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Synthetic Cells in Biomedical Applications

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Abstract

Synthetic cells are engineered vesicles that can mimic one or more salient features of life. These features include directed localization, sense-and-respond behavior, gene expression, metabolism, and high stability. In nanomedicine, many of these features are desirable capabilities of drug delivery vehicles but are difficult to engineer. In this focus article, we discuss where synthetic cells offer unique advantages over nanoparticle and living cell therapies. We review progress in the engineering of the above life-like behaviors and how they are deployed in nanomedicine. Finally, we assess key challenges synthetic cells face before being deployed as drugs and suggest ways to overcome these challenges.

Graphical Abstract

Introduction

Living cells offer many impressive capabilities that nanoparticle engineers often seek to imitate. These include directed localization (e.g. chemotaxis), sense-and-respond behavior, gene expression, metabolism, and high chemical and serum stability. Recent years have

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seen considerable advances in the bottom-up engineering of synthetic cells. Though several aspects of this technology are still in their formative stages, this field has made substantial headway into the realm of clinical applications.

The phrase “synthetic cell” has been used widely and requires disambiguation. Here, we use “synthetic cell” to mean an aqueous compartment bounded by either a polymer or lipid membrane that comprises molecular machinery sufficient to mimic one or more of the above features of living cells. Moreover, in the context of medicine, these features can induce desirable therapeutic outcomes. Synthetic cells can range from 100 nm to 10’s of μm in size. However, most synthetic cell studies work at scales >1μm due to the technical difficulty of generating nano-sized vesicles. Synthetic cells can be built from defined, synthetic components (“bottom-up”) or a combination of synthetic and cell-derived components (“semi-synthetic”). Here, we consider “top-down” engineering or harnessing of extant living cells a distinct technology and beyond the scope of this review.

The above definition of synthetic cells (SCs) emphasizes the compartmentalization of an aqueous interior. This is because physical segregation of an aqueous core enables many of the functions of cellular life, including aqueous biochemistry involved in everything from gene expression to enzymatic reactions. The presence of a distinct amphiphilic membrane structure is also key, as it enables signal transduction, selective transport, and the anchoring of functional moieties on and within the membrane. The aqueous interior and amphiphilic membrane distinguishes SCs from solid polymer nanoparticles, lipid nanoparticles and micelles with hydrophobic interiors, which cannot perform many of these functions that are critical to living system.

In terms of complexity, synthetic cells can be thought of as an intermediate between passive nanoparticle drug delivery systems (e.g. liposomes) and engineered living cell therapies (e.g. Chimeric Antigen Receptor T cells or synthetic beta cells for diabetes treatment (Chen et.al. 2018)). Compared to nanoparticles, SCs generally have many more unique components. This greater complexity makes them more capable than nanoparticles but also more difficult to manufacture and control. Compared to living cells, SCs are much simpler and well-defined. Despite the incredible progress of modern cell biology, cells are still in large part black boxes -- they are incompletely understood and therefore inherently unpredictable, especially when faced with the wide diversity of environments in human physiology. Hence, engineered living cells present inherent risks when deployed as therapeutics. SCs, on the other han d, are assembled bottom-up from known components and are therefore better defined and more predictable. Additionally, SCs will not replicate (unless they are programmed to). Therefore, SCs can offer considerable safety advantages over living cells.

SCs can also perform tasks that living cells cannot (Figure 1). For example, SCs can utilize non-natural and toxic molecules to a degree not possible in extant living cells (Martin et al. 2018) . Non-natural amino acids have been shown to endow proteins with valuable properties such as longer half-lives and enabling biochemistry (Wei Gao et al. 2019; H.-N. Chang et al. 2015) . Highly toxic molecules that would otherwise kill living cells can also be produced with in vitro transcription/translation (IVTT) systems encapsulated in SCs (Orth et al. 2011; Salehi et al. 2016; Dondapati et al. 2018) . Composing SCs of other exotic

chemistry can therefore endow them with unique advantages in terms of stability as well as the functions they can enact inside the body.

Additionally, while manufacturing of SCs presents its own challenges (more below), it promises certain advantages over the manufacturing of engineered living cell therapies. Autologous cell therapies currently require extensive handling. The process of purifying, engineering, amplifying, and re-administering autologous cell therapies makes their manufacture extraordinarily expensive and time consuming(R. K. Iyer et al. 2018) . Synthetic cells, on the other hand, could in theory be generated at centralized facilities, lyophilized or otherwise preserved, and shipped to hospitals, much like current biotherapeutics. Their manufacturing is scalable via microfluidics and can be generalized across different indications and compositions. At the same time, their manufacture at a smaller scale would still allow customization for personalized medicine.

Here, we review how the capabilities of synthetic cells can be leveraged for therapeutic effect. First, we describe the structure and components of SCs and review some of the manufacturing approaches developed to date. We then discuss how the life-like processes that can be engineered in synthetic cells -- directed localization, sense & respond behavior, gene expression, metabolism -- can offer unique therapeutic strategies. Finally, we discuss both the technical and regulatory hurdles that challenge the development of SC therapeutics.

Composition & Structure

Synthetic cells mainly comprise a membrane and an internal payload. In the context of drug delivery, the membrane has several key functions. First, it protects the payload from destabilizing factors in the external environment. Second, it prevents the payload from getting out too quickly and triggering physiological responses (e.g. anaphylaxis). Third, it concentrates the payload in the interior, enabling critical biochemistry or the delivery of the payload to its target at a high concentration. Lastly, the membrane itself allows for the anchoring of membrane proteins or other moieties that further stabilize or functionalize the SC.

The composition of the membrane is critical to the SC’s stability and function and must be carefully considered. The membrane may comprise phospholipids, proteins(Huang et al. 2014) , polymers(Kuiper et al. 2008) , peptides(Fatouros et al. 2014) , colloids(S. Sun et al. 2016) , virus membrane or capsids(H. Liu et al. 2015; Y. Wang et al. 2020), or other amphiphilic molecules as well as small molecules such as cholesterol that have a stabilizing effect(Briuglia et al. 2015) . As such, the membrane composition can be tuned to endow the SC with varying stability in different environments. This, in effect, enables the SC to “sense” the environment (see below). The composition of the membrane can also affect the function of embedded proteins(Elmore and Dougherty 2003) . During formation of liposomes, the inner and outer leaflets can be produced separately, making their composition unique (Doktorova et al. 2018; de Matos et al. 2019) . This enables unique functions to be incorporated at the interior and exterior of the membrane.

