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Trends in Biotechnology

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Review

Can Bottom-Up Synthetic Biology Generate Advanced Drug-Delivery Systems?

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Creating a magic bullet that can selectively kill cancer cells while sparing nearby healthy cells remains one of the most ambitious objectives in pharmacology. Nanomedicine, which relies on the use of nanotechnologies to fight disease, was envisaged to fulfill this coveted goal. Despite substantial progress, the structural complexity of therapeutic vehicles impedes their broad clinical application. Novel modular manufacturing approaches for engineering programmable drug carriers may be able to overcome some fundamental limitations of nanomedicine. We discuss how bottom-up synthetic biology principles, empowered by microfluidics, can palliate current drug carrier assembly limitations, and we demonstrate how such a magic bullet could be engineered from the bottom up to ultimately improve clinical outcomes for patients.

Highlights

Nanomedicine has demonstrated the potential of nanotechnology in treating diseases by selectively targeting pathogenic cells and releasing their cargo on site, but the complexity of molecular engineering such drug-delivery vehicles impedes their broad application and clinical translation. New methodologies to generate more advanced and intelligent systems are required.

Bottom-up synthetic biology, empowered by microfluidics, allows the conception of multifunctional cell-mimicking structures – such as synthetic exosomes – that showcase its ability to create sophisticated systems.

Recently, considerable progress has been made towards the assembly of complex structures that can dynamically release therapeutics, sustain protein biosynthesis, and sense and interact with the nearby environment. These functionalities will propel the creation of advanced drug-delivery platforms.

Drug-Delivery Challenges: Opportunities for Bottom-Up Synthetic Biology

Paul Ehrlich, considered to be the pioneer and founder of modern chemotherapy, envisaged a therapeutic capable of directly interreacting with its intended disease-causing cellular structure while remaining harmless to the surrounding healthy cell population. Depicted as a magic bullet, his idea has greatly influenced and fascinated various fields of science for more than a century [1]. Among them, the field of **nanomedicine** (see Glossary) – which relies on nanotechnologies to improve passive and active accumulation of drugs nearby target pathogens or cell populations – was expected to achieve this highly coveted goal. Despite its major focus on oncology, nanomedicine has also triggered the engineering of an arsenal of novel nanotechnologies and functionalization strategies. Overall, these innovations have resulted in a variety of enhanced bio- and physico-chemical properties for inorganic-, polymer-, and lipid-based nanometric carriers.

Despite recent progress, nanomedicine is often synonymous with modest clinical translation and remains the focus of significant debate (as reviewed extensively [2–4]). Isolated examples of success, namely Doxil® [5,6], Abraxane® [7], and most recently Onpattro® [8], are few, and greater success in the design of nanoformluated drugs in the near future remains unlikely because several barriers will need to be overcome to achieve effective and specific delivery of drug-loaded carriers.

Targeted delivery of therapeutics may be organized into different levels, referred to as primary, secondary, and tertiary targeting. These levels are defined by the degree of target specificity and control over release dynamics, which increase along the chain [9]. This targeting hierarchy is summarized and illustrated in Boxes 1 and 2, and also in Figure 1. Briefly, primary targeting, or the targeting of specific organs, is highly size-dependent since smaller nanomaterials are expected to transit through the blood–brain barrier [10–12], or navigate through the fenestrated vessels in the liver endothelium or tumor environment more easily [13]. However, this size discrimination may be circumvented by meticulously engineering the physicochemical properties of the carrier such as molecular composition, surface charge, and mechanical properties (Box 1). To

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Box 1. The Targeting Hierarchy of Cell-Selective Drug Delivery: Primary Targeting

Primary targeting implies the specific, in most cases passive, accumulation of therapeutics in the desired organ where disease-causing cells reside (see Figure 1, left in main text). So far, primary targeting has mostly focused on organs that have high levels of mononuclear phagocyte system (MPS components, lower blood velocity (i.e., low blood pressure), and small blood capillary diameters. These characteristics, which are common for the liver, spleen, kidney, and lymph nodes, lead to an increase in the passive accumulation of therapeutic carriers in these organs. Consequently, primary targeting of these organs enabled the acquisition of important trends and criteria that needs to be considered when assembling a drug-delivery system. To reach the desired targeted site, a drug-delivery system must have a long circulation time inside the bloodstream, meaning that the carrier must be able to evade the immune system, minimize opsonization (i.e., nonspecific adsorption of serum proteins onto the carrier surface), and, most importantly, evade the MPS. The latter is the greatest obstacle to successful primary targeting. To put it in numbers, the MPS has been made responsible for the fact that only 0.1% of nanomedicines accumulate at the target site [14]. Multiple interdependent parameters modulate the interaction of a drug carrier with the MPS and affect its biodistribution and accumulation during its journey towards a specific organ [68,69]. These parameters include surface charge, particle size, geometry, porosity, chemical composition, and mechanical properties. All of these need to be optimized to maximize delivery efficiency and thus therapeutic outcome. Furthermore, in the case of cancer therapy, additional optimization to account for tumor type and stage needs to be included [70,71].

Typically, the targeting efficiency of specific organs – such as the brain and the tumor environment – is highly size-dependent. Despite the general observation that smaller delivery vehicles lead to higher uptake, various reports also showed that size might be less important than other surface properties [72,73]. For example, the transport of larger carriers across the blood–brain barrier was facilitated by either conjugating lipophilic moieties such as apolipoprotein E [74,75] or by adjusting charge polarity and molecular composition [76]. For instance, lipid nanoparticles were recently reported to enable specific charge-mediated targeting to the lungs, liver, and spleen by changing their lipid composition [77]. These observations highlight the interdependence of physical parameters such as size, charge, and mechanics.

achieve efficient secondary and tertiary targeting, the carrier needs to harbor various biochemical moieties to discriminate and selectively interact with the targeted cellular population – such as cancer cells – that are typically surrounded by a plethora of healthy cellular entities (Box 2). To this aim, functional groups sensitive to local endogenous changes – such as pH, redox states, or enzyme overexpression – are employed to promote interaction of the carrier with the target

Box 2. The Targeting Hierarchy of Cell-Selective Drug Delivery: Secondary and Tertiary Targeting

Secondary targeting brings up additional and more intricate challenges compared to primary targeting. First, cell type-specificity needs to be established as part of the carrier to selectively target disease-causing cells, which are usually surrounded by several distinct types of healthy cells. This selectivity is typically introduced by incorporating various molecular receptors such as antibodies, aptamers, peptides, or proteins on the carrier. Upon recognition with the targeted molecular target, the interaction may result in a prolonged lingering of the carrier in close proximity to the target cell population. Ultimately, this selective interaction will promote the internalization or fusion of the carrier with the target cell membrane. Once internalized, the carrier must release its therapeutic cargo.

