ARTIFICIAL CELLS:

An Overview of Main Approaches and Applications

Matura paper

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List of Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AWC	artificial water channel
Cas9	CRISPR-associated protein 9
gRNA	guide RNA
GUV	giant unilamellar vesicle
HDR	homology directed repair
NHEJ	non-homologous end joining
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
RBC	red blood cell
SGC	synthetic gene circuit
TX-TL	transcription-translation
w/o/w	water/oil/water

Abstract

It has been about 20 years since extended research on artificial cells kicked off around the world. Since then, many different developments on the preparation of artificial counterparts of cells and their machinery have been formulated by using a variety of strategies and building blocks. Cells are not of simple nature, and their study is not straightforward either, as research on artificial cells concentrates on separately deciphering the cell's numerous components one by one, rather than the entire lot at once. Furthermore, this research can be conducted by either altering and examining natural organisms and their parts or by attempting to recreate them from scratch. The central aim of this Matura paper is to provide comprehensive descriptions and summaries of the most efficient and commonly utilized methods for the creation of artificial cell-like structures that are used to recreate and study life on the cellular level. A few real-world applications of artificial cells that are currently relevant will also be considered.

1 Introduction

It would not be wrong to say that we currently have a reasonably advanced understanding of cellular life. The structures and functions of many cellular components, processes, and mechanisms have been investigated in depth and unraveled, and some of these can even be artificially replicated in a laboratory with great efficiency. Even so, it is the staggering extent of the intricacy with which an entire cellular system is put together and coordinated that renders artificial replication of even the simplest unicellular organisms a distant goal as of now. Still, as intimidating of a goal as it is, the creation of a synthetic equivalent of a natural cell is also a highly tempting aspiration that would certainly be of great use in many fields. Synthetic biology attempts to further understand the machinery and organization of cells by recreating single components and systems of cells either by editing the genome of a natural organism (top-down approach), or by assembling a cell from individual building blocks (bottom-up approach).

1.1 What Are Artificial Cells?

Synthetic biology is a field of research that, in the context of artificial cells, strives towards the development of an ideal artificial cell for the sake of research. This ideal artificial cell involves two main connotations. First, it would behave just like a natural, living organism, i.e., it would be able to capture, process, and make use of energy, regulate and balance its own resources, store and translate genetic information, and even be capable of reproduction and adaptivity to its environment, among other things. Secondly, it would be assembled completely from artificially created parts. Each part of the ideal artificial cell, from the membrane to the genetic material, would be constructed entirely via artificial means. Naturally, construction of such an ideal artificial cell is a distant goal as of now, and much of the research on ideal artificial cells is conducted by experimentation with living organisms. Still, by altering and attempting to replicate natural cellular processes and parts, much can be discovered about the fundamental systems that drive all life on earth, and it is hoped that this field of research will also uncover some of the mysteries on the origin of the very first lifeforms (Deamer, 2005, C. Xu et al., 2016).

In the field of biotechnology, which involves usage of organisms and biological parts for the development of various types of useful products, the concept of artificial cells typically refers to simple and pragmatic artificially engineered cell-like structures that can house a variety of biological or synthetic components, such as genes and enzymes, inside of a membrane that can also be either directly borrowed from nature or formed via artificial means. Thus, it would be incorrect to think that “biotechnological” artificially engineered cells are made up entirely of components that are built in a laboratory, since many parts can be taken from living cells to

maximize efficiency. These artificial cells are often built specifically to mimic a real cell's particular set of functions, often for medical purposes, and are sometimes referred to as "cell mimics" (C. Xu et al., 2016). This Matura paper will predominantly focus on the former research-oriented aspect, rather than the latter application-oriented one.