Inside a patient, SCs experience diverse, challenging environments. Once injected, a unique protein corona will form around the SC(Francia et al. 2020) . The composition of the protein corona is dynamic and depends on many variables, including the membrane composition, charge, zeta potential, and size, among others(Baimanov, Cai, and Chen 2019; Giulimondi et al. 2019; Pattipeiluhu et al. 2020) . Undecorated SCs are typically recognized as non-self and cleared by the reticuloendothelial system (RES), mainly in the liver and spleen(Sercombe et al. 2015) . Once opsonized, the SCs are recognized by macrophages and cleared via phagocytosis(Sercombe et al. 2015) . SCs can also be damaged via enzyme activity, which further reduces their half-life. The use of PEG to shield the outside of the liposome from the surrounding matrix has been a highly successful strategy in lengthening nanoparticle half-lives *in vivo*(Maruyama et al. 1992; Photos et al. 2003) . Many strategies have been developed to improve on current shielding effects(Boyer and Zasadzinski 2007; He et al. 2019; Gulati, Stewart, and Steinmetz 2018) .

The interior of the SC contains the payload, which can be drug or aqueous solutions that further endow the SC with function. In the simplest case, the compartment houses only a small molecule drug to be delivered to the site of action. In one of the most complex cases, the compartment houses an *in vitro* transcription/translation (IVTT) reaction mixture that can generate genetically encoded RNA or protein(Silverman, Karim, and Jewett 2020) . In some cases, SCs can house entire particles, vesicles(N.-N. Deng et al. 2017) , or condensates(Niederholtmeyer, Chaggan, and Devaraj 2018; S. Liu et al. 2020) that can further endow the SC with functions such as sensing, metabolism(Leduc et al. 2007) and movements (more below)(Siton-Mendelson and Bernheim-Groswasser 2016) .

A variety of methods have been developed to manufacture synthetic cells. These include thin-film hydration(H. Zhang 2017), reverse emulsion(Pautot, Frisken, and Weitz 2003; Huang et al. 2013; Thompson, Williams, and Armes 2015), electroemulsion(Angelova and Dimitrov 1986), and others(Fatouros et al. 2014; Y. Hu and Qiu 2019; H. Liu et al. 2015). Recently, microfluidic platforms and other techniques have been developed to improve the efficiency, speed, and reliability of SC generation: electroformation and hydration(Girard et al. 2004), extrusion(Dittrich et al. 2006) , hydrodynamic focusing(Jahn et al. 2004), pulsed jetting(Funakoshi, Suzuki, and Takeuchi 2007; Kamiya et al. 2016; Gotanda et al. 2018) , double emulsion templating(Shum et al. 2008), transient membrane ejection(Matosevic and Paegel 2011), droplet emulsion transfer(Ota, Yoshizawa, and Takeuchi 2009), reverse emulsion (cDICE)(Abkarian, Loiseau, and Massiera 2011), droplet-supported dGUV (dsGUV)(Weiss et al. 2018; Haller et al. 2018), octanol-assisted liposome assembly (OLA)(Deshpande et al. 2016), wedge splitting(Deshpande et al. 2018) , and toroidal mixing(Webb et al. 2020). This rapid innovation suggests that many hurdles to microfluidic manufacturing may soon be overcome, which could prove catalytic to the field(Shah et al. 2020) . The synthetic cells that result from these processes tend to range from 500 nm to several tens of microns in size. The larger the particle, the faster it is cleared from the body, so therapeutic nanoparticles are generally made as small as possible, on the order of 10–100 nm(Hoshyar et al. 2016) . The more complicated the SC composition, the more difficult the manufacturing process becomes and places considerable technical and cost constraints on SC therapeutics.

Applications of synthetic cells

How synthetic cells are utilized in nanomedicine is determined by their capabilities (Figure 2). Life-like capabilities that can be engineered include directed localization, sense-and-respond behavior, gene expression, metabolism, and high *in vivo* stability. Applications of these properties range from diagnostics to therapeutics.

Directed Localization

A key strategy of living things is to move towards resources that benefit them. Similarly, there is great value in engineering synthetic cells that move to or localize at a target site. This physically concentrates SC's activity at the target and reduces off-target toxicity. Targeted localization can be accomplished either actively (by energy-consuming movement) or passively (by increasing their affinity to the target tissue).

Significant progress has been made in endowing SCs with active chemotactic systems. While liposomes have been used to study natural systems of cell locomotion, these systems have proven difficult to employ due to their complexity(Pontani et al. 2009; Siton-Mendelson and Bernheim-Groswasser 2016) . Recently, relatively simple synthetic systems that leverage biophysical principles have provided more traction(Gentile et al. 2020) . Conjugation of enzymes to the surface of liposomes has been shown to endow them with the ability to move up or even down a pH or metabolite gradient(Ghosh et al. 2019; Somasundar et al. 2019; Hortelão et al. 2020) . This strategy has also proven effective in polymersomes(J. Wang et al. 2020) . Battaglia and colleagues created polymersomes with asymmetrically thick membranes that use encapsulated enzymes to generate a motor force(Joseph et al. 2017) . Another type of polymersome used a platinum nanoparticle to catalyze peroxide oxidation to chemotax towards neutrophils(Peng et al. 2015) . Other mechanisms for directed chemotaxis include the conjugation of complementary DNA oligomers or light-activated protein binders to a surface(Pan et al. 2019; Bartelt et al. 2018) . However, such strategies are more difficult to implement *in vivo*. Another method for directed localization is the conjugation of nanoparticles to living cells as "cellular backpacks"(Jones et al. 2017; Klyachko et al. 2017; Layek et al. 2018; Xie et al. 2017), but this does not require life-like behavior from the nanoparticle itself.

