifically DOX, from thermosensitive liposomes *in vitro* across a clinically relevant temperature range of 30 to 45 °C. This effect was attributed to increased packaging defects in small vesicles with significantly increased membrane curvature [126]. Similarly, Hatzakis *et al.* showed that vesicle sizes below 150 nm increased in size with increased occurrences of packaging defects [127]. Characterization of these types of relationships have likely facilitated development liposomal chemotherapies with of temperature-controlled drug release, such as ThermoDox® which is currently in phase III clinical trials for hepatocellular carcinoma in the US [128].

The thermosensitive DDSs discussed above are generally stable at some temperature lower than body temperature, but then begin to degrade and release their contents around 37°C. Thus, the drug release mechanism is relatively passive. Alternatively, active delivery mechanisms, where an external trigger is used to induce drug release specifically at the tumor site (e.g., to which a DDS may be targeted. For example, Yan *et al.* developed liposome-microbubble complexes targeted to breast cancer cells [129]. They then applied focused ultrasound to the tumor area to trigger release of PTX. This approach significantly increased *in vitro* and *in vivo* antitumor efficacy in murine mammary carcinoma models. This is an attractive method of drug delivery, however, any method that depends on a priori knowledge of tumor location is limited to treating only large detectable cancers (1 cm^3), and the undetectable small metastatic lesions will likely remain. The untreated metastatic lesions could be eliminated by an immune response that is activated by killing the primary lesion, however this cannot be predetermined, and relapse will likely occur and lead to death. Therefore, delivery methods that are directed to all cancer cells without a priori knowledge of location will be more effective.

The tumor microenvironment typically has a relatively low pH, inspiring the development of nanocarriers responsive to pH as a method to improve targeting drug release to the tumor without a priori knowledge of location [130]. In a study published in 2018, Yuba *et al.* developed a pH-sensitive, liposomal DDS for the chemotherapeutic agent, bleomycin (BLM) [131]. Liposomes were fabricated from egg yolk-derived phosphatidylcholine modified with N-[methoxy (polyethyleneglycol) 2000]-distearoyl phosphatidylethanolamine (PEG-PE) to achieve prolonged circulation in the bloodstream. While phosphatidylcholine is inherently pH sensitive, addition of 2-carboxycyclohexane-1-carboxylated polyglycidol-having distearoyl phosphatidylethanolamine (CHexPG-PE) provided a spacer group between the carboxyl group and ester group in the polyglycerol moiety to increased sensitivity to a pH-triggered change from a coiled-to-globule conformation when carboxylates are protonated. Intravenous administration of these liposomes lead to the suppression of growth of colorectal cancer cells (Colon-26 cells) more effectively in mice than did the free drug [131]. Bode *et al.* reported that cationic, PTX-encapsulating liposomes led to a reduction in the primary tumor mass and reduced angiogenesis, as compared to the use of conventional treatment, in a model of prostate cancer in rats [132].

Beyond liposomal formulations, a redox-responsive, virus-mimicking polymersome surface-decorated with Angiopep-2 peptide (ANG-PS) was developed to selectively target glioblastoma tumors and efficiently deliver the chaperone plant toxin saporin (SAP) (Figure 7B). Polymersome-based DDSs had anti-tumor activity against the U-87 MG glioblastoma cell line *in vitro* and efficiently delivered the drug across the blood-brain barrier with demonstrable accumulation in human xenografts of U-87 glioblastoma cells in mice [133]. Furthermore, they found that the systemic administration of SAP-loaded ANG-PS to these glioblastoma-bearing mice led only to mild side effects but had effective tumor inhibition, and significantly improved survival [133].

Protein-based drugs have unique properties relative to small molecule chemotherapeutics, including improved therapeutic specificity and reduced side effects. Liposomes and polymersomes have been used successfully for the delivery of peptides, synthetic lipopeptides, or proteins as cancer therapeutics. For example, asparaginase-encapsulated liposomes were reported to improve survival in a mouse model of lymphatic leukemia [134]. Similar to how studies of targeted chemotherapeutics have found that liposome encapsulation mitigates systemic toxicity, Gasper *et al.* showed that liposome encapsulation prevented the production of anti-asparaginase antibodies in animals treated with asparaginase-encapsulated liposomes. In another study, Wang *et al.* designed chimeric biodegradable polymersomes targeting hepatocellular carcinoma, where drug release is triggered by the reducing environment of the cytoplasm (Figure 7C) [135]. Galactose-PEG-PCL, PEG-PCL-poly(2-(diethylamino)ethyl methacrylate) (PEG-PCL-PDEA, asymmetric), and PEG-SS-PCL polymersomes were loaded with bovine serum albumin (BSA) and ovalbumin (OVA) as model biological therapies, or cytochrome C or granzyme B as potential therapeutics. *In vitro* studies showed that therapeutic proteins released by chimeric polymersomes could induce apoptosis of the HepG2 human hepatocellular carcinoma cell line better than free protein [135]. Furthermore, addition of galactose to polymersome surfaces enabled targeting of protein-loaded polymersomes to hepatocellular carcinoma cells through binding to the asialoglycoprotein receptor [135].

In addition to galactose, various molecules have been explored as tumor-targeting moieties for the decoration of nanosized delivery systems, including anisamide, a benzamide derivative, which shows high affinity to sigma receptor. The sigma receptor is a membrane bound protein known to be overexpressed on several human malignancies, including those in lung and prostate cancers [136]. Anisamide-targeted, PEG-based liposomes delivered DOX to DU-145 prostate cancer xenografts in mice and effectively inhibited tumor growth with minimal toxicity [137]. Free DOX was also effective at inhibiting tumor growth, but had significant toxicity. Lu *et al.* developed pH-sensitive degradable chimeric polymersomes decorated with anisamide to target lung cancer. When loaded into PEG-b-poly(2,4,6-trimethoxybenzylidene-1,1,1-tris(hydroxymethyl)ethane methacrylate)-b-PAA (Anis-PEG-PTTMA-PAA) and PEG-PTTMA-PAA polymersomes, granzyme B release was accelerated under mildly acidic conditions (pH:5) [138]. Furthermore, anisamide appeared to effectively target sigma receptor-overexpressing cells, including H460 lung cancer cells and PC-3 prostate cancer cells, through which polymersomes were quickly internalized. In another example of targeting specific tumor cell receptors, Linuma *et al.* investigated the therapeutic effects of DDS consisting of transferrin-modified PEG liposomes, to target human gastric adenocarcinoma cells, and encapsulated cisplatin, a common chemotherapeutic, in a mouse mode of xenografted MKN45 cells [139]. Their results showed that these liposomes might be useful in the treatment of gastric cancer with peritoneal spread [139].