1.2 History

The first simple artificial cells were developed in the 1960s by Thomas Chang at McGill University, Canada (Chang, 1964). These early structures were made of a synthetic polymer membrane and were able to encapsulate biological components such as enzymes and other proteins in a stable manner. Like natural membranes, the artificial cells' membranes were semi-permeable and allowed diffusion of certain molecules in and out of the cell. The author's research showed that his encapsulated enzymes were able to act almost as efficiently as under natural conditions (Chang, 2007). Since most of this research took place before the modern era of synthetic biology, it has only been about 20 years since many of the originally presented ideas have been explored and developed more thoroughly. Since then, however, there has been much rapid progress and innovation. In the year 2000, the first simple artificial genetic systems involving a few genes, called artificial gene circuits, were created. In 2010, a self-replicating bacterial cell whose genome had been replaced with one that had been synthesized entirely by artificial means was made, and, in 2019, a fully new, viable artificial variant of the bacterium *Escherichia coli* was reported (Gardner et al., 2000, Gibson et al., 2010, Fredens et al., 2019).

2 Building an Artificial Cell

Currently, there are two main approaches for building an artificial cell: the top-down approach and the bottom-up approach (Figure 1) (C. Xu et al., 2016). The top-down approach has a biological organism with a simpler genome, typically a bacterium, at its basis. This organism can either have its genes knocked away one by one until it only has enough genetic material for its intended purpose or properties, or its genome removed and completely replaced by a chemically synthesized one (Gibson et al., 2010, C. Xu et al., 2016). The bottom-up approach, on the other hand, seeks to build a living organism from scratch by assembling biological and non-biological parts and molecules (Forlin et al., 2012). In other words, the aim of the top-down approach is to re-shape life in a controlled way, although typically to the point where the original organism is not considered to be alive anymore, while the ultimate aim of the bottom-up approach is to create something that is alive with only dead parts as building blocks.

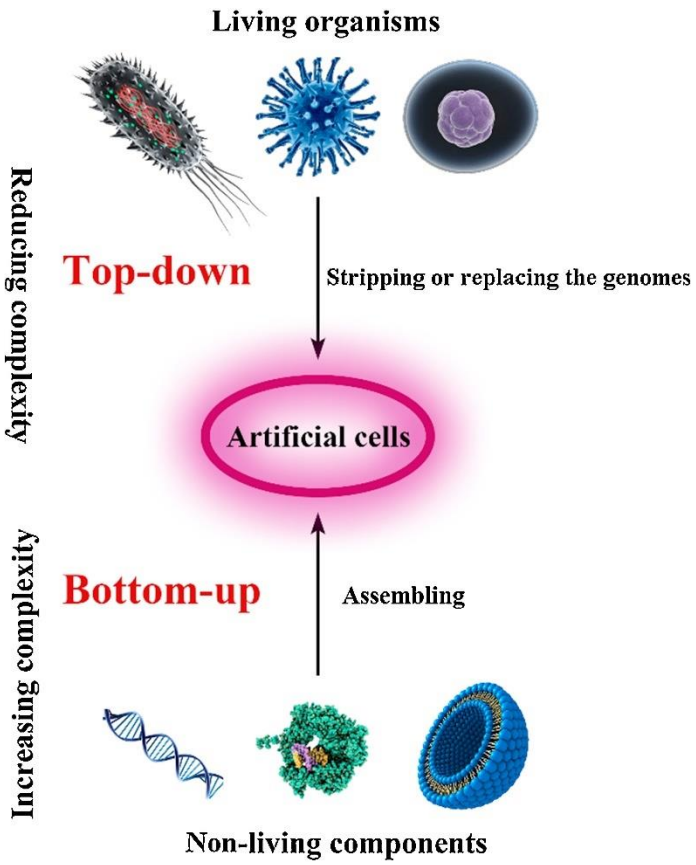

Figure 1: ***The two main approaches for the creation of artificial cells.*** *The top-down approach consists of altering natural organisms' genomes to study their inner workings, while the bottom-up approach attempts to create something that resembles a living organism by assembling components. (C. Xu et al., 2016)*

2.1 Top-down Approach

In synthetic biology, the focus of the top-down approach is not yet to create cells that exhibit particular functions, but rather to build a “minimal cell”, a conceptual cell with only the minimum amount of genetic material needed to fulfil every living being’s most fundamental goal: to survive. All the genes that remain in this cell are, by definition, essential (Lachance et al., 2019). It is thought that the study and creation of such simple cells will help to understand the vital processes that regulate and define life, much like how the hydrogen atom was first used by physicists to understand the basic rules of atomic structures. Experiments with the goal of developing something close to a minimal cell are, as of now, limited to prokaryotic cells, since eukaryotic cells exhibit genomes that are too complex to be reliably tampered with (Glass et al., 2017).