In addition to selective interaction, the drug carrier must be stimulus-responsive. The molecular composition of the immediate extracellular environment is often determined and altered by the cellular phenotype [78]. Common endogenous biochemical changes in proximity to tumor cells include a decrease in the extracellular pH, a change in the redox potential owing to hypoxia promoted by poor vascularization of the tumor environment, and increased expression of matrix metallopeptidases. Accordingly, recent studies have worked on improving drug carrier formulations to integrate ionizable motifs that undergo charge modulation as a function of pH [79–81]. The charge modulation triggered by the ionizable motif can promote the destabilization of the carrier. This can either increase the permeability of the carrier, thereby facilitating the ability to release its cargo on site [79,82], or enhance its fusogenicity to promote fusion with the cancer cell membrane [83]. Increased fusogenicity allows the intracellular delivery of therapeutics and minimizes drug toxicity to neighboring healthy cells.

Following carrier internalization, tertiary targeting requires the efficient release of its cargo and selective interaction with the intracellular target structure, for example, specific organelles, RNAs, DNAs, or ribosomes (Box 1). The intracellular fate of the drug is directly dictated by its capacity to overcome additional biological barriers. Major intracellular obstacles include the entrapment of therapeutics in endosomes and lysosomes – organelles that degrade foreign materials through the action of enzymes and low intraluminal pH [84]. Therefore, carriers need to recognize specific surface receptors to allow them to distinguish between healthy and malignant cells, and perceive subtle changes in the local extracellular environment.

Glossary

Bottom-up synthetic biology: an emerging interdisciplinary scientific field that aims to create minimalistic cellular replicates through the precise assembly of molecular building blocks.
Droplet-based microfluidics: microfluidic platforms that allow the generation and manipulation of discrete droplets (femto- to pico-liter volume) in an immiscible carrier fluid. These platforms enable the utilization of various modules to manipulate each individual droplet with high precision.
Exosomes: a subtype of extracellular vesicles that are produced by various cell types. Owing to the mechanism of their biogenesis, exosomes harbor a variety of membrane-bound and soluble constituents – such as cytosolic proteins and RNAs – from their cellular origin. Common membrane-bound proteins include the tetraspanins, such as CD9, CD63, and CD81; MHC I, MHC II; membrane-transport and -fusion proteins (Rab, GTPases); and lipid raft-associated proteins [67]. This intricate molecular composition gives exosomes the ability to activate multiple signaling pathways that, in concert, initiate, drive, and regulate the single and collective responses of tissues. These responses in turn underlie nearly all facets of multicellular organisms, underscoring the central physiological role of exosome-mediated intercellular communication and their involvement in a wide variety of disease states. These natural attributes thus make exosomes, *a priori*, ideal natural carriers for drug-delivery applications.
Giant unilamellar vesicles (GUVs): micrometer-sized lipid-based vesicles that are commonly employed as an artificial cell-membrane mimetics.
Microfluidics: a technology that relies on the fabrication of microscopic channels in a polymeric substrate (i.e., PDMS) or the use of glass microcapillary to precisely manipulate various fluids at the picoliter scale.
Nanomedicine: as a broad definition, nanomedicine implies the use of various forms of nanotechnology to diagnose or treat (or a combination of both referred to as theranostics) different health diseases. More specifically, but not exclusively, nanomedicine has received significant attention in the field of oncology owing to the enhanced physicochemical and biochemical properties conferred by nanomaterials.

cell population, facilitate its internalization, and ultimately release its molecular payload intracellularly.

However, a central question remains open: is the current focus on nanoscale formulations necessary to improve the clinical performance of drug-delivery systems? This question builds on the observation that a restricted number of nanomaterials have provided only minimal benefit to cancer therapy over the past decades. This mostly reflects their poor efficacy in improving delivery efficiency [14] as well as the structural and chemical complexity of the biological barriers that need to be overcome for efficient drug delivery [15–17]. Conversely, a growing body of research supports a shift of focus. For example, preliminary results showed a specific potential for primary targeting of the brain by delivering rivastigmine to treat Alzheimer's disease via oral and intraperitoneal administration of micron-scale liposomes ranging from 3 to 10 μm in diameter [18,19]. These observations show that micron-scale lipid carriers, such as **giant unilamellar vesicles** (GUVs), might have great potential for primary targeting. An additional advantage of micron-scale lipid-based vehicles is that the cell–carrier interaction can be modulated by incorporating various ligands, or repellant motifs, while also incorporating stimulus-sensitive motives for improved fusogenicity and internalization [20]. Moreover, several recent studies have demonstrated that micron-scale lipid-based vesicles can interact with cells and their intracellular components [21] as well as directly release therapeutics inside the cytoplasm [22]. These examples highlight that micron-scale vesicles can reach specific organs, be internalized by cells, and interact with intracellular constituents.

Expanding the selection of tools to include micron-sized materials and systems incorporating multi-scale compartments opens up entirely new possibilities for the generation of more advanced carriers. However, prerequisite for this ambitious task is that the carriers need to have a long circulation time, minimal passive release of its cargo during transit, and evade both the immune system and mononuclear phagocyte system (MPS) [23]. Considering all these criteria, natural living cells may represent the ideal drug-delivery system owing to their ability to transport therapeutics and minimally interact with the immune system and MPS by acting as nonforeign carriers. Consequently, the use of naturally occurring entities or naturally derived systems to deliver therapeutics has become a growing focus of research. First attempts towards more natural systems have included the use of hybrid materials based on either isolated cell structures or on fully synthetic motives to improve drug-delivery efficiency. Following recent technical innovations in the field of **bottom-up synthetic biology** – where mostly GUV-based artificial cell-like compartments have been exploited – improved cell mimicry could be engineered to further improve drug delivery. Such a paradigm shift from nano- to micron-sized materials would also allow the utilization of **microfluidic** technologies (Figure 2, Key Figure) that offer the notable advantages of excellent reproducibility, controllability, complexity, and production capability. In particular, **droplet-based microfluidics** is ideally suited to engineer micron-scale complex lipid vesicles through sequential and precise manipulation of water-in-oil droplets [20,21,24–26].

This review describes the potential advantages of employing bottom-up synthetic biology principles for the construction of advanced drug-delivery carriers. We illustrate how naturally derived systems can facilitate and improve drug delivery. In addition, we present examples that apply bottom-up synthetic biology principles to generate bioinspired synthetic systems with well-controlled biophysical and biochemical properties for advanced drug release. We also describe the generation of complex systems with various microfluidic platforms, and critically evaluate the drawbacks and challenges that these platforms must overcome before successful clinical translation. Finally, we present a short perspective on the potential of the advanced bottom-up assembly of lipid-based carriers for improved therapies.