Many biotherapeutics are inherently sensitive to their surroundings and susceptible to biodegradation. Such agents require tailored DDSs. For example, Tran *et al.*, developed a microfluidic-based methods for highly efficient production of amphiphilic, heparin-folate-retinoic acid (HFR) bio-conjugates for selective and efficient delivery of the drug to cancer cells *in vitro* [140]. Another anti-tumor agent requiring an alternative liposome formulation because of rapid changes in bioavailability and frequent side effects is all-*trans* retinoic acid (ATRA). Cristiano *et al.* developed a liposomal carrier for ATRA and reported greater anti-tumor activity, compared to the free drug, on two out of three thyroid carcinoma cell lines (FRO and B-CP, but not PTC-1) after 24 h of incubation *in vitro* [141].

Artificial cells are in development with the dual purposes of tumor imaging/identification and treatment. Belwal and Singh developed a nano-sized, silica-supported PEG liposome for the controlled release of vincristine sulfate, a chemotherapeutic drug used to treat leukemia [143]. In addition to vincristine sulfate, these protocells could be loaded with various dyes to provide different color contrast in during tumor imaging. Notably, addition of silica supports to PEG liposomes increased residence times in circulating body fluids, and thus the number of protocells reaching tumors. Dagar *et al.* modified stabilized liposomes with vasoactive intestinal peptide (VIP), receptors for which are overexpressed in breast cancer cells, and loaded them with both the chemotherapeutic n-methyl nitrozourea (MNU) and the radionucleotide Tc99m. These DDSs were able to actively target breast cancer, delivering MNU and providing a contrast agent for nuclear imaging [144]. Finally, DDSs have been developed to facilitate photodynamic therapy (PDT) for cancer, where cancer cells are selectively heated using a combination of a photosensitive reagent and applied light [145, 146]. Theranostic approaches enable monitoring delivery of the therapeutic agent and often

times can be used to monitor therapeutic outcomes such that image guidance can be used to optimize therapy and determine efficacy.

3.2. Nucleic acid-based therapy

Nucleic acids can be used as therapies, and quite recently as vaccines, and have shown promise for treatment of several human diseases, cancer [147], viral infections [148], and diseases that result from genetic disorders [149]. However, since nucleic acids are positively charged, unstable and targeted for degradation by multiple cellular mechanism complex delivery strategies are necessary for their effective use as therapeutics. Artificial cell-based systems seem ideal for nucleic acid delivery, and have been investigated for the delivery oligodeoxynucleotides, plasmid DNA (pDNA), ribozymes, siRNA, and miRNA [150–152]. Efficiency of targeted delivery of nucleic acid-based therapeutics depends on size, lipid-to-nucleic acid ratio, chain length, and surface charge, the presence of ester bonds, and nature and chain length of ligand composition and their clinical utility will be determined by all of these features [46].

Poor lipid solubility and electrostatic repulsion generally prohibit nucleic acids from spontaneously crossing lipid-rich, negatively charged cell membranes. Thus, design of efficacious carriers for delivery of nucleic acids is essential and a focus of intense investigation [153]. Owing to their opposite surface charge, cationic liposomes interact strongly with negatively charged DNA and RNA molecules and act as good carriers, but with nucleic acids, delivery alone can be inadequate if the nucleic acid must be transcribed and/or translated into their functional macromolecules. DNA-loaded liposomes are positively charged so that they avoid the electrostatic barrier faced by naked DNA to enter natural cells [154]. In addition, the micellar structure of liposomes sequesters water-soluble nucleic acids to the center, leaving lipid-soluble surface groups which can pass through a cell membrane.

The first cationic pDNA-liposome system was developed by Felgner *et al.* in 1987 [155]. They demonstrated improved DNA transfer, also known as transfection, efficiency by utilizing a synthetic cationic lipid, N- [1- (2,3-dioleoyloxy) propyl] -N, N, N-trimethylammonium chloride (DOTMA), as a unilamellar liposomal carrier. DOTMA facilitated the fusion of the lipid-DNA complexes with the plasma membrane of cultured cells, resulting in both uptake and expression of DNA. Since then, many liposome-gene complex systems have been developed and optimized for use in a large number of delivery applications. Phospholipids commonly used for the stabilization of the liposome/DNA complexes include dioleoil phosphatidylethanolamine (DOPE) [156], dioleoyl phosphatidyl choline [157], DC-cholesterol HCl (3 β - [N- (N', N'-dimethylaminoethane)-carbonyl] cholesterol hydrochloride) [158], DOTAP (1,2-dioleoyl-3-trimethylammonium-propane [chloride salt]) [159], DOBAQ (N- [4-carboxybenzyl] -N, N-dimethyl-2,3-bis (oleoyloxy) propan-1-aminium) [160], DDAB (dimethyldioctadecylammonium [bromide salt]) [161] and MLV5(N1-[2-((1S)-1-[(3-aminopropyl)amino]-4-[di(3-amino-propyl)amino]butylcarboxamido)ethyl]-3,4-di[oleoyloxy]-benzamide) [162]. The ability of cationic liposomes to complex with nucleic acids to increase their ability to transfect animal target cells has led to their pervasive use for non-viral gene delivery. Double

stranded nucleic acid is more stable than single stranded nucleic acid, and the prevalence of ribonucleases that cut RNA can limit the use of RNA as a therapy—protecting the nucleic acid while also delivering a functional molecule is a challenge and a number of successful strategies have been reported.

The design and synthesis of biodegradable polymeric materials that provide suitable polymeric carrier properties for temporal and spatial distribution of nucleic acids in the body have been described [163, 164]. Polymeric carriers targeting based on particle size and surface charge represent a critical issue for material selection and design; however, the release of the active agent will also depend on the distribution in the carriers and the rate of degradation of the polymer. An ideal gene delivery system should show high transfection levels for long-term administration, be non-toxic and biodegradable. Polyethyleneimine (PEI) is one of the most effective cationic compounds used for nucleic acid delivery into mammalian cells [163]. In 2009, Koh *et al.* developed PEI/pDNA complexes using a microfluidic, hydrodynamic focusing technology to efficiently condense pDNA. Compared to bulk mixing, this method improved size control, transfection efficiency, and cytocompatibility of the PEI / pDNA complexes [164]. This method was particularly useful for assembling complexes with small plasmids and oligonucleotides, which are less susceptible to chain breakage at higher flow rate ratios.