Passive targeting can be accomplished by increasing the affinity of SCs to the target tissue while relying on diffusion and circulation to do the work of carrying them to the tissue. In these cases, the surface of SCs can be modified with polymers, protein ligands, or antibodies that increase their targeting specificity(Leo et al. 2018; J. Cao et al. 2018; Kim, Niidome, and Lee 2019). When multiple targeting moieties are used, the specificity of the targeting increases (Khoshtinat Nikkhoi et al. 2018; Qu et al. 2014; Gray, Li, and Brown 2013; S. Oliveira et al. 2010; P. Guo et al. 2019) . While implementing these strategies, engineers must carefully consider the conjugation chemistry and orientation of the targeting molecules.

The physical properties of synthetic cells can also passively target them to specific tissues. For example, the small size of nanoparticles tends to concentrate them in tissues in which the enhanced permeability and retention (EPR) effect is observed, such as tumors and inflamed or otherwise damaged tissue(van den Hoven et al. 2011; Lobatto et al. 2015; Allen

and Cullis 2013; Lammers et al. 2012) . However, recent work has shown that the EPR effect is dynamic and depends greatly on the composition and structure of the tissue(J. Fang, Islam, and Maeda 2020; Natfji et al. 2017; Danhier 2016). As another example, the strong positive charge of cationic lipid nanoparticles leads them to be taken up by the liver at high efficiencies(Witzigmann et al. 2020) . This has made cationic lipid nanoparticles excellent delivery vehicles for nucleic acid-based gene therapies such as Patisiran(Adams et al. 2018) .

Another approach to achieve localization is to coat nanoparticles with natural membranes(Esteban-Fernández de Ávila et al. 2018) . For example, nanoparticles decorated with red blood cells (RBC) membranes can target macrophages(Wan et al. 2018). To target cancer cells, nanoparticles can be decorated with cell membranes of platelets(Sarkar et al. 2013). Platelet membranes can be additionally labeled with anti-CD22 monoclonal antibodies to precisely deliver drugs to tumor cells(P. Xu et al. 2017; Q. Hu et al. 2015). As an alternative anti-cancer approach, one can use cancer cell membranes to coat nanoparticles that will be presented to antigen-presenting cells and promote anticancer immune response (R. H. Fang et al. 2014).

Sense and Respond

Living organisms can sense key environmental cues and subsequently respond with the appropriate action. Such seemingly “smart” behaviors are key to survival and are a highly desirable function in nanomedicines for several reasons. First, they enable more precise delivery to target tissues. Second, they enable more “analog” responses in which the output can be titrated to the strength of the input signal, thereby avoiding undue toxicity in neighboring tissue. The landscape of such “smart” sensing in vesicles has been extensively reviewed elsewhere(Abraham, Mao, and Tan 2018; Leduc et al. 2007; Majumder and Minko 2020; Torchilin 2014).

Sense-and-response functions can be facilitated directly via changes in SC membrane structure. Membranes have been composed of molecules sensitive to temperature(Yatvin et al. 1978; Needham et al. 2000; Tagami, Ernsting, and Li 2011; Ta et al. 2014; F. Liu et al. 2015; Xi et al. 2020; Jose et al. 2019), light(Miranda and Lovell 2016; Carter et al. 2014; D. Luo et al. 2016; Peyret et al. 2017; Enzian et al. 2020) , magnetism(Babincová et al. 2002; Amstad et al. 2011; H. Guo et al. 2015; H. Oliveira et al. 2013; Geilich et al. 2017), acoustics(Shekhar et al. 2017; Z. Deng et al. 2016; Rwei et al. 2017), pH(Naziris et al. 2017; Abri Aghdam et al. 2019; Leo et al. 2018), redox states(X. Yin et al. 2017; Chi et al. 2017; Mirhadi et al. 2020) , and enzymes(Thamphiwatana et al. 2014; Haas et al. 2015) . By combining these materials, the membrane can sometimes be made sensitive to multiple types of stimuli(Tran et al. 2017; S. Feng et al. 2019) , enabling even more precise targeting. The major limitation of these materials is that few respond to the specific molecules of interest, such as certain cell surface receptors or cancer metabolites. For this, more specific sensors such as protein receptors are required.

Sensing can also be accomplished by membrane-embedded amphiphiles or proteins that are sensitive to specific molecules or conditions. These can transduce detected signals into the interior of the SC through various mechanisms(Langton 2020) . Protein and peptide pores that respond to osmotic pressure, heat, pH, and electrical potential by creating selective

and nonselective pores have been shown to work in liposomes(Louhivuori et al. 2010; Kisovec et al. 2017; Garamella et al. 2019; Aimon et al. 2011; Yanagisawa et al. 2011; Kreir et al. 2008). One group developed a membrane-spanning amphiphile that responds to changes in pH or protein unbinding by localizing at the interior leaflet of the liposome and inducing catalysis that results in the release of drug(Langton et al. 2017; Ding, Williams, and Hunter 2019) . Several bacterial 2-component systems(Ravikumar et al. 2017) have been functionally reconstituted in liposomes(Sanowar and Le Moual 2005; Ito et al. 2009; Jung, Tjaden, and Altendorf 1997; Pflüger et al. 2018), though none of them have been used to induce downstream protein production. Additionally, few other natural or engineered protein transducers (e.g. the SynNotch receptor),Morsut et al. 2016) have been successfully tested in synthetic cells(. This speaks to the sensitivity and complexity of many membrane-bound protein transduction systems and the difficulty with which they can be implemented in synthetic systems.

Another strategy is to embed nonspecific, permanently open pores into the membrane to enable passage of small molecule signals that then stimulate activity inside the SC. To that end, Staphylococcus aureus α-hemolysin has become a favorite tool for synthetic cell engineers due to its ability to spontaneously insert into a wide diversity of membranes and form nonspecific pores. This has been used to allow nutrients(Noireaux and Libchaber 2004) , chemical inducers (e.g. IPTG)(Lentini et al. 2014) , and other small molecules(Wu et al. 2011; Soga et al. 2020) to traffic the SC. Furthermore, SNAREs and DNA oligos have been used to facilitate SC fusions(Schuette et al. 2004; W. Xu et al. 2015) and thereby deliver molecular messengers into the interior of the target SC. Once inside the SC, these messengers can then induce catalysis or even protein production via gene expression.