Surfactants: amphiphilic molecules (such as lipids and block-*o*-polymers) that exhibit amphiphilic interactions with both an oil (organic) phase and an aqueous phase. Lipids or block-*o*-polymers can be supplemented in the oil phase of a droplet-based microfluidics system to stabilize water-in-oil droplets, thus enabling their manipulation with ease. In addition, surfactants can be supplemented in the aqueous phase to further stabilize lipid-based vesicles or promote surface dewetting by altering the surface and interfacial tension.

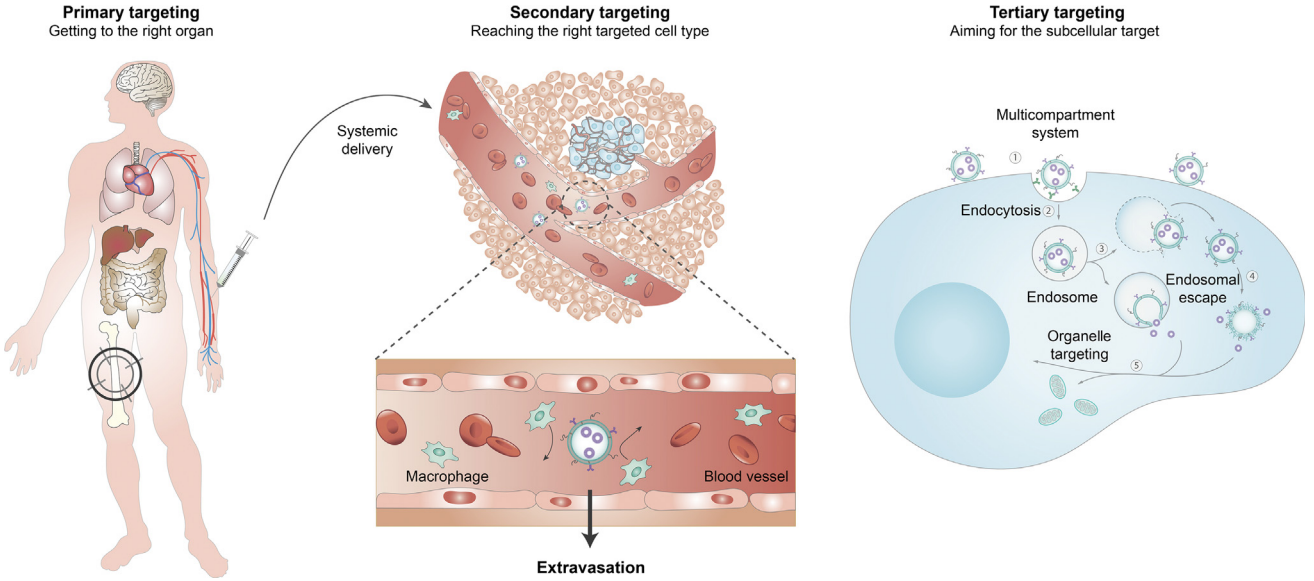

Figure 1. Hierarchy of Targeted Drug Delivery. To achieve a higher efficiency of drug delivery to disease-causing cells, the therapeutic carrier is engineered to maximize its passive accumulation in the primary target organ. This can be facilitated by a suitable means of administration (shown on the left). Therapeutic carriers are preferably administrated intravenously, upon which the secondary targeting journey towards the appropriate cell type begins. During transit, the carriers steer towards the disease-causing cells while evading various histological barriers such as MPS components, non-specific adsorption of serum proteins, and the immune system, and then extravasate from the blood vessel (middle). The therapeutic carriers then need to selectively interact with the nearby target cell population, ultimately leading to their adsorption or internalization. Once internalized, tertiary targeting is initiated. The carriers need to evade endosomes before finally releasing the therapeutic into the cytosol of the designated cell. The final release of the therapeutic, which targets only a specific organelle within the cytosol, from the carrier is influenced by various endogenous or external stimuli (right).

Naturally Derived Systems to Improve Drug-Delivery Efficiency

Extracellular vesicles (EVs) are lipid-based vesicles that are secreted by various cell types and range from 100 nm to several microns in size. EVs have recently gained significant attention as therapeutic vehicles [27] and biomarkers [28,29] owing to their pivotal roles in intercellular communication and their involvement in various disease states. For instance, encapsulation of paclitaxel into EVs can achieve both increased accumulation and improved targeted delivery to cells [30,31]. To benefit from the qualities of advanced EVs in terms of targeting, Rayamajhi and colleagues developed hybrid **exosomes** (HEs) by fusing synthetic liposomes with cell-extracted exosomes [32]. The HEs produced presented various membrane proteins, such as CD63, CD81, and CD9 (see Exosomes in the Glossary), derived from macrophage-derived exosomal preparations. HE application was associated with both improved cancer targeting and cell internalization. In addition, Liang and coworkers recently demonstrated the importance of the mechanical properties of doxorubicin-loaded EVs for drug delivery to tumor cells [33]. The authors report that the mechanical properties of microparticles secreted by cells – vesicles 1–5 μm in diameter – depends on the mechanical characteristics of the production cell culture environment. Soft microparticles isolated from tumor cells (i.e., from cells cultured in a soft environment) led to improved delivery of encapsulated doxorubicin *in vivo*.

In addition to improved targeting by using cell-derived receptors, natural systems also feature immune-signaling ligands. For example, CD47 glycoprotein expressed on mammalian cell membranes is a putative marker of 'self'. This signal is a 'don't eat me' message to macrophages and other phagocytes, and prevents an autoimmune reaction. By employing liposomes with D-peptide CD47 mimicry, Tang and coworkers reported that these 'self'-labeled liposomes can

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Key Figure

Schematic Illustration Presenting the Bottom-Up Assembly of Multicompartment Lipid-Based Vesicles for Advanced Drug Delivery.