Using pH-sensitive poly(2-(methacryloyloxy)ethylphosphorylcholine)-co-poly(2-(diisopropylamino)ethylmethacrylate) (PMPC–PDPA) diblock copolymers, Lomas *et al.* developed polymersomes for encapsulation and delivery of pDNA as a non-cytotoxic synthetic vector for the rapid and efficient intracellular delivery of active agents [165, 166]. DNA-loaded polymersomes have biomimetic surfaces due to the presence of PMPC chains and minimize any interaction with blood plasma proteins and hence prolonged the average polymer circulation time in the blood. The PMPC-PDPA polymersomes are able to retain encapsulated DNA for at least two weeks, indicating that their membranes have a very low degree of “leakage”. Also, unlike cationic liposome transfection agents, PMPC-PDPA polymersomes led to very low cytotoxicity and significantly reduced levels of cellular damage [165, 166].

Small interfering RNA (siRNA) and siRNA mimetics have the advantages of being inherently functional without needing to be translated into proteins, and being catalytic offering efficiency for turning genes and pathways on or off. As such, these molecules are potentially useful therapeutic tools for silencing disease associated genes or activating beneficial pathways, however, use of any nucleic acid is limited by instability. The challenges of using siRNA as a therapy are maintaining stability in the circulation, directed delivery to the target cells, and effective release within the target cell for function. Discher and colleagues showed delivery of siRNA and antisense oligonucleotides using biodegradable block copolymers, including PEG-b-PCL, PEG-b-PLA, and PEG-b-PBD-based non-ionic polymersomes [167]. By varying the copolymer molecular mass, the mechanical properties and permeability of the polymersomes can be adjusted, and generally they circulate *in vivo* for much longer time than lipid vesicles and cationic carriers. The results indicated that non-ionic polymer vesicles are both biocompatible and

immunocompatible, offering advantages over viral-mediated delivery of siRNA encoded as short hairpin RNA (shRNA) or use of cationic compounds.

While targeted delivery can selectively and preferentially deliver therapeutic agents to disease sites to improve therapeutic efficacy, the reticuloendothelial system comprised of phagocytes and the renal clearance pathway are frequent dead end destinations of systemically delivered nanoparticles, and represent the greatest obstacles to successful delivery [168]. Wilhelm *et al.* analyzed 117 published studies that reported the delivery efficiency of various nanoparticles to tumors [169]. Overall, a median of about 0.7% of the injected nanoparticles reached their targets. Moreover, the percentage of nanoparticles reaching cancer cells *in vivo* maybe be even less than 0.7% because of the dense tumor stroma. Addressing this low efficiency is a central challenge for translating nanomedicines into the clinic.

Extracellular vesicles (EVs), which are natural cell-derived vesicles important for intercellular communication between adjacent and distant cells in the body [170–174], and they present the opportunity to create artificial cells as DDSs that overcome these issues with synthetic nanoparticles. EVs have been shown to be traverse dense extracellular matrix (ECM) environment, while liposomes or synthetic nanoparticles with similar sizes cannot diffuse through the ECM [175]. Deformability of EVs appears to be essential for their superior ability to transverse tissue [176]. The naturally produced lipid bilayer membrane of EVs varies in composition from the cell membrane, rendering them highly stable [177]. In addition to the advantages of good tissue penetration and stability in circulation and tissues, biocompatibility and low immunogenicity have led researchers to explore the use of EVs as nanocarriers for various cargo therapeutics, from chemotherapeutics to genes [178–180].

EVs can be engineered to carry therapeutic cargo, including proteins, drugs, or nucleic acids (miRNA, DNA), using active and passive bottom-down approaches. In active approaches, therapeutic cargo can be incorporated into donor cells, and subsequently packaged into EVs, through co-incubation with drugs or transfection [181], and loaded EVs can then be isolated. In passive approaches, EVs are isolated prior to loading with therapeutic cargo using various physical methods including incubation, sonication, electroporation, extrusion, permeabilization, and freeze-thaw cycling [181–183]. EVs are also uniquely suited as a platform for development of targeted therapies since they are produced with cell receptors that interact with cells and sometimes preferentially with specific cell types [184–186]. Additional engineering of EVs, such as hybrid carriers fusing liposomes with EVs [187–189] or surface decoration with PEG [190], can be performed to potentially improve delivery efficiency and circulation times. Despite the exciting potential of EVs as clinical therapeutics, additional work is needed to fully characterize their biodistribution and pharmacokinetics as well as to achieve controlled, reproducible EV production [191].

The limited number of EVs produced by cells hampers their applications. Therefore, artificial EV-mimetics have been generated and investigated as a therapeutic tool for drug delivery [192]. However, most of the artificial EV-mimetics proposed or studied to date are actually liposomes. Small unilamellar vesicles (SUVs) are ideal precursors for the preparation of vesicles that can mimic exosomes due to their similarities to natural exosomes

(size range and membrane disposition). Lu *et al.* developed exosome-mimicking liposomes for delivery of VEGF siRNA to A549 cancer cells and HUVECs. These exosome-mimetics had lower cytotoxicity and higher storage and physical stability (reduced aggregation) in serum. It was also reported that they can endocytosis into A549 cells and HUVECs, and their oligonucleotide delivery efficiencies are much lower compared to those of cationic lipids such as Lipo-2000 and DOTAP [193].

3.3. Vaccine Delivery

Vaccines evoke immune responses in patients leading to the production of antibodies and protection against future disease [194]. Vaccine delivery systems based on artificial cells may offer significant improvements to more traditional systems. Traditional vaccines generally contain inactivated or attenuated disease-causing agents, such as antigenic proteins of pathogens, which are generally associated with toxicity, allergic responses, hypersensitivity, or inflammation [194]. Subunit vaccines, are prepared from a fragment of the pathogens (e.g. carbohydrates, recombinant DNA, RNA or synthetic proteins) and reduce the danger of inducing the disease by living viruses or bacteria as vaccines [195]. Liposomes and their derivatives have intensively been studied as potential drug carriers for the effective delivery of peptides as antigenic subunits [194–210]. Liposomes can also improve the stability of antigens by providing protection against enzymatic degradation, thus reducing the required dose [211]. Antigens can be encapsulated within the liposomes at different stages during liposome preparation [198, 212] or, alternatively, covalently conjugated [195] or physically adsorbed [198, 202] to the liposomal surface. The method by which antigens are incorporated can have distinct effects on their immunogenic potential. For example, Quer *et al.* reported that the adsorption of hemagglutinin antigens to a liposomal surface was more effective in evoking an immune response than antigenic encapsulation inside liposomes [198]. More recently Lanza *et al.* demonstrated that Leishmania hypothetical (LiHyp)-liposomal vaccines incorporated into a dissolvable microneedle patch provide unprecedented control over antigen release and long-term protection against parasitic leishmaniasis infection [210].