Transcription factors, RNA riboswitches, and enzymes can also act as sensors inside the SC compartment. These sensors are limited to sensing the interior of the SC, so only molecules that can pass through the membrane or pore can be detected. Enzymes will detect their substrates, and the resulting reactions can change the ambient conditions, such as the pH(Peters, Nijemeisland, and van Hest 2015) . Various transcription factors and riboswitches that are sensitive to the presence of diverse small molecules(Salehi et al. 2017; X. Liu et al. 2020; L. Zhang, Guo, and Lu 2020; Dwidar et al. 2019) and even light(P. Zhang et al. 2020; Schroeder et al. 2012) can be used to control transcription and translation in SCs (see below). Combining sensor signals into transcriptional logic can enable powerful programming of Boolean behaviors(S. Iyer et al. 2013; Shis et al. 2014; Adamala et al. 2017) . Communication between SCs and natural living cells has also been engineered and can give rise to complex interactions between synthetic and natural populations(Lentini et al. 2014, 2017) . Furthermore, the communication that results from mass exchange and sensing among SCs enables the formation of complex multicellular “synthetic tissue” (Niederholtmeyer, Chaggan, and Devaraj 2018; Villar, Graham, and Bayley 2013; Aufinger and Simmel 2018; Ding, Williams, and Hunter 2019; T.-Y. D. Tang et al. 2018; Schwarz-Schilling et al. 2016; Toda et al. 2018; Adamala et al. 2017) . Transcriptional and translational responses to chemicals, however, are relatively slow compared to other sensing mechanisms. To program rapid responses such as those needed for a SC-based bionic jellyfish(Nawroth et al. 2012) (ref), electromechanical sensors and actuators still need to be developed.

Gene Expression

Another powerful capability of synthetic cells is their ability to express genes. Producing protein or small molecules *in situ* is advantageous in drug delivery when the drug is unstable, needs to be titrated or is so reactive that it would kill living cells. Through the action of encapsulated *in vitro* transcription/translation (IVTT) reaction mixture, SCs can produce RNA, proteins, or even small molecule drugs *in situ*. For this, nucleic acids encoding the desired gene must be co-encapsulated. IVTT reaction mixtures may include purified cell extract(Z. Z. Sun et al. 2013; Kwon and Jewett 2015) or defined mixtures of recombinant protein such as the PURE system(Y. Shimizu et al. 2001; Yoshihiro Shimizu and Ueda 2010; Lavickova and Maerkl 2019) . Gene expression can be induced when a triggering signal is sensed (see above). RNA that is produced could serve as a diagnostic signal that can be detected by sequencing RNA extracted from whole blood(Pös et al. 2018) . Translated proteins can be enzymes that together comprise a metabolic pathway that generates a small molecule drug(Dudley, Anderson, and Jewett 2016; Grubbe et al. 2020) . SCs can also synthesize membrane proteins that will spontaneously insert into membranes and act as uptake signals to target cells .(Kaneda et al. 2009; Lu et al. 2019)

Previously, *in situ* protein production could only be accomplished by living cells. These were delivered into the body encapsulated in polymer membranes, where they could survive a long time while ameliorating chronic conditions such as diabetes(Lim and Sun 1980; Soon-Shiong et al. 1994; Y. Sun et al. 1996; Calafiore et al. 1999; de Vos, Hamel, and Tatarkiewicz 2002), neurological diseases(Bloch et al. 2004), haemophilia(Basic, Vacek, and Sun 1996), or cancer(Löhr et al. 2001) and are reviewed elsewhere(Thomas Ming Swi Chang 2005, 2019) . Recent development of synthetic cells that encapsulate IVTT reactions offers an alternative approach to encapsulated whole cells. Schroeder and colleagues pioneered SC therapy by demonstrating that liposomes containing IVTT could be used to synthesize anti-cancer proteins inside tumors. In this work, they showed that synthetic cells producing *Pseudomonas* exotoxin A killed most cancer cells in culture and caused robust apoptosis when injected into 4T1 tumors in mice(Krinsky et al. 2018).

Beyond its utility in direct therapeutic intervention, cell-free gene expressions has enabled other biomedical technologies and novel research tools. For instance, the high stability of freeze-dried IVTT reactions has enabled the development of on-demand biotherapeutic manufacturing platforms(Pardee et al. 2016; Adiga et al. 2020, 2018; Jaroentomeechai et al. 2018). Another example is liposome display, a technology that uniquely enables *in vitro* selection and directed evolution of protein pores(Fujii et al. 2014; Uyeda et al. 2016). Membrane-bound IVTT protein production can also control the orientation of integral membrane proteins(Ando et al. 2018; Ohta et al. 2016). Challenges these technologies still face include relatively low titers of protein produced, reproduction of critical post-translational modifications, and the limitations on controlling the insertion and orientation of membrane proteins. Nonetheless, due to the close ties between the fields of cell-free biochemistry and synthetic cell engineering, each will doubtless benefit from the other's continued advancement.

Metabolism

Living cells maintain their functions through active metabolism. This allows them to act against entropy repeatedly or continuously over extended periods of time, a behavior that is challenging to engineer in nanoparticle drug delivery systems. SCs, however, can be loaded with complex biochemistry that can mimic many of the metabolic processes that living cells perform. These include production of energy molecules such as ATP, the regeneration of essential cofactors, or chemical transformation of target metabolites. The value of this is both to sustain the therapeutic function of the SC as well as directly metabolize toxic metabolites.

By actively generating ATP or other energy molecules, SCs can maintain a sustained response instead of generating only a short burst of activity from the ATP encapsulated during production. Several different approaches have been taken to endow SCs with the ability to generate ATP. A common strategy is to create a proton gradient that can then be used by ATP synthase to drive ATP production. To generate the proton gradient, light-activated bacteriorhodopsin(Choi and Montemagno 2005; Dhir et al. 2018; Z. Chen et al. 2019) or other proton pumping systems(X. Feng et al. 2016; Steinberg-Yfrach et al. 1998; Cladera et al. 1996; Altamura et al., n.d.) can be embedded in the membrane. The resulting light-dependent ATP synthesis can then drive IVTT protein production(Berhanu, Ueda, and Kuruma 2019)or other ATP-dependent processes. Though this approach has been fruitful, it is challenging to implement *in vivo* because light only penetrates a few millimeters into the skin(Sabino et al. 2016). Instead, ATP synthesis can be driven via catabolic chemistry on ambient energy-rich molecules(Jewett and Swartz 2004; Calhoun and Swartz 2005; Biner et al. 2020; Caschera and Noireaux 2015). The feedstocks for these pathways are also substantially cheaper than the high-energy molecules used in some batch reactions(Calhoun and Swartz 2007).