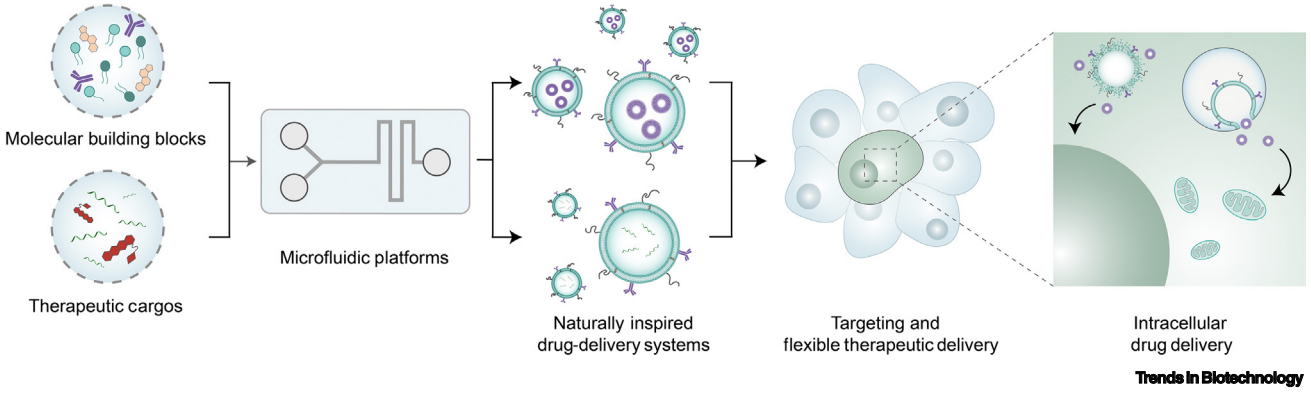


Figure 2. By employing various microfluidic platforms, naturally inspired drug-delivery systems encapsulating various therapeutic cargos can be easily assembled from elementary building blocks. This bottom-up engineering approach offers the possibility to incorporate enhanced biochemical functionalities into the drug carrier to enable selective interaction with disease-causing cells and efficient intracellular release of its therapeutic payload.

act as a mask by coating the hepatic phagocyte membrane. As a result, phagocytic clearance of subsequently injected drug-loaded polymeric nanoparticles was significantly reduced [34]. Overall, this 'self' signaling mechanism showed potential to lower the MPS barrier for subsequently injected drugs *in vivo*. Another example based on the assembly of EVs presenting various immunological ligands involved immobilization of recombinant Fas ligand (FasL). FasL can induce target cell apoptosis by activating a Fas signaling cascade [35]. Yerneni and colleagues recently applied this strategy by anchoring FasL in the lipid bilayer of cell-isolated EVs by means of cholesterol-tagged DNA to selectively induce apoptosis of cancer cells upon interaction with the FasL-bearing EVs [36].

These results highlight the combinatorial impact of physical properties (e.g., stiffness and size) and biochemical properties (e.g., harboring various cell-derived ligands) on improving the targeting and drug-delivery efficiency. These key features can be incorporated into drug-delivery systems by using EVs as building blocks. However, EV extraction and isolation procedures remain irreproducible, technically challenging, and time-consuming, and these obstacles impede their broad clinical translation [37]. Moreover, in addition to selectively targeting specific cells and evading the MPS, frontier drug-delivery systems will need to be able to sense and respond to changes in their immediate environment during drug release – a property referred to as intelligent and dynamic release of therapeutics. In sum, the principles of a bottom-up approach can be implemented by the high-throughput generation of bioinspired multifunctional synthetic systems with well-controlled biophysical and biochemical properties for advanced drug release.

Bottom-Up Assembly of Synthetic Cells as Advanced Drug-Delivery Systems

The modular engineering approach for the bottom-up assembly of synthetic cells can be employed to construct more complex systems that can control the amount of cargo released based on external physiological triggers (Box 3). Chen and colleagues developed an ingenious synthetic β cell (AβC) that is capable of dynamic insulin secretion as a function of the extracellular glucose concentration [38]. An interdigitation–fusion method of assembly was employed to

Box 3. Synthetic Vesicles versus Synthetic Cells

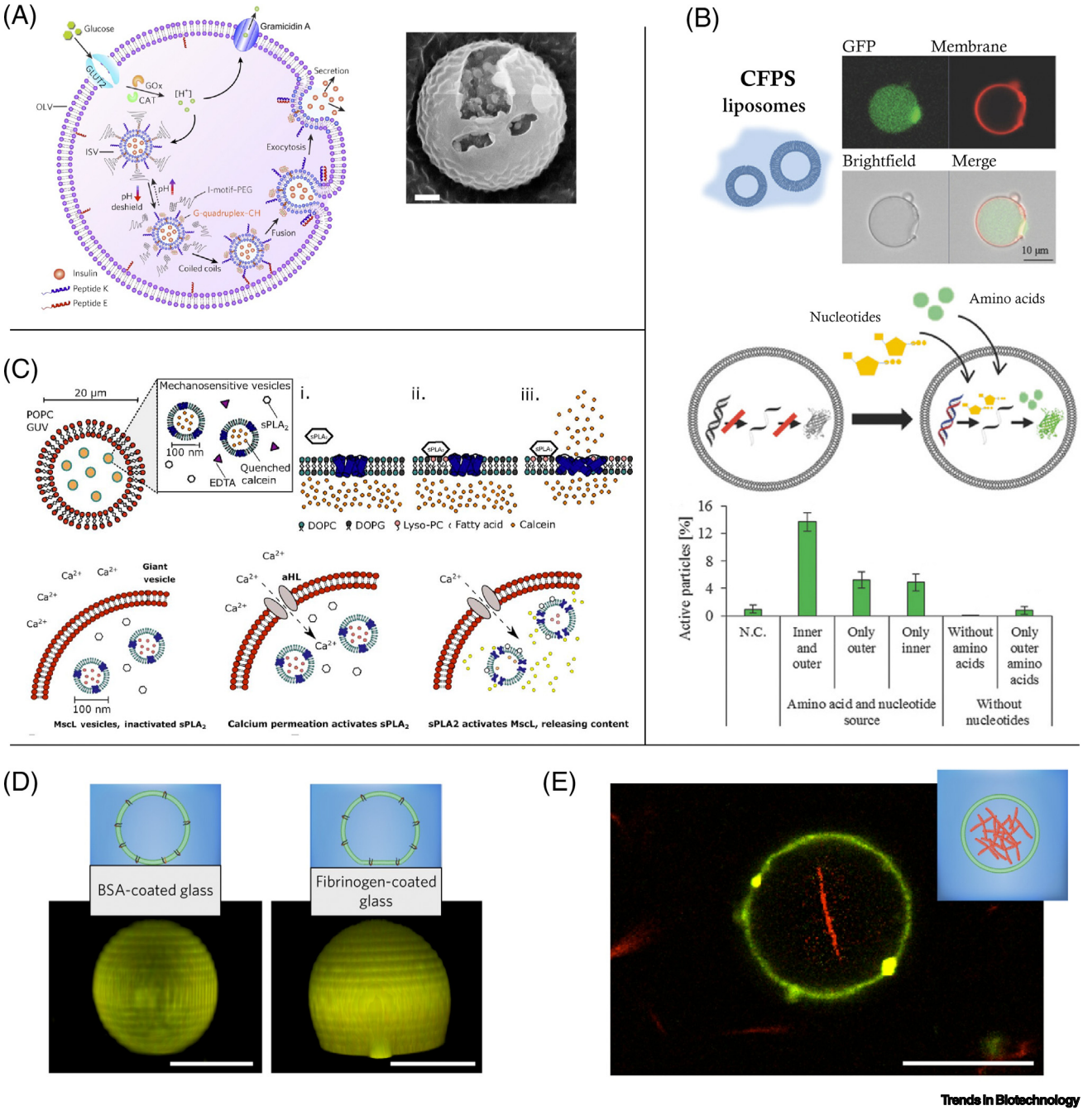
In general terms, a synthetic vesicle may be defined as the sequestration of discrete volume (typically in the pico- to femtoliter range) through the self-assembly of a physical boundary from fundamental building blocks. For instance, liposomes and larger lipid vesicles employ lipids as elementary units to enclose constituents through the assembly of a lipid bilayer. In addition to lipids, polymers, proteins, or even colloids may be employed as fundamental building block to generate various synthetic vesicles – respectively polymersomes, proteinosomes, and colloidosomes. Although their use must be tailored to individual needs, their physicochemical properties can be meticulously engineered to tackle various biotechnological challenges. In the field of drug delivery, these synthetic vesicles typically encapsulate therapeutic drugs, transport their cargo, and release their payload upon endogenous or exogenous stimulation once the designated site is reached. Various biochemical ligands and chemical functionalities are incorporated into the synthetic vesicles to confer sensitivity and selectivity. However, despite advances in the generation of complex architecture, synthetic vesicles all face the same challenge: lack of dynamics and feedback, or, in other words, an inability to adapt the release of therapeutics as a function of local environmental changes.