The composition of liposomes is also important in inducing immune responses to antigens [213, 214]. Phillips *et al.* showed that liposomes prepared from phosphatidylethanolamine (PE) or phosphatidyl glycerol (PG) were more effective than phosphatidylserine (PS)-based liposomes in inducing immune responses [213]. More recently, Kaur *et al.* showed that the presence of cholesterol in liposomes increases bilayer rigidity, resulting in prolonged residence *in vivo* [215]. However, Nakano *et al.* showed that cholesterol in liposomes can adversely affect the immunoglobulin (IgG) response [216], indicating that cholesterol may also reduce the immunogenicity of liposomes [195].

As liposomes include an inner aqueous compartment and hydrophobic outer surface (Figure 8A–C), they are capable of incorporating both hydrophilic or hydrophobic components; for example, to create dual antigen delivery [194]. In another example, the outer membrane of liposomes can be modified with APC-targeting molecules, such as mannose to facilitate antigen delivery (Figure 9A) [195, 207, 208]. The surface charge also plays a critical role in liposomal interactions with APCs (Figure 9B). Conventional wisdom indicates

that cationic liposomes are more likely to interact with anionic cell membranes and efficaciously deliver the antigen payload into cells into antigen-presenting cells (APCs; e.g., macrophages and dendritic cells), evoking a stronger immune response. However, some studies have shown a higher efficacy of negatively charged liposomes to induce Th1- and Th2-biased cellular immune responses, implicating other factors, such as the type of phospholipids on liposomes, may substantially affect APC-liposome interactions [195]. Other liposome-based vaccine delivery systems include, but are not limited to, virosomes and vesosomes. Virosomes are unilamellar liposomes based on viral membrane proteins and have been used widely for vaccination against hepatitis A and influenza (Figure 9C). Vesosomes, or “double-bagged liposomes”, have dual phospholipid bilayers to provide enhanced protection of encapsulated antigens (Figure 9D) [195]. Boyer *et al.* showed that the external membrane of vesosome can provide a physical barrier to protect the internal membrane from degradation, thus increasing the drug retention time from minutes to hours [217].

Polymerization of liposomal nanocarriers can improve their stability under physiological conditions. For example, vaccines for SARS-CoV2 (the virus that causes COVID-19) that have recently developed by Pfizer and Moderna and widely distributed among the human population, contain an mRNA that encode a viral subunit that is wrapped in liposomal nanoparticles, which are then coated by a layer of PEG to increase their residence times *in vivo* [218, 219]. The challenge of using mRNA as a therapy or vaccine is that it must be delivered to the cytoplasm of cells in a way that it can escape the endosome and be translated into protein. This presents a significant challenge that was overcome by this double coating approach by Pfizer and Moderna. An alternative approach is to use charge-altering releasable transporters (CARTS) where the encapsulated mRNA has a net charge that allows binding and internalization by the cells, and then the charge changes to escape the endosome and release functional mRNA that can be translated into functional proteins [220].

A wide variety of polymerized liposomes have been developed as oral or nasal vaccines as they exhibit high stability, controlled antigen release and mucosal antiviral responses in mice immunized with these liposomes [197, 201, 204, 209]. Beyond liposomes, other types of vesicle-based artificial cells such as polymersomes have also shown some potential for vaccine delivery. For example, polymersome nanocarriers have been shown to enhance the immunogenicity of influenza subunit vaccine at *in vivo* [221] and humoral immunity against Lassa virus (LASV), which is responsible for severe hemorrhagic fever with high mortality [222]; however, these polymeric nanostructures have not reached clinical trials [223].

Subunit vaccines made from highly purified antigens often show poor immunogenicity, however, subunit vaccines exhibit some distinctive structural features, particularly defined as the pathogen/damage/danger-related molecular patterns (PAMPs) exhibited on microbe surfaces. To overcome the poor immunogenicity, subunit vaccines are often combined with an adjuvant or included in a carrier to direct antigens to APCs and enhance the immunostimulatory effect [194]. These adjuvants may include liposomes, emulsions, polymeric and inorganic nanoparticles, or pattern recognition receptors such as squalene, saponin and insoluble aluminum salts [224, 225]. Liposomes have long been validated

as intrinsic adjuvants which, unlike most adjuvants, have minimal reactogenicity [226]. Other advantages of liposomal adjuvants are biodegradability, good biocompatibility and controlled release of encapsulated payloads [227]. Liposomes can exert strong immunomodulatory effects due to their particulate structure and their ability to interact with APC lipid receptors through targeting molecules on their surfaces, thus fusing with the wall of target cells and getting rapidly incorporated into the reticuloendothelial system [194]. Liposomes can also be used to encapsulate structurally different epitopes to induce various immune responses [228–230]. For example, Cole *et al.* showed that TLR7/8 and TLR4 agonists co-encapsulated liposome delivery systems used with a split-flu vaccine resulted in stronger immune responses against heterologous influenza infections [203].

4. Perspective and Conclusion

As engineered biological systems, artificial cells can be developed to offer unique opportunities for drug delivery with improved safety and efficiency. An artificial cell can be fabricated using either top-down or bottom-up approaches, where each strategy has its advantages and limitations. Among the artificial cell fabrication techniques, microfluidic-based methods represent one of the most advanced tools for bottom-up assembly of artificial cells. In general, bottom-up production of artificial cells as DDSs, especially with the use of microfluidic systems, is expected to lead to the development of autonomous microcarriers with narrow polydispersity that can monitor their environment and intervene when necessary. However, DDSs currently approved for clinical use are typically made using traditional emulsion methods, because microfluidic platforms suffer from the limited quantity of synthesized particles that they can produce [231, 232]. Alternatively, EVs provide a promising platform for top-down fabrication of artificial cells. In general, bottom-up approaches have been more successful. Artificial cells that use liposomes, polymersomes, or dendrimersomes to create a membrane component have been developed as advanced DDSs for cancer treatments, gene therapies, and vaccines. Surface modification of these carriers can improve payload delivery, through increasing circulation times and preventing degradation, and enable systems with active-targeting and stimulus-responsive capabilities. Overall, artificial cells can be fabricated with various sizes, deformability, sustained/controlled payload release profiles, and versatile targeting functions.