Regeneration of cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH) is often essential to maintain biochemical reactions. This can be accomplished by encapsulating enzymes that catalyze the regenerating reaction(Meeuwissen et al. 2011). Hirst and colleagues recently demonstrated sustained ATP synthesis by coupling ATP synthase to NADH oxidation(Biner et al. 2020). Integrating novel ways to import or regenerate cofactors and other reagents is critical for longer sustained reactions in synthetic cells.

Chemically transforming metabolites can provide vital therapeutic benefits. Vesicles that contain enzymes have been developed for therapeutic application since the 1960s. Seminal work by Thomas Chang demonstrated that compartmentalized enzymes could provide therapeutic effects in animals lacking normal enzyme activity(T. M. S. Chang and Poznansky 1968) . Since then, therapeutic encapsulations of urease(Cattaneo and Chang 1991; Gu and Chang 1990; Lvov et al. 2001; Miele et al. 2020) , catalase(R. Zhang et al. 2017; Shi et al. 2020) , superoxide dismutase(Riedl et al. 2005; Shazeeb, Feula, and Bogdanov 2014; Niesman, Johnson, and Penn 1997) , β-galactosidase(Rao, Chawan, and Veeramachaneni 1994) , bacterial DNA repair enzymes(Berardesca et al. 2012; D. Yarosh et al. 1996; D. B. Yarosh, Rosenthal, and Moy 2019), alcohol oxidases(Pratsinis et al. 2017; C. Lizano et al. 1998; Whitmire, Chambers, and Dillon 1991), glucose oxidase(S. Liu et

al. 2020) among others, have opened doors to novel therapies. Many of these formulations aim to remove membrane-permeable metabolites from the body. Pratsinis, et al. employed liposomes bearing either alcohol oxidase or catalase in their membranes in peritoneal dialysis to remove ethanol from the blood of rats(Pratsinis et al. 2017). This work follows older efforts in which alcohol dehydrogenase and aldehyde dehydrogenase are encapsulated together to break down ethanol in vivo. In these systems, another enzyme (e.g. malate dehydrogenase) is used to regenerate the NAD+ cofactor required to maintain the oxidation reaction, highlighting the importance of cofactor regeneration to maintaining high catabolic rates(Campbell and Chang 1978; T. M. S. Chang 1987; Carmen Lizano, Teresa Pérez, and Pinilla 2001).

Encapsulated enzymes provide value to an impressive diversity of indications. A recent clinically tested example include Lipoxysan, a transdermal liposomal encapsulation of superoxide dismutase, which was recently tested in Peyronie's disease in Phase 2 clinical trials (Riedl et al. 2005). Mann and colleagues demonstrated that complex assemblies of glucose oxidase-containing coacervate and hemoglobin-containing red blood cell-derived membranes were able to generate nitric oxide in vivo, inducing vasodilation(S. Liu et al. 2020) . An example in which SCs serve to aid in diagnosis is in the work by Molina and colleagues. In this work, the authors generated different SCs containing mixtures of three or more different enzymes. Based on the metabolites present, SCs would generate different colored products. Incubating these SCs in urine aided in the diagnosis of pre-diabetic states in patients(Courbet et al. 2018) .

Multilamellar liposomes, vesosomes, and different species of liposomes can work together to control reactions. Incompatible enzymes can be separated in defined compartments allowing the spatial organization and segregation of the multistep tandem reaction(Klermund, Poschenrieder, and Castiglione 2017) . Different liposomes containing varied enzymes can be connected via α-haemolysin channels . Polymer SCs containing two distinct populations of enzyme-encapsulating vesicles have been demonstrated to function inside living cells(Godoy-Gallardo et al. 2017). To prevent the deactivation of catalysts in water or avoid unwanted cross-reactions, catalysts are often site-isolated in nanopockets or separately stored in compartments. These examples show that control of the localization of enzymes within an SC can be as valuable to the SCs function as the enzyme activity itself.

High stability

One of the most desirable characteristics of living cells is their ability to remain intact in the blood for long periods of time. Nanoparticles, on the other hand, are typically less stable and are rapidly cleared by the reticuloendothelial (RES) system(Sercombe et al. 2015). This is typically due to both their physical nature (large, spherical, stiff objects are more quickly removed from the blood) and to the fact that they do not display proteins that mark the nanoparticle as the body's own cell(F. Chen et al. 2017; Vu et al. 2019; Zahednezhad et al. 2019).

Numerous strategies have been taken to endow nanoparticles with longer half-lives in blood. The decoration of the particles with polyethylene glycol (PEG) is perhaps the most successful "stealth" strategy. PEGylation, however, is falling out of favor due to

the production and presence of anti-PEG antibodies(L. Yin et al. 2015), PEG tissue accumulation(Lane et al. 2017; Rippe et al. 2019) , evidence of lack of PEG degradation, potentially creating vacuoles(Baumann et al. 2014; Ivens et al. 2015) , and alteration of enzyme activity(Leuzzi et al. 2016). As such, alternatives to PEG such as heparosan are being developed(Lane et al. 2017; Rippe et al. 2019).

Decoration of particles with membranes derived from living cells is a powerful strategy to shield nanoparticles. This “semi-synthetic” approach, pioneered by Hu and colleagues, has proven highly versatile(C-M J. Hu et al. 2011). All manner of cell membranes and cell membrane proteins have been used to coat nanoparticles and SCs(Corbo et al. 2017; Liang et al. 2018; Che-Ming J. Hu et al. 2013; Weiwei Gao et al. 2015; L. Luo et al. 2017; J. Tang et al. 2017; H. Cao et al. 2016; Pitchaimani, Nguyen, and Aryal 2018). This effectively shields the SCs from the RES and endows them with some of the signaling properties of the cells from which their borrowed membranes derive. As mentioned above, this strategy also enables targeting.