A synthetic cell – also referred as a protocell – is a minimalistic replicate of a living cell that attempts to mimic key cellular functionalities by enclosing biological machinery in a cell-like compartment. These functions may imply (i) continuous energy and biomaterial harvesting or genesis to sustain metabolic activities, (ii) compartmentalization to isolate distinct reagents and/or to perform independent intraluminal reactions, (iii) the capacity to growth and divide, (iv) sensing and communication with the immediate surroundings, and finally (v) motility [85]. In contrast to synthetic vesicles, synthetic cells enable the introduction of feedback mechanisms – or dynamics – by encapsulating various biological machineries to address tasks that cannot be achieved by simple synthetic vesicles. For instance, synthetic cells are able to autonomously harvest molecular components from their extracellular environment to sustain their own energy production [86]. Empowered by compartmentalization of the system, molecular cargos can be spatially isolated and released in response to physicochemical changes in the vicinity of the synthetic cell. Consequently, by applying these synthetic cells to drug delivery, dynamic release of therapeutics can be achieved for smarter drug therapy.

create multicompartment vesicles loaded with glucose oxidase, catalase, and insulin-loaded liposomes (Figure 3A). Upon exposure to high extracellular glucose concentrations, glucose enters the synthetic AβC through the membrane-incorporated recombinant GLUT2 importer protein. Once inside, glucose degradation by glucose oxidase begins. This degradation process leads to a decrease in pH inside the GUV lumen, which in turn unshields the insulin-loaded liposomes. Once exposed, peptide K can associate with peptide E, which is located inside the lipid membrane of the homing GUV. This reaction triggers the secretion of insulin mediated by recombinant SNARE proteins. The AβC has an impressive ability to regulate blood glucose levels *in vivo* in a streptozotocin (STZ)-induced type 1 diabetic mouse model. A second example was recently published by Krinsky and coworkers. The researchers encapsulated a cell-free protein system in GUVs to directly express and release *Pseudomonas* exotoxin A into their immediate surroundings [39]. By taking advantage of essential building blocks in their local environment, such as ATP, GTP, UTP, and amino acids, the GUV-based therapeutic synthetic cells could autonomously produce and release *Pseudomonas* exotoxin A (Figure 3B). The released toxin induced the efficient killing of cancer cells *in vitro*, and also *in vivo* when the GUVs were directly injected intratumorally into BALB/c mice bearing orthotopic 4T1 tumors. Interestingly, the therapeutic synthetic cell exhibited higher toxicity than purified toxin. This may be attributed to the improved stability of the protein expressed directly in the synthetic cells or possibly to the prolonged release of protein in disease-causing cells. This novel platform, which merges synthetic biology and drug delivery with elegance, highlights the potential of cell-like advanced carriers to directly synthesize therapeutics at the disease site. Moreover, Hindley and colleagues reported bottom-up assembly of calcium-sensitive GUVs (Figure 3C). These were used for the release of low molecular weight compounds, such as calcein loaded within small unilamelar vesicles, in response to exogenous calcium influx [40].

In addition to the ability to sense and react to their local environment, advanced drug-delivery systems will need to overcome several barriers (see section on Drug-delivery Challenges: Opportunities for Bottom-Up Synthetic Biology). This will require mechanical stability, energetic autonomy,

and the possibility of external control. By co-reconstituting photosystem II and proteorhodopsin with ATP synthase, Lee and coworkers created proteoliposomes that can dynamically facilitate ATP synthesis steered by independent photoactivation [41]. These photosynthetic organelles were subsequently encapsulated inside GUVs and were used to activate F-actin polymerization upon illumination with red light to achieve local membrane deformation without rupture. The F-



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actin cortex is an important mechanical support for improved mechanical stability of GUVs. In addition, Weiss and colleagues reported the reconstitution of ATP synthase directly inside the GUV membrane to produce ATP controlled by an external pH gradient [26]. They also showed that sequentially assembled GUV-based synthetic cells were capable of sensing and responding to the extracellular environment by creating specific interactions with the extracellular matrix via transmembrane integrin proteins (Figure 3D) to reconstitute an F-actin cortex (Figure 3E). These synthetic cells showcase the potential of the bottom-up approach to engineer complex systems that are sensitive to external stimuli such as light and/or pH to provide energy-independence and to create specific interactions with their extracellular environment.

Microfluidic Generation of Micro-Scale and Multicompartment Synthetic Cells

The key advantages of bottom-up synthetic biology lie in the modularity and intercompatibility of the methods of synthesis. By means of droplet-based microfluidic platforms (Box 2), various compartments can be independently assembled by three distinct methods: charge-mediated assembly, emulsion phase transfer, and double-emulsion. Each method offers unique advantages. Charge-mediated assembly provides the ability to generate GUVs with various lipid compositions, thus enabling the fine-tuning of physicochemical properties such as surface charge, stiffness, and fluidity [26,42]. Moreover, it allows the encapsulation of precise amounts of biologically active small molecules, permits the reconstitution of transmembrane proteins into the lipid envelope, and enables controlled assembly of multicompartment systems [20,26,42,43]. Emulsion phase transfer enables the formation of an asymmetric lipid bilayer [44], the direct isolation of vesicles in aqueous medium, and does not require the use of dewetting **surfactants** or solvent extraction step [40,45–48]. Moreover, this method can encapsulate complex protein machinery [39]. This is an advantage over charge-mediated assembly because it could potentially impede small unilamelar vesicles fusion to the droplet periphery. The double emulsion-based method also allows the generation of asymmetric lipid vesicles [49,50]. It is also compatible with organic solvents in glass capillary microfluidic devices, which are typically incompatible with poly(dimethylsiloxane) (PDMS)-based devices [51]. Importantly, double-emulsion methods allow the flexible assembly of a multicompartment system either through successive encapsulation of the vesicles generated or through the coencapsulation of distinct vesicles [52]. A summary of the respective methods is presented in Table 1.