However, most attempts to create artificial cells have focused on recreating biological-like activity under laboratory conditions in the absence of other living cells. But a few laboratories have begun to put together more complex ecosystems of different artificial cells or artificial cells mixed with natural living cells [233, 234]. Artificial cells that integrate with the wider cellular community can help reveal the physicochemical underpinnings of cellular behavior and provide opportunities for designing advanced drug delivery systems. Therefore, despite the potential and future implications of this technology, further studies on the interaction of artificial cells with eukaryotic cells under physiological conditions are needed.

Other promising approaches to consider as DDS are Cellular hitchhiking [235, 236] and cell-particle hybrid systems [237, 238]. Cellular hitchhiking method involves nanoparticle attachment to circulating blood cells to improve the delivery capabilities of the nanoparticles

(e.g. targeting and circulation). Unfortunately, these delivery systems are still in their inception and therefore not generally used and much less researched, but systems developed and tested in preclinical settings have demonstrated improved targeting and stealth capabilities over their chemically and physically modified counterparts. The combination of these strategies with artificial cells could pave the way for the development of new DDSs.

Despite extensive progress over the last few decades, before artificial cells capable truly dynamic and interactive relationships with natural biological systems are created, important questions must be answered regarding: (i) communication between artificial cells and their local environment; and (ii) interactions between different types of artificial cells with different functions. There are still remarkable differences between the living cellular membranes and their artificial compartments, and studies to develop new materials as well as basic experiments to better understand how a membrane's composition contributes to its physical properties and biological functions are important to bridge this gap. In fact, artificial cells will likely facilitate these basic biological studies. To date, a limited number of materials have been used for the production of artificial cells. Therefore, the development of alternative and new smart and improved properties such as nanoscale efficiency, self-organization, and adaptability for therapeutic and diagnostic applications is needed. Ultimately, creating artificial cells with properties and functions of living cells and have broad clinical utility will require extensive cross-disciplinary cooperation among the fields of biophysics, biochemistry, medicine, biomedical engineering, materials science and molecular/cellular biology.

Acknowledgements

The authors have no **competing interests**. We acknowledge funding from National Institutes of Health (1UG3TR003148-01, N.A.), the American Heart Association (COVID-19 Rapid Response Award 20203858, N.A.), and generous support from the James and Kathleen Cornelius Endowment (C.H.C). S.E.D. would like to thank the Scientific and Technological Research Council of Turkey (TÜB TAK) for financial support. M.T. would like to acknowledge financial support from Fonds de recherche du Québec - santé (FRQS).

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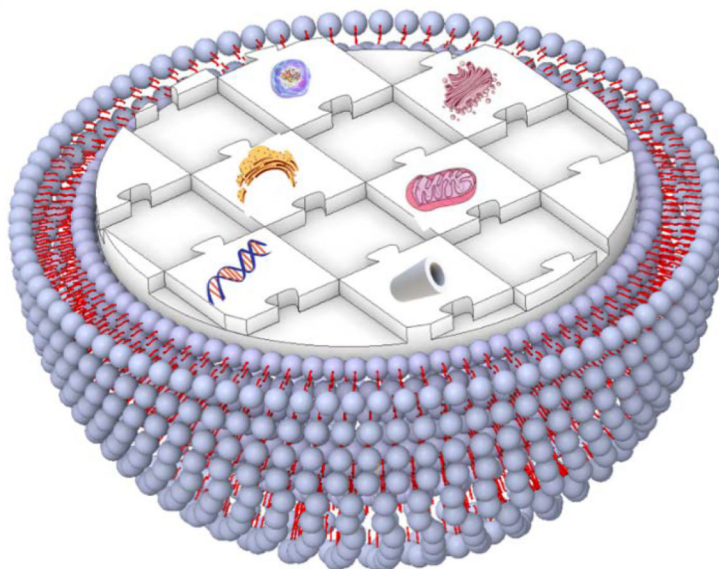


Figure 1. Schematic illustration representing a human-made artificial cell comprising of nucleic acids, cytoskeleton, small biomolecules, and cytoplasmic organelles in a giant unilamellar vesicle.