When altering a genome by the top-down approach to create a minimal cell, distinctions need to be made regarding the essentiality of genes. A gene is deemed essential if, when disrupted or otherwise inactivated, one or more of the cell’s essential functions is impaired to the point where the cell cannot indefinitely continue to sustain itself or propagate. Essential gene functions consist mainly of functions related to maintaining a working metabolism and cellular structure, replicating DNA, transcribing RNA and translating it into proteins, engaging in cell division, and a few other basic processes common to all organisms (P. Xu et al., 2011). On the other hand, a gene is non-essential if it can be inactivated without affecting any of the cell’s most vital functions in any significant way. Furthermore, there is also a third class of genes called quasi-essential genes, which describe functions a cell needs for effective self-sustenance or propagation, but which are not necessarily essential (Glass et al., 2017). Still, it should be noted that, in practice, which genes are truly essential, non-essential, or quasi-essential is highly dependent on the cell’s circumstances such as its environment, as it can among other things determine which resources and building blocks the cell would need to synthesize and recycle by itself and which would be provided by the environment (C. Xu et al., 2016).

2.1.1 Genome Minimization

One method to develop something close to a minimal cell is to strip an organism of its non-essential genes to examine its viability. With the help of experimental and computational strategies based on this concept, Gil and colleagues were able to propose an amount of only 206 strictly essential genes needed by the bacterium *E. coli* to produce proteins for its essential functions (Gil et al., 2004). For comparison, the biological organism capable of self-reproduction with the simplest known genome, the pathogenic bacterium *Mycoplasma genitalium*, has

525 genes, 482 of which are protein-coding (Fraser et al., 1995). There are two main strategies for identifying and inactivating non-essential genes: transposon mutagenesis and site-directed mutagenesis. Transposon mutagenesis makes use of randomly induced mutations via transposon insertion. A transposon is a segment of DNA that can change position within a cell's chromosomes. In nature, they play a significant role in the evolution of a cell's genome, as their movement can alter gene expressions and cause mutation, and they even make up a considerable amount of the mass of DNA in eukaryotic cells (Gao et al., 2015, Bourque et al., 2018). However, in this case, a harmful foreign transposon is forcefully inserted into a bacterial cell's chromosome, which causes it to damage and effectively inactivate the gene it inserts itself into**.** With this concept in mind, a bacterial cell culture is bombarded with foreign transposons, which insert themselves randomly in the organisms' genomes and inactivate the genes they interact with. If a transposon interacts with an essential gene bound to a function that is essential to the cell, the gene and thus the function will be disrupted, the cell will lose viability and either die or be unable to pass on its genetic material any further. On the other hand, if a transposon interacts with and disrupts a non-essential gene, the cell will not experience any significant impairment and will keep living and reproducing. So, cells that have been mutated by transposons and that are still capable of survival and growth suggest that no transposon inserted itself in any gene that is crucial for that cell's viability, i.e., in any essential gene. After this process is concluded across the entire cell culture, the locations of the transposon insertions can be discerned through microarrays, transposon sequencing or inverse polymerase chain reaction (inverse PCR) (Glass et al., 2017). A microarray is a tool used in the laboratory that can determine the expressions of thousands of genes of a given sample at the same time, while transposon sequencing makes use of another sequencing technology, massive parallel sequencing. After sequencing, it is possible to discern the insertion sites of transposons whose sequences are known (Sassetti et al., 2003, Van Opijnen et al., 2009). Finally, inverse PCR is a variation of traditional PCR that makes use of "inverse" primers that amplify DNA in the opposite direction as traditional PCR, i.e., in the direction away from the known sequence (the transposon) and towards the flanking unknown sequences (the transposon insertion site), causing amplification of the unknown sequences (Ochman et al., 1988). The resulting product can then be sequenced and compared to a DNA database to recognize spots of DNA with interruptions caused by the transposons, thus allowing identification of transposon insertion sites. The results of the entire surviving cell culture received from any of the described methods are then cross-referenced to identify the organism's essential genes (Glass et al., 2017).