Challenges Facing Synthetic Cell Therapeutics

The field of synthetic cell engineering is relatively new, and many challenges still remain to be solved or even identified. These challenges include need for molecular tools, integration of disparate technologies, difficult manufacturing, and regulatory frameworks that disfavor complex drug formulations (Figure 3).

To coordinate release of therapeutic agents at the right location (e.g. at a tumor), more robust sense & respond mechanisms are needed. Currently, there is a relative lack of sensors that activate SCs in response to specific molecules, such as membrane proteins overexpressed on cancer cells. Such tools are now readily available in living cell therapies, such as CAR-T cells. Transferring natural membrane transduction systems to synthetic membranes is difficult due to their size, complexity and requirements for post-translational modification and membrane insertion machinery. While bacterial transduction systems are smaller and relatively more robust proteins, they are likely immunogenic and cannot recognize eukaryotic membrane proteins. To enable reliable sensing of eukaryotic molecular markers of disease, a concerted effort is needed to engineer membrane transduction systems that function specifically in SCs.

A similar problem is the limited repertoire of trans-membrane channels available in synthetic cell systems. Living cells tightly control the flow of molecules in and out of the cell through channel proteins or membrane budding mechanisms. SCs, however, currently lack most of these systems. This is reflected in the prolific use of α-hemolysin, a simple and robust bacterial toxin that enables passive transport of small molecules. Only a few active transporters have been demonstrated in synthetic cells, including proteins as complex as ATP synthase. If more reliable membrane transporters could be identified and engineered to spontaneously insert into the membrane in the desired orientation, it would greatly broaden the sensing and delivery repertoire of SCs.

An issue for the entire field of synthetic cell engineering, including all therapeutic applications of this technology, is the integration of subsystems into one robust entity. As described in earlier sections of this review, many SC subsystems have been developed to demonstrate specific functionalities. Because each subsystem is engineered ad hoc, it is often difficult to reconcile their diverse chemistries and structures. To address this, several approaches can be taken. First, engineers in the field could standardize the chemical and structural framework in which they develop SCs. To encourage this, funding agencies could require adherence to these frameworks when it is sensible. Second, subsystems could be engineered with integration in mind by reporting subsystem performance across a variety of contexts. Lastly, computational models rooted in empirical data could be developed to guide the integration, much like what was done to guide the integration of synthetic genetic circuits (Nielsen et al. 2016).

To use synthetic cells as human therapeutics, other issues will need to be solved as well, like the toxicity of cell-free IVTT systems. While defined systems such as PURE contain mostly purified proteins, cell-derived fractions of ribosomes still contain some amount of endotoxins (i.e. lipopolysaccharides), which are highly pyrogenic. So far, only direct tumor injection was demonstrated as a method for localizing synthetic cells into a solid tumor in mice (Krinsky et al. 2018), and there is a relative lack of available data on half-lives and dose-dependent toxicity of synthetic cell formulations in animals.

As therapeutic applications of synthetic cells progress through foundational research and commercial R&D pipelines, the field will need to face technical challenges related to scaling up production of those novel therapeutics. Among those challenges, two areas present the most well-defined focus points: the compartment and the chemicals inside it.

Scaling up manufacturing of lipid vesicles to create membrane encapsulating synthetic cells will require progress in current microfluidic technology, or perhaps development of entirely new class of liposome formation technologies. Synthetic cells are typically larger than liposome drug delivery vehicles (single microns vs tens of nanometers in diameter), and enzymes encapsulated inside synthetic cells can not be encapsulated via remote loading used for some liposomal drugs. This creates a need for a whole new class of reliable, reproducible and scalable encapsulation techniques.

Similarly, producing large amounts of proteins and small molecules needed to provide therapeutic quantities of synthetic cells might require adjustment in supply chains. Production of cell-free protein expression systems is already scalable to 100-liter reaction volumes (Zawada et al. 2011), but availability of certain high value reagents remains a limiting step. We as a field anxiously await the invention of “PURE that makes PURE” -- a cell-free IVTT reaction that can make every one of its own functional components and need little more than raw material as feedstock -- as an idealized solution to the scaling problem.

Once synthetic cell technologies pass into animal testing, the need to fulfill FDA approval requirements will become critical. Currently, there are few guidelines for developing therapies as molecularly complex as synthetic cells . FDA approval is granted either for drugs with precisely controlled chemical composition, or for natural cell therapeutics.

Synthetic cells, being made from synthetic components but not being descended from known living cells, may require a new framework by which such systems are evaluated . Uniformity of the formulation will remain critical, putting pressure on the above mentioned supply chain and scalability of membrane formulations. The FDA is already aware of the needs that might arise with progress of novel therapies using untested chassis, facilitating development of new oversight rules through the FDA Emerging Technologies Program. (Center for Drug Evaluation and Research 2019) It will be critical that the synthetic cell community works closely with regulatory agencies to develop a supply pipeline and draft new frameworks for the testing and eventual deployment of those therapies for patient use.

Discussion & Outlook

Many have noted in recent years that as increased resources are devoted to development of drugs, ever fewer result in approved therapies. (Scannell et al. 2012) This phenomenon, known as Eroom's law (the reverse of Moore's law), highlights the need for new therapeutic modalities and approaches. Here we describe how synthetic cells offer a unique, engineerable platform for achieving a range of valuable therapeutic behaviors. These offer to augment existing means of drug delivery as well as tools for research and drug discovery pipelines. Though significant technical progress has been made in mimicking several advantageous features of living systems, the integration of these features remains a challenge, as do the development and manufacturing of such systems. Given recent progress, the vision of a synthetic cell that can identify and ameliorate disease in a programmable manner without adding risk of adverse effects looks less like a moonshot than an inevitable next step for medicine.