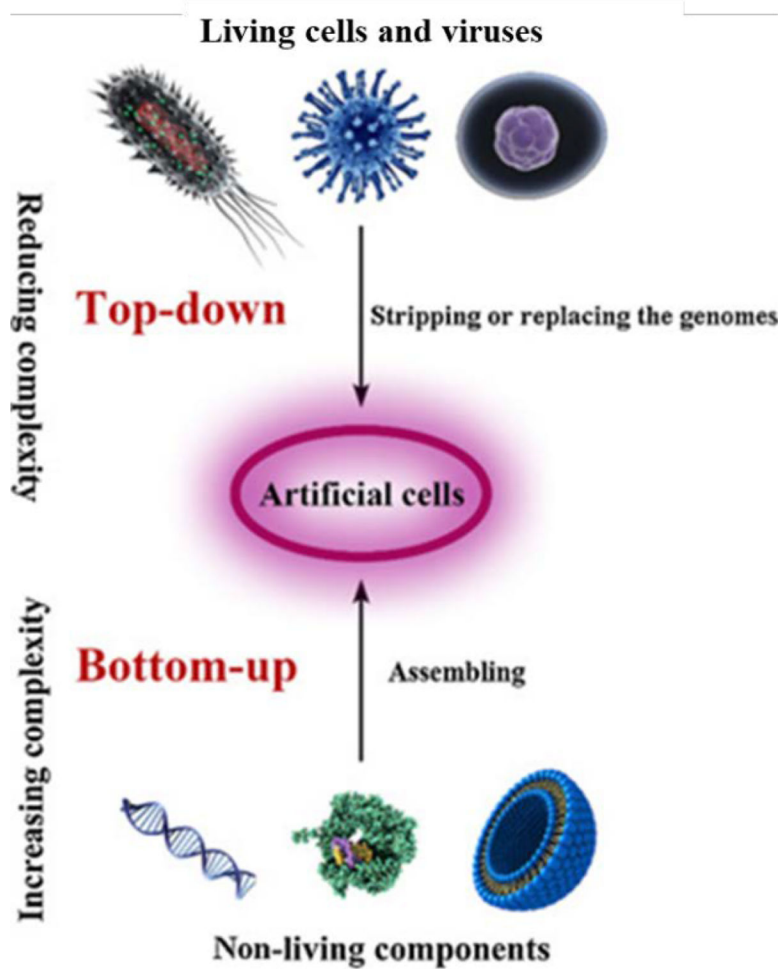
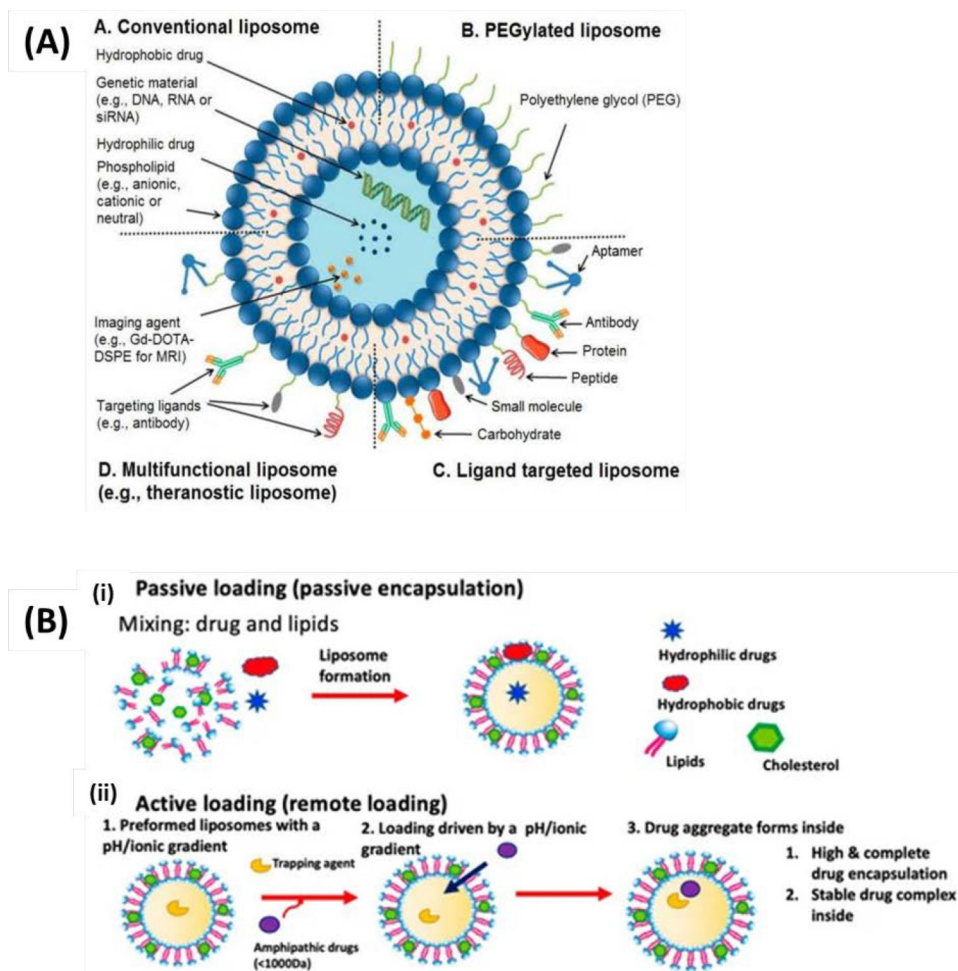


Figure 2. The construction of artificial cells with top-down or bottom-up approaches. Adapted from Xu *et al.* [17], with permission from Elsevier.

**Figure 3.**

(A) Liposomes: Conventional liposomes (A); PEGylated/stealth liposomes (B); ligand targeted liposomes (C); and multifunctional liposomes, (D). Reproduced from Riaz *et al.* [50] with permission from MDPI (B) The two major methods for liposomal drug loading. (i) Passive loading involves co-current loading and liposomal formation. (ii) In the active loading, liposomes are formed containing a gradient used to load drugs. Reproduced from Pauli *et al.* [47]. is an open access article distributed under the Creative Commons Attribution License.

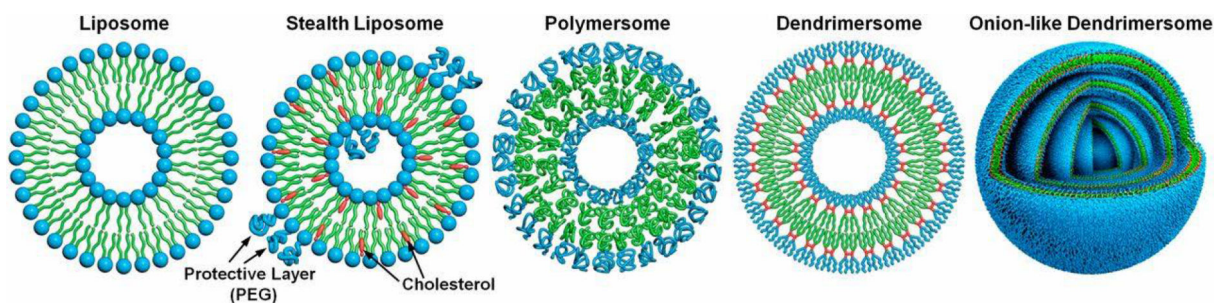


Figure. 4. Strategies for the preparation of single-bilayer vesicles and multilayer onion-like vesicles. Reproduced from Zhang *et al.* [60], with permission from the National Academy of Sciences of the United States of America.



Figure 5. Microfluidic geometries are used to generate droplets. (A) T-junction, (B) co-flow, (C) flow-focusing. (1: continuous phase, 2: dispersed phase). Reproduced from Zhang *et al.* [75], with permission from Elsevier.