Site-directed mutagenesis, the other main strategy to strip an organism of its non-essential genes, consists of systematic deletion of specific targeted genes. With this method, the natural organism's genes are inactivated step by step, and developments in the organism's viability

and other properties are recorded after each gene cluster deletion. This process continues until there is no gene left that can be disrupted without fundamentally impairing the organism's viability (Glass et al., 2017). There are various methods and technologies that can be used for site-directed mutagenesis, but the one that is the most relevant and effective presently is CRISPR (clustered regularly interspaced short palindromic repeats) gene editing. CRISPR gene editing is derived from the antiviral defense system of certain bacteria and archaea. If, for example, such a bacterium is attacked by a bacteriophage and survives, it has the ability to store segments of the genetic material that was injected by the bacteriophage as part of its CRISPR adaptive immune system. If that same bacterium is ever infected by a bacteriophage again, the stored sequences are copied and transported to proteins called Cas9 (CRISPR-associated protein 9) as guide RNA (gRNA). Cas9 then interrogates the genetic information in the bacterium until it finds a segment of nucleic acids that matches the gRNA's target sequence, which is usually about 20 bases long, at which point it will proceed to cleave the viral DNA, thus rendering it inactive and interfering with the virus's replication process (Figure 2) (Barrangou et al., 2007). Furthermore, to make sure that Cas9 does not attack the originally viral sequence that is now stored as part of the CRISPR system, a 2-6 base pair long sequence, called the protospacer adjacent motif (PAM), must be present just after the viral sequence that matches the gRNA in order for Cas9 to proceed with the cleaving. The CRISPR system will never include the PAM, but it is a very generic and short sequence that occurs frequently and naturally in viral genomes. Cas9 first probes for the PAM to confirm that it is not interrogating a CRISPR sequence, and only then does it actually check the DNA. The PAM sequence needed for Cas9 to operate is dependent on the specific species of bacterium and on the type of Cas protein. To provide an example, *Streptococcus pyogenes*, a bacterium whose CRISPR-Cas9 system has been studied in depth, requires the PAM sequence 5'-NGG-3', where "N" is a placeholder for any nucleobase, for its Cas9 protein to take effect (Shah et al., 2012, Anders et al., 2014).

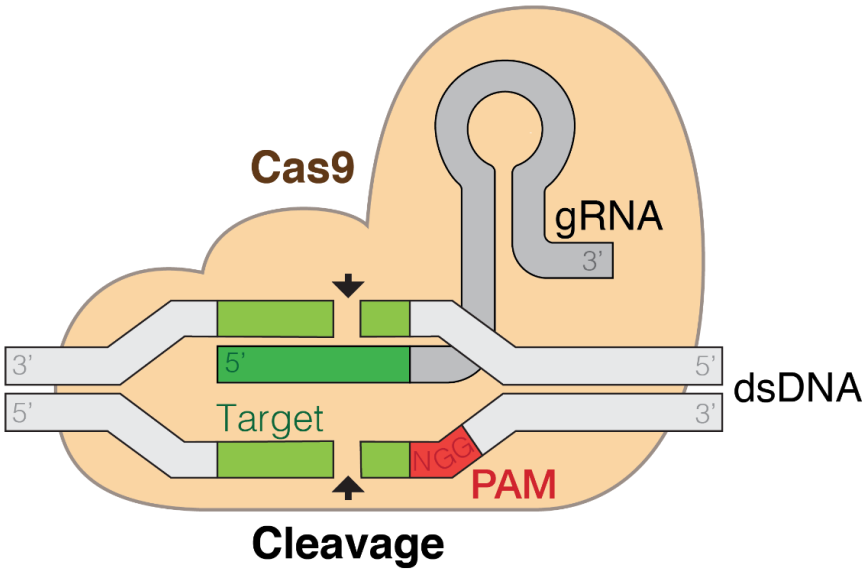

*Figure 2: **Cas9.** With the help of a sequence complementary to the target sequence as part of its gRNA complex (dark green area), Cas9 can identify and disrupt invasive genetic material (light green area). By also requiring a viral PAM sequence (red area), Cas9 makes sure that it does not damage its host organism's CRISPR system. dsDNA: double stranded DNA. ("Cas9", n.d.)*