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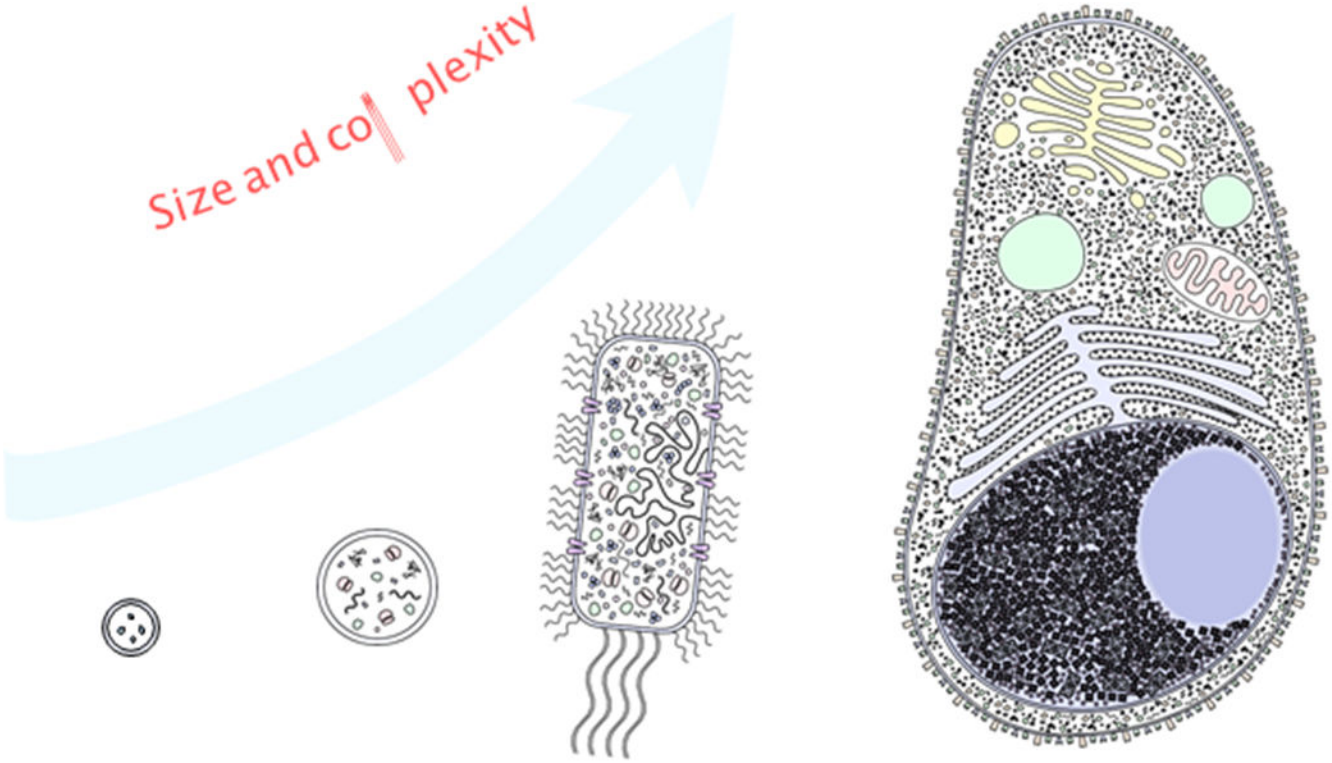

Liposomes	Synthetic Cells	Bacteria	Eukaryotes
Not programmable	Programmable	Programmable	Programmable
Defined	Defined	Undefined	Undefined
No reproduction	No reproduction	Reproduces	Reproduces
Nontoxic	Nontoxic	Toxic/immunogenic	Nontoxic
No lateral gene transfer	No lateral gene transfer	Lateral gene transfer	No lateral gene transfer
No metabolism	Limited metabolism	Extensive metabolism	Extensive metabolism
Difficult to produce	Difficult to produce	Easy to grow	Slow to produce/grow
No steady state	No steady state	Steady state	Steady state

Figure 1:

Comparison of Synthetic Cells to Other Drug Delivery Systems. Synthetic cells tend to fall between liposomes and living cells in terms of complexity. Their capabilities extend beyond those of liposomes but cannot match the sustained, nuanced behaviors of living cells. This relative simplicity also endows them with advantages, such as complete programmability and zero risk of uncontrolled replication.