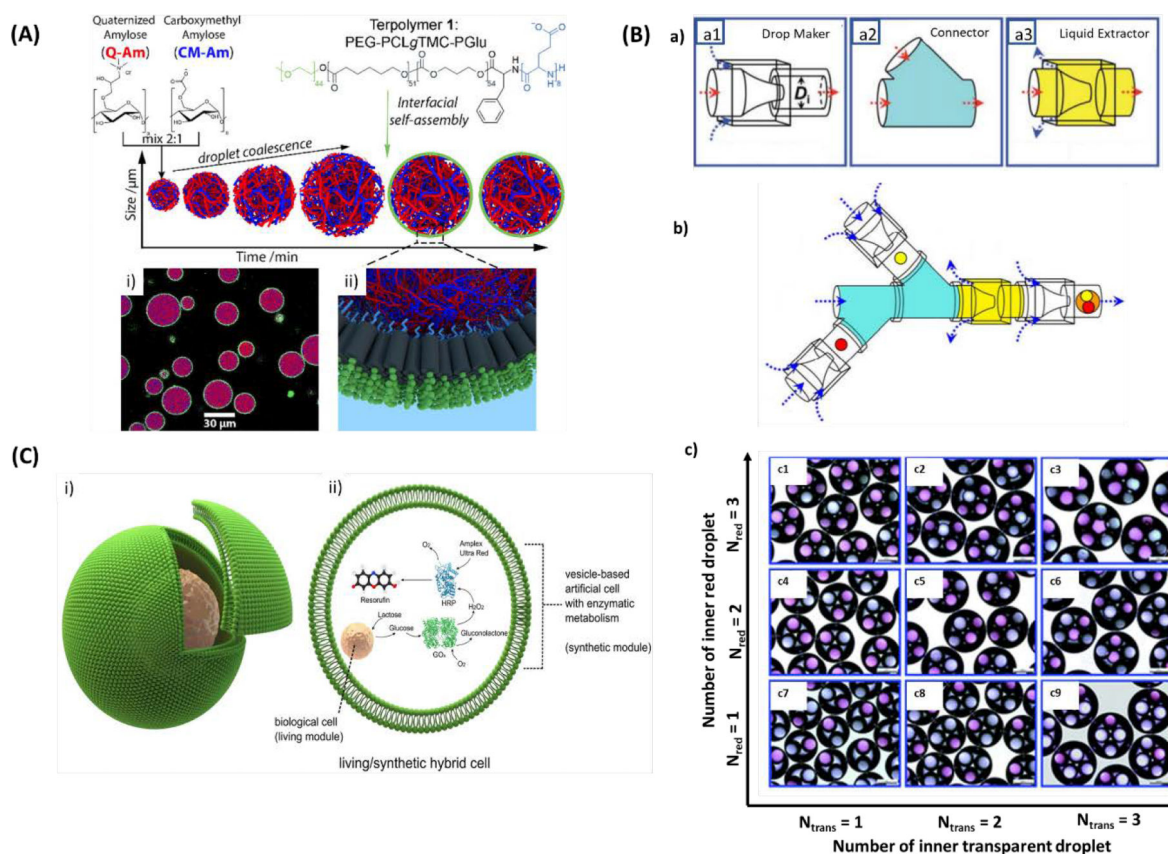
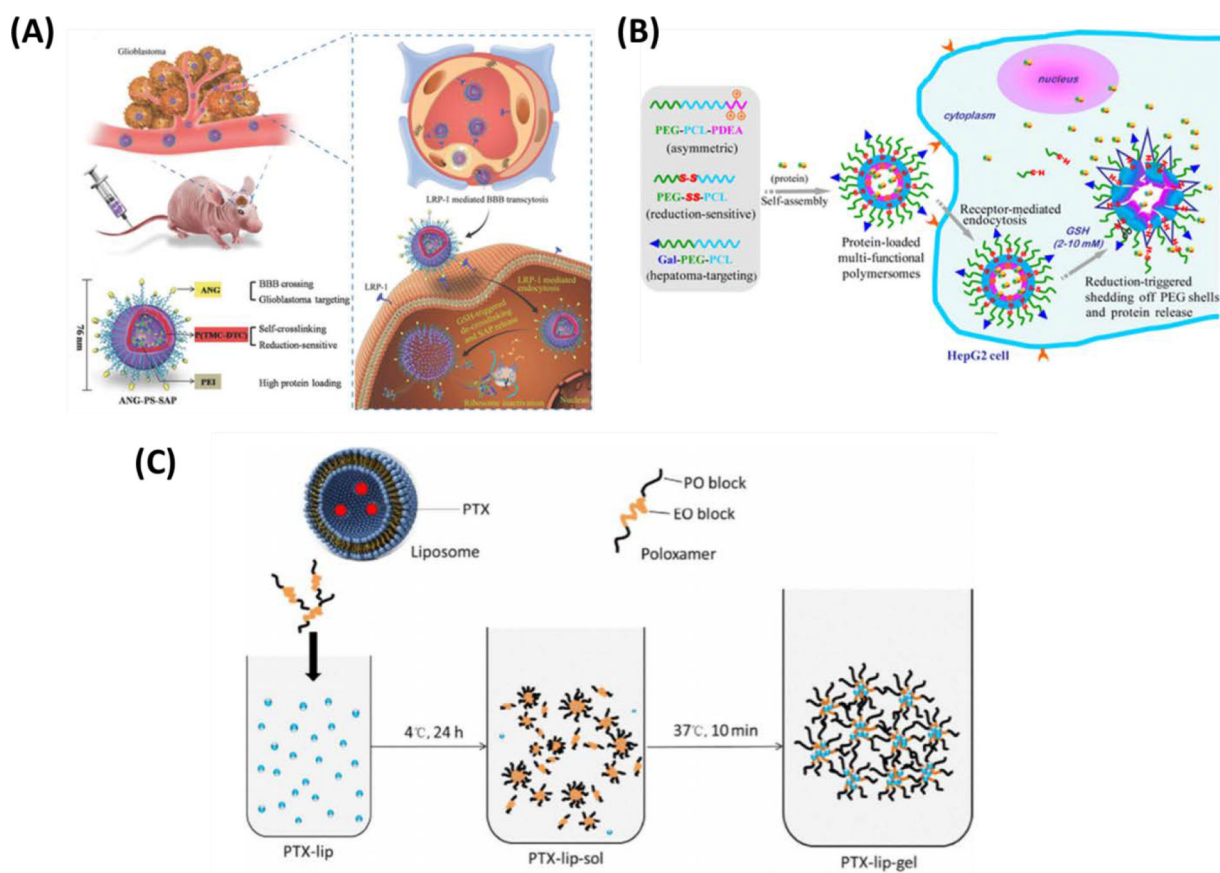


Figure 6.

(A) Hierarchical self-assembly of a biodegradable triblock copolymer-stabilized cell-sized coacervate microdroplets. (i) Confocal micrograph of terpolymer/coacervate microdroplets with absorbed BSA-FITC (purple) and terpolymer membrane (green, Nile Red). (ii) 3D representation of the interfacial assembly of terpolymers. Reproduced from Mason *et al.*[82], with permission from the American Chemical Society. (B) (a) The microfluidic device parts to generate multicomponent multiple emulsions, (b) The schematic of the constructed microfluidic from a combination of three functional building blocks, (c) Optical micrographs of quadruple-component double emulsions exhibiting the precise control over the number and ratio of inner red and transparent droplets. Scale bars are 200 μm . Reproduced from Wang *et al.* [83], with permission from the Royal Society of Chemistry. (C) The construction of living/synthetic hybrid cells. (i) Diagram of a biological cell encapsulated artificial cell. (ii) The encapsulated biological cell has an organelle-like function in the vesicle reactor. Reproduced from Elani *et al.* [87], with permission from Springer Nature.