Notably, in the case of double emulsion-based methods, the addition of a specific surfactant to the outer aqueous phase promotes dewetting (i.e., the spontaneous removal of excess oil surrounding the inner aqueous core). This strategy enables the total elimination of potential residual oil pockets that can affect the stability of the vesicles. Deng and coworkers reported the use of Pluronic® F68 surfactant to initiate and promote the dewetting of chloroform/hexane residual oil in a capillary-based microfluidic system [51–54]. Nonetheless, the use of chloroform as an

Figure 3. Bottom-Up Assembly of Synthetic Cells That Mimic Key Cellular Functionalities. (A) Schematic depicting the biochemical processes inside an artificial β cell (AβC). Letter in square brackets indicates the concentration. The inset shows a cryo-scanning electron microscopy (cryo-SEM) micrograph of multicompartment assembly (scale bar, 5 μm). Image adapted, with permission, from [38]. (B) A synthetic cell encapsulating a cell-free protein system. By sourcing essential nutrients such as nucleotides and amino acids from the extracellular environment, the synthetic cell can sustain independent synthesis of the therapeutic protein. Image adapted, with permission, from [39]. (C) A giant unilamellar vesicle encapsulating mechanosensitive liposomes and secretory phospholipase A2 ($sPLA_2$). The liposome contains reconstituted mechanosensitive channels of large conductance (MscL). Ca^{2+} influx into the nested vesicles through α-hemolysin pores activates the conversion of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipids to their 1-oleoyl-2-hydroxy-glycero-3-phosphocholine (lyso-PC) form by $sPLA_2$. This conversion results in local mechanical deformation of the liposomes situated inside the vesicle and ultimately leads to the release of their content. Image adapted, with permission, from [40]. (D) Reconstitution of $\alpha_{IIb}\beta_3$ integrin into giant unilamellar vesicles that exhibit selective interaction with fibrinogen-coated glass, demonstrating the ability of a synthetic cell to maintain the function and activity of reconstituted proteins. (E) Actin cytoskeleton containing a synthetic cell. Images adapted, with permission, from [26]. Abbreviations: BSA, bovine serum albumin; CAT, catalase; CFPS, cell-free protein synthesis; GFP, green fluorescent protein; GOx, glucose oxidase; N.C., negative control referring to synthetic cells with all required components, but excluding the DNA template.

Table 1. Current Methods for High-Throughput Generation of Micron-Sized Lipid Vesicles[a]

Method	Possible lipid composition	Multicompartment generation	Suitable for charged lipids	Use of surfactant?[b]	Refs
Interdigitation-fusion	DPPC	Liposomes	Not mentioned	No	[38,87–89]
Emulsion phase transfer	DOPC, POPC, Ch	Liposomes	Not yet demonstrated	No	[39,40,44–48]
Capillary-based double emulsion (W/O/W)	Egg PC, DOPC, DPPC, and Ch	Liposomes or coacervates	Not yet demonstrated	Pluronic F68	[49,51–53,55]
PDMS-based double emulsion (W/O/W)	Soy PC, egg PC, DOPC, DOPG	Coacervates	Up to 25 mol% anionic lipid	Pluronic F108 or poloxamer P188	[50,56-58,90-92]
Charge-mediated (droplet-stabilized)	DOPC, DOPE, DOPS, egg PC, egg PG, POPC, DOPG, DOTAP, and DOBAQ	Oppositely charged liposomes and GUVs	Suitable for anionic (DOPG) and cationic (DOTAP) lipids	PFO	[20,21,26,42,43]
Electroformation	PC-rich lipids and Ch	Lipo- and proteoliposomes	Up to 20 mol% anionic lipid	No	[41,93]

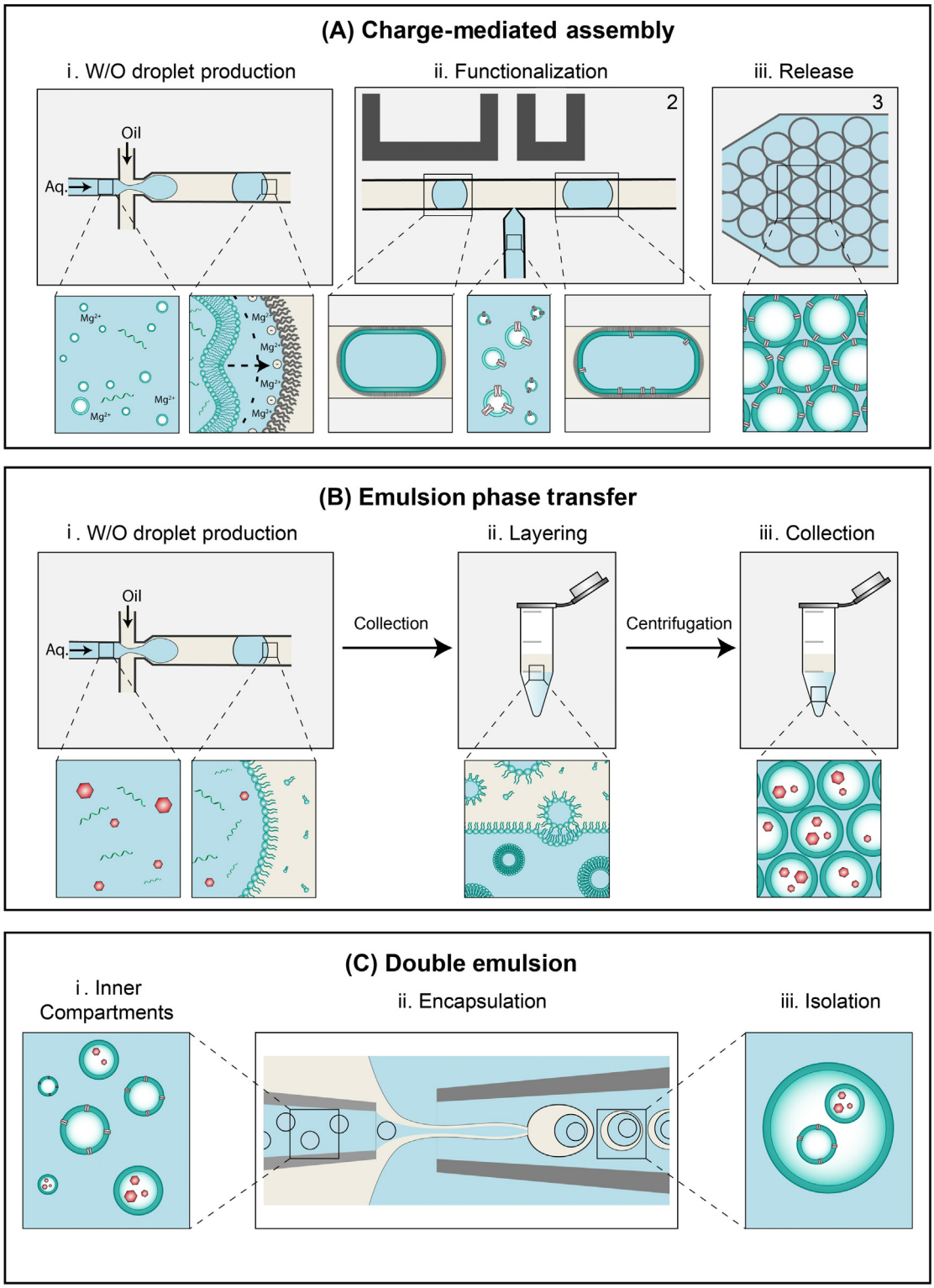
[a]Abbreviations: Ch, cholesterol; DOBAQ, *N*-(4-carboxybenzyl)-*N*,*N*-dimethyl-2,3-bis(oleoyloxy)propan-1-aminium; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol); DOPS, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine; DOTAP, 1,2-dieleoyl-3-trimethylammonium-propane; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; GUVS, giant unilamellar vesicles; PC, phosphocholine; PFO, 1H,1H,2H,2H-perfluoro-1-octanol; PDMS, poly(dimethylsiloxane); PG, phosphatidylglycerol; POPC, 1-palmotoyl-2-oleoyl-glycero-3-phosphocholine; W/O/W, water/oil/water.
[b]The surfactant was employed for one of the following reasons: to promote/initiate dewetting, stabilize the resulting GUVs once released into the aqueous phase, or enable transfer from the oil phase to the water phase.