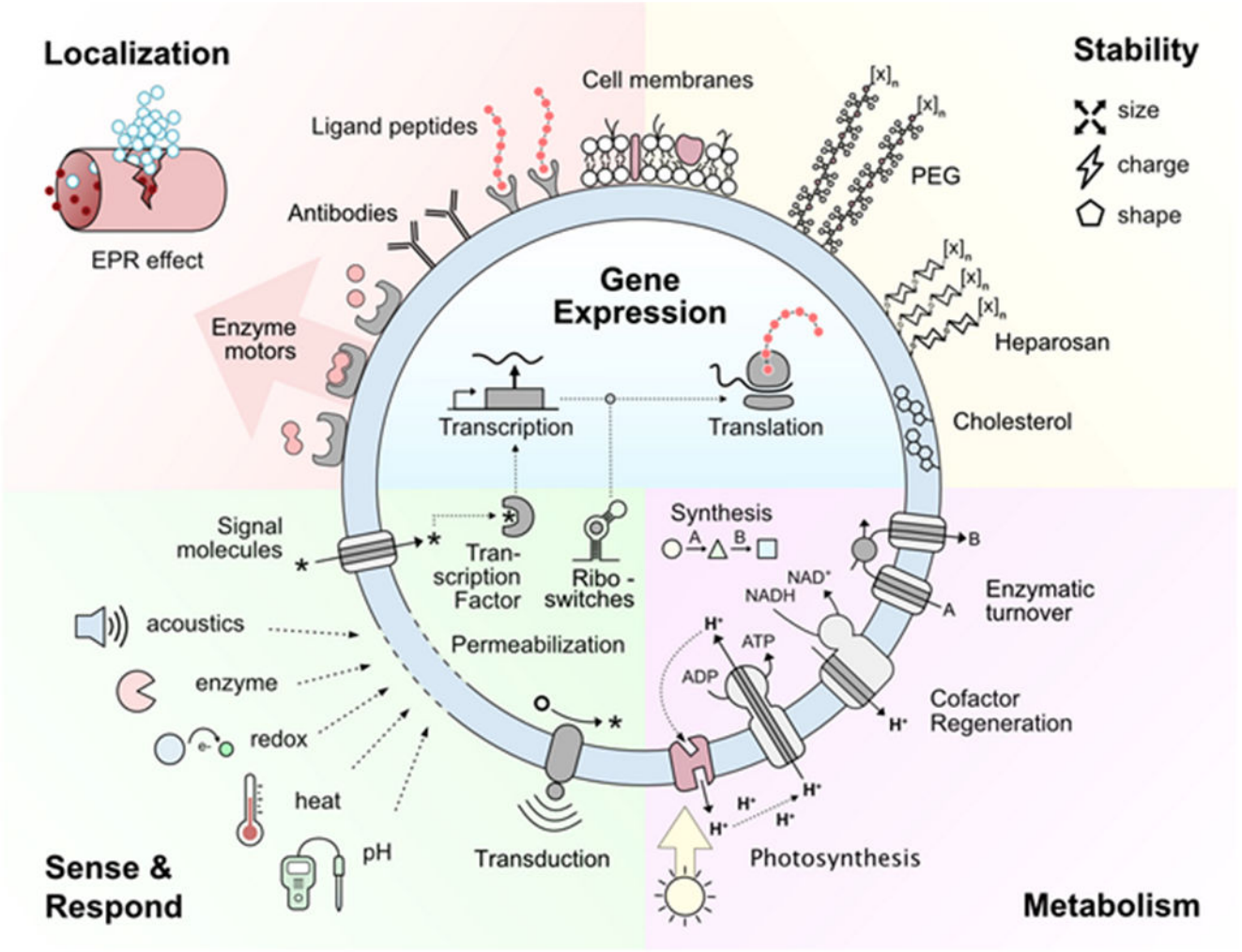

Figure 2: Life-like Functions of Synthetic Cells in Nanomedicine.

Synthetic cells are engineered with various chemical tools to mimic one or more functions of living cells. The physical properties of the SCs affect both their stability and localization through the EPR effect.

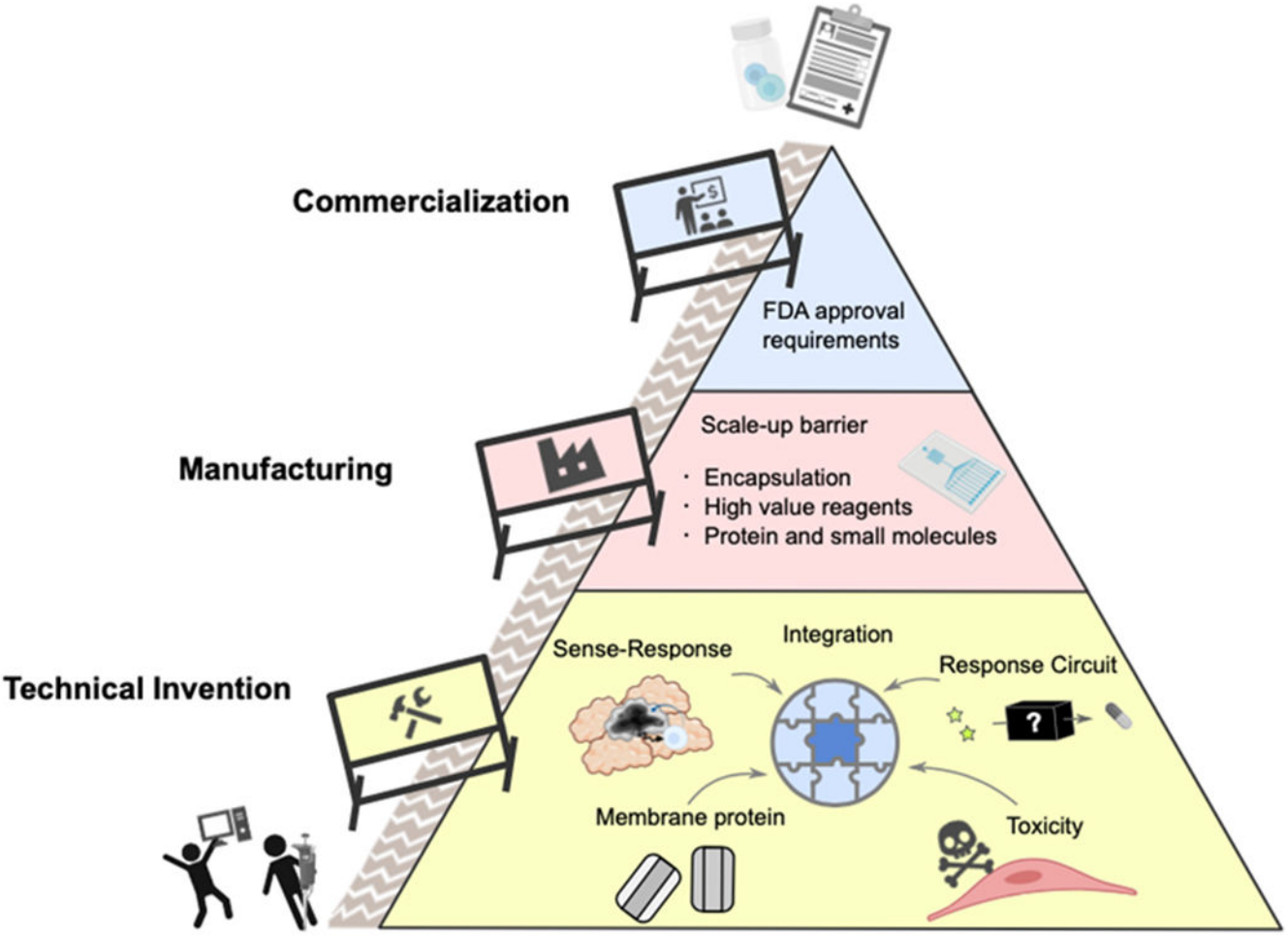

Figure 3:

Barriers to Synthetic Cell use in nanomedicine. Most fundamentally, new innovations and solutions are needed to endow SCs with modular functions that can perform reliably in physiological conditions. Next, production and cost barriers need to be addressed. Finally, therapeutic SCs will encounter regulatory hurdles that may require adapting current frameworks.