**Figure 7.**

(A) Schematic presentation of ANG-directed and redox-responsive virus-mimicking polymersomes (ANG-PS) for efficient loading and selective chaperone of SAP to orthotopic human glioblastoma xenografts in nude mice. Reproduced from Jiang *et al.* [142], with permission from WILEY-VCH Verlag GmbH & Co. (B) Schematic illustration on hepatoma-targeting reduction-sensitive biodegradable chimeric polymersomes for active loading and intracellular release of proteins. Reproduced from Wang *et al.* [135], with permission from the American Chemical Society (C) The loading process of PTX liposome in the hydrogel. Reproduced from Mao *et al.* [124], with permission from Elsevier.

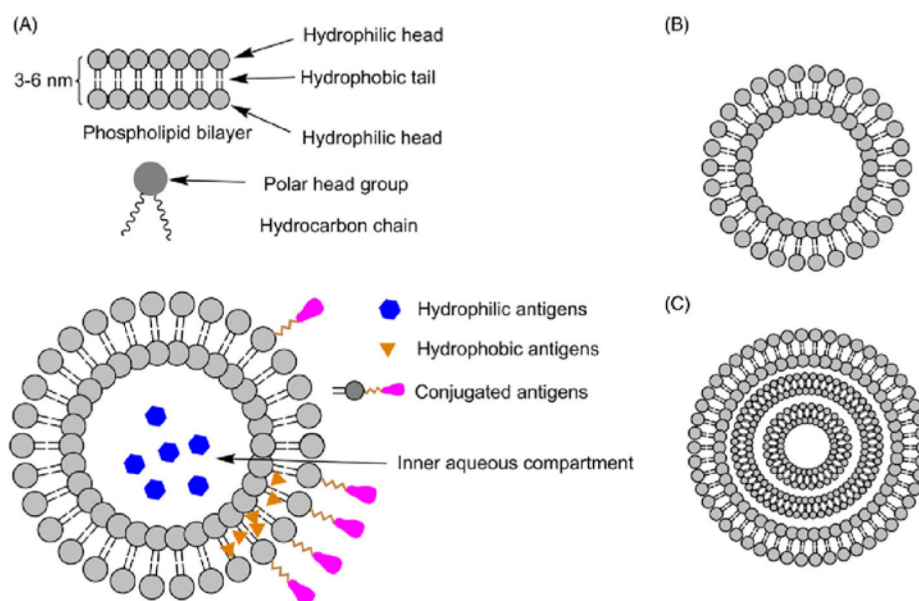


Figure 8. (A) Schematic illustration of liposomes as a vaccine delivery system. (B) Unilamellar liposomes. (C) Multilamellar liposomes. Reproduced from Marasini *et al.* [195], with permission from Elsevier.

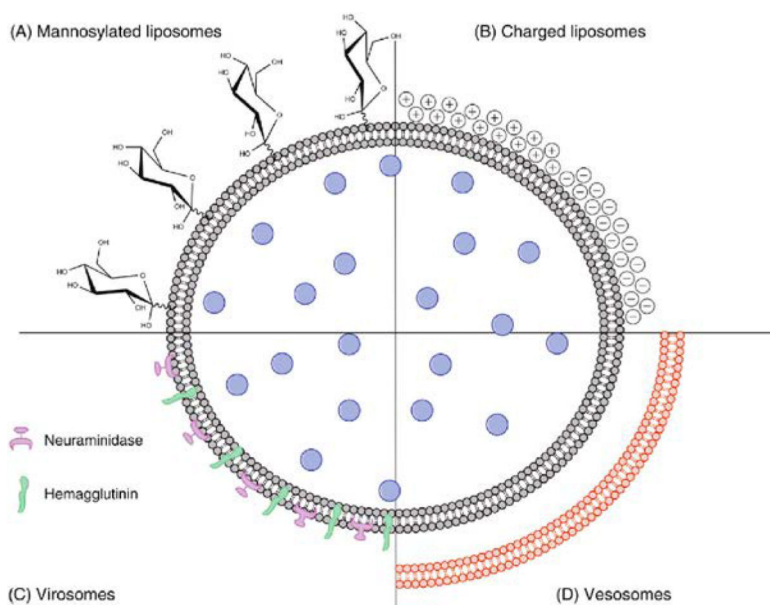


Figure 9. Schematic presenting various types of liposomes for vaccine delivery. (A) Modified liposome with a targeting moiety (e.g. mannose). (B) Cationic or anionic liposomes, (C) virosomes, and (D) vesosomes. Reproduced from Marasini *et al.* [195], with permission from Elsevier.

Table 1.

Comparative overview of advantages and disadvantages of different vehicle as artificial cells.

Vehicle	Advantages	Limitations	Reference
Liposomes	<ul style="list-style-type: none"> - Various sizes with either single or multiple lipid bilayers, - Diversity of lipid compositions - Simple fabrication process - Active clinical research 	<ul style="list-style-type: none"> - Potential complement activation and low cellular uptake - Insufficient drug loading and leakage - Premature drug release - Limited storage conditions 	[88, 89]
Polymersomes	<ul style="list-style-type: none"> - More chemical stability lifetime than liposomes - Hydrophilic core and a hydrophobic bilayer allowing encapsulation of both hydrophilic and hydrophobic drugs - Functionality for targeted and stimuli-responsive (pH, redox, enzyme, ultrasound, magnetic field, light) drug delivery 	<ul style="list-style-type: none"> - Residual organic solvent - Incompetent control of the early drug release - Cumbersome fabrication steps, and toxicity concerns 	[90, 91]
Dendrimersomes	<ul style="list-style-type: none"> - Modifiable branches of dendrimers - Controllable size and monodispersity - High penetration into cell membranes 	<ul style="list-style-type: none"> - High cost of productions - Improved quality control - Lack of clinical practice 	[92]
Droplet-based artificial cells	<ul style="list-style-type: none"> - Simple and reproducible - Amenable to automation - Narrow size distribution - Large scale manufacturing - Small volumes 	<ul style="list-style-type: none"> - Manufacturing and microfluidic skills required - Limitations in generating nano-sized structures 	[93, 94]