essential component of the oil phase may not be compatible with all types of cargo owing to its partial water solubility [55]. By using an oil phase that is both compatible with PDMS-based microfluidics and has minimal solubility in water, Deshpande and colleagues employed high concentration of poloxamer 188 (i.e., Pluronic® F68) to promote the fast dewetting of excess octanol [56,57]. Referred to as octanol-assisted liposome assembly (OLA), this double emulsion-based method enables the high-throughput generation of small GUVs (down to 5 μm in diameter) directly on a chip. Although featuring clear advantages, these recent platforms rely on the use of an outer aqueous mixture of high viscosity, such as glycerol:water or polyvinyl alcohol:polyethylene glycol:water. These aqueous phases impede the use of centrifugation as a purification method. As an alternative, Krafft and coworkers developed a simplified system relying solely on sucrose/salt solution as the inner and outer aqueous phases [58]. To completely dewet the

Box 4. Droplet-Based Microfluidics for the Assembly of Advanced Molecular Systems

In contrast to conventional microfluidics, which involve the continuous flow of various fluids in a laminar flow regime (i.e., low Reynolds number), droplet-based microfluidics allows the manipulation of discrete volumes through the generation of emulsions such as water-in-oil (W/O) or oil-in-water (O/W) droplets. These microdroplets can act as independent picoliter reactors and have found numerous applications in various scientific fields.

Droplet-based microfluidics features various functional units: a droplet production unit to encapsulate a variety of chemical and biochemical contents [26]; a pico-injector for the sequential injection of picoliter volumes into individually produced droplets [94]; a fusion module to provoke the fusion of two distinct droplets, thus enabling the mixture of various chemical and biochemical reagents with high temporal control [95]; a mixing module to improve convection and thereby facilitate molecular reactions within the droplet [96]; a sorting module to precisely separate individual droplets based on various analytical signals such as fluorescence [62] or mass spectrometry [97]; a releasing unit to liberate the entrapped mixture from the droplet into an aqueous continuous phase by applying an electrical field [98] or by using a passive trapping structure [26]; and a detection or observation module that facilitates the assessment of a large droplet population [43,99]. Adding to the various possibilities of droplet-based microfluidics, capillary microfluidics – which in contrast to PDMS-based microfluidics uses glass capillaries – is even compatible with the use of organic solvent [52]. Capillary microfluidics is therefore ideally suited to generate both simple and complex emulsions [100].



(See figure legend at the bottom of the next page.)

water/oil/water double emulsion, their approach relied solely on an osmotic gradient generated by encapsulation of sucrose at concentrations lower than the outer phase, thus rendering it possible to use centrifugation to purify the obtained GUVs. Nevertheless, most double emulsion-based methods have an inherent drawback: adsorption of surfactant molecules onto the lipid bilayer. This concomitant adsorption is likely to affect membrane dynamics and mechanical properties. For instance, in the case of capillary-based microfluidics, the proportion of Pluronic® F68 molecule adsorbed on a liposome surface was approximated to follow a ratio of 1 mol surfactant to 17 mol lipids [51]. Thus, the impact of the adsorbed surfactant on the mechanical properties and dynamics of the lipid bilayer will need to be addressed, especially with regards to possible therapeutic applications. On a positive note, a recent study comparing GUVs produced by OLA to those created by electroformation found that the presence of surfactant molecules during OLA production only had a limited impact on the lateral diffusion of lipids in the GUV membrane compared to membranes generated by electroformation [59].

Microfluidic platforms for micron-scale carrier assembly make it possible to minutely engineer complex lipid-based vesicles, comprising multifunctional targeting and compartmentalization modules, from the bottom-up (Box 4). Moreover, the available methods can be combined to design advanced drug-delivery systems (Figure 4). This enables the usage of dedicated protocol for the encapsulation of hydrophobic/hydrophilic molecules, cells, and protein machineries [46,60], or even short interfering RNA [61], but also to create compartments that exhibit complex spatial arrangements such as inner and outer membrane leaflets that differ in composition [49,50]. Protein machineries, for instance, can be encapsulated by applying emulsion phase transfer [39], and other compartments such as proteoliposomes can be generated using a charge-mediated lipid vesicle formation. Both methods can be supported by droplet-based microfluidics and pico-injection to achieve high throughput and low polydispersity [26]. The resulting individual compartments can then be assembled within a homing GUV by capillary microfluidics, a method that allows the number and stoichiometric composition of individual compartments to be finely tuned [52]. Finally, the enrichment and quality control of drug carriers produced, that comprise the desired number and type of compartments, can then be performed by using currently established microfluidic platforms such as fluorescence-activated droplet sorting [62].

Outstanding Questions

How can we generate synthetic systems whose functional complexity is comparable to that of living systems? More specifically, how can we further tailor these systems towards advanced drug-delivery applications?

Can bottom-up synthetic biology help to create a fully synthetic EV? Moreover, would these synthetic vesicles exhibit similar biological properties and induce similar cellular responses as natural vesicles?

Can both bottom-up synthetic biology and microfluidics personalize therapy for individual patients by rapid and reliable re-engineering of therapeutic vehicles?

The use of surfactants to stabilize GUVs seems to be essential to maintain their integrity and prevent their uncontrolled rupture in aqueous environments. Can alternative lipid compositions, or even fully synthetic lipids, palliate the need for surfactants and thus circumvent their use?

Can these microfluidic platforms be incorporated into current industrial pipelines for large-scale production of advanced therapeutic carriers?

Concluding Remarks

Nanomedicine has provided novel strategies for improving drug delivery towards specific pathogens and disease-causing cells. However, assembling these carriers requires exquisite control over their chemical composition, structure, and functionalization with a multitude of biological ligands to achieve selective drug release. These engineering challenges thus require the development of high-throughput, well-controlled, and novel platforms to manipulate both chemical and biological constituents. Inspired by natural living systems, the design and development of advanced drug carriers with well-defined biophysical and biochemical properties can be facilitated

Figure 4. Engineering a Multicompartment Drug Carrier by Microfluidics. (A) Charge-mediated assembly of lipid-based vesicles. By using droplet-based microfluidics, the aqueous phase containing small unilamellar vesicles (SUVs) and therapeutic cargo is entrapped within a water-in-oil (W/O) droplet. Addition of a negatively charged surfactant to the oil phase promotes SUV fusion with the periphery of the droplet in the presence of Mg^{2+}. This process enables the assembly of a micron-scale vesicles that entrap the drug cargo (i). Following their generation, membrane proteins may be incorporated into the lipid bilayer by introducing proteoliposomes by pico-injection (ii). The vesicles can then be releases into the physiological environment where their physicochemical and biochemical properties can be assessed (iii). (B) Emulsion phase transfer allows encapsulation of large and complex protein machinery through the generation of a water-in-oil emulsion, where herein, the oil phase is supplemented with lipids of the desired composition (i). The emulsion can then be layered onto a lipid monolayer formed beforehand at the water/oil interface (ii). By applying centrifugation forces, the W/O droplets migrate to the lower aqueous phase, enabling the generation of a full lipid bilayer (iii). (C) These two approaches enable the generation of distinct functional lipid-based vesicles that act as inner compartments (i). These inner compartments can then be encapsulated, at the desired stoichiometry, by the use of capillary microfluidics (ii) to achieve the assembly of a multicompartment drug carrier (iii).

by bottom-up synthetic biology principles and associated microfluidic technologies. By imitating key cellular functionalities – such as extracellular sensing, intracellular protein synthesis, and dynamic release of small molecules – novel carriers can achieve dynamic release of therapeutics. Moreover, empowered by microfluidics, the precise and sequential assembly of multifunctional multicompartment systems can be achieved with unprecedented control (see Outstanding Questions).

By focusing on creating stimulus-responsive multicompartment systems, the order of release and the properties of the molecular cargo can be engineered to allow sequential on-site release that maximizes therapeutic benefits. In addition, multicompartment systems make it possible to encapsulate entities dedicated to preparing the cellular environment before the release of a therapeutic, another step towards optimizing therapeutic outcome. For example, Zinger and coworkers recently encapsulated collagenase inside liposomes, resulting in so-called collagozomes. These were used to dissemble extracellular matrices as a means to improve drug penetration of subsequently injected therapeutics [63]. Collagozomes were applied to degrade the dense collagen stroma typically found in pancreatic ductal adenocarcinoma, followed by injection of paclitaxel micelles. This strategy led to a significant reduction in the level of fibrotic tissue surrounding the tumor. Simultaneously, improved drug penetration – assessed by size reduction of the pancreatic ductal adenocarcinoma tumors – was observed. Supported by these recent innovations, we envisage that the sequential release of various inner compartments can promote and facilitate a potential hierarchical targeting, from primary to tertiary, resulting in overall improved intracellular delivery of therapeutics.

Nevertheless, the toxicity and the clinical benefits of multicompartment-based drug carriers has not yet been assessed. The use of microfluidics to produce and assemble these advanced carriers will likely facilitate such assessments by allowing unprecedented control over size, reproducibility, and throughput. Cytotoxicity investigations will be essential, especially when microfluidic platforms rely on surfactant to either stabilize GUVs or initiate a dewetting process. The evaluation of the absolute amount of surfactant and the impact of the mechanical properties of the lipid bilayer, such as rigidity, fluidity, and stiffness, need to be investigated. This is especially true because softer materials have been shown to improve drug penetration. Furthermore, the nature of these surfactants may compromise clinical translation. Charge-mediated assembly of multicompartment systems using novel synthetic fluorosurfactants will surely offer interesting alternatives to double emulsion-based methods of assembly.

Taken together, the pharmacokinetics of micron-sized lipid-based carriers should be investigated in greater detail because they could not only transport considerably larger drug quantities, but also deliver drugs that are larger in size. The past decades of pharmacological research have generated ever more complex and larger therapeutics, ranging from low molecular weight compounds [64] to supramolecular antibodies [65] and therapeutic viruses [66]. In addition, because larger delivery systems carry increased drug quantities, they are also more likely to function as durable drug reservoirs in the target tissue, thereby improving the longevity of future biomedical applications. This emphasizes the need to create larger vehicles that have sufficient cargo capacity for next-generation therapeutics. The possibility to engineer ever more complex systems inspired by living cells provides a framework for the development of carriers whose functional diversity is comparable to that of cells. For instance, several immunological cells possess the ability to identify, interact with, and eventually kill specific targets. They subsequently take up target leftovers to carry them to lymph nodes for presentation and conditioning of other immune cells. Similarly, we foresee systems that combine complex therapeutics and diagnostic functionalities, each isolated inside specific inner compartments, in a single vesicle. Such a

theranostic vesicle could be engineered to identify patient-specific targets, unload their molecular cargo, and afterwards take a 'molecular biopsy'. The theranostic vesicle could directly transduce an analytical signal *in vivo* or could be collected from the body fluids of the patients for further diagnostic and treatment-evaluation procedures. Such a drop-off/pick-up system for automated storage and retrieval at the micron-scale could transform modern medicine.

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