Of note is the fact that a synthetic gRNA can easily be presented to Cas9 to allow targeting of any genomic site. If the goal of the procedure is to edit an organism's genetic code by removing and then replacing genetic material, a DNA repair template will be presented to the cell to allow it to naturally conduct homology directed repair (HDR) after Cas9 has cleaved the targeted site, thus incorporating the new genetic material into the organism after removing the old undesired material. With this method, precisely defined DNA replacements can be conducted (Ran et al., 2013). On the other hand, if no repair template is presented, the cell will attempt to repair the often lethal double-stranded breaks in the DNA through the error-prone non-homologous end joining (NHEJ). It has been proven that NHEJ is not random in the way it chooses to repair DNA lesions, but it is highly unpredictable, and the procedure typically results in deletion mutations known as indels, which are the goal of site-directed mutagenesis (Lin & Luo, 2019). Traditional CRISPR-Cas9 induced targeted deletions can only inactivate short segments of DNA at a time, however, new methods, which involve co-delivery of Cas9 proteins and pairs of gRNA to target multiple genes at once, have been developed that can efficiently generate DNA deletions up to 10 kb in length, although the results can also be highly dependent on whether the procedure is carried out on a prokaryotic or eukaryotic cell, as not all prokaryotes make us of NHEJ (Neldeborg et al, 2019, Weller et al., 2002). In site-directed mutagenesis, these procedures are employed for serial deletions of non-essential gene clusters based on known and unknown gene functions to test an organism's viability and properties after each cluster deletion. Eventually, as more and more genes are inactivated, the size of the clusters is reduced to the point where only single gene deletions take place. This continues

until the genome has been reduced to a desired size, or until further gene deletions would result in the organism's death or in an unacceptable decrease in cell functionality (Glass et al., 2017).

2.1.2 Synthetic Genome

Although stripping an organism of its unwanted genes is not ineffective, there exists another, more advanced method to create a top-down artificial cell by completely replacing the organism's natural genome with a synthetically fabricated one, whose structure and sequences are based off current understanding of genetic codes. This method is considerably more expensive and complicated, but shows much potential, and if developed, would be much more efficient than stripping a cell of its genetic code step by step in a cumbersome process (C. Xu et al., 2016). Artificial gene synthesis consists of two main stages: DNA synthesis and DNA assembly. DNA synthesis, or less commonly referred to as DNA printing, concerns itself with the construction of short DNA chains called oligonucleotides, while DNA assembly is needed to assemble oligonucleotides into functional genes, multi-gene circuits, or even entire chromosomes or genomes. A variety of methods exist to create oligonucleotides with a desired sequence, but the most commonly used one is the phosphoramidite method first developed in the 1980s (Caruthers, 2011). Since naturally occurring nucleotides are not reactive enough to make efficient building blocks for artificial DNA synthesis, nucleoside phosphoramidites, typically simply called phosphoramidites, are used as the building blocks of oligonucleotides. Due to the greater reactivity and selectivity of these nucleotide variants, they experience considerably greater ease and rate of formation of links between each other than what would be possible between regular nucleotides, thus also increasing DNA chain formation rates (Beaucage & Caruthers, 1981). The first step of phosphoramidite preparation consists of making sure that the number of undesired side reactions during the whole procedure remains as small as possible. To do this, natural nucleosides' functional groups are coated with protective groups to render the functional groups inactive during the whole phosphoramidite preparation and oligonucleotide synthesis process (Figure 3). Typically, the 5'-terminal hydroxy group is protected with DMT, i.e., dimethoxytrityl, a substance that is used almost exclusively for this specific purpose as part of oligonucleotide synthesis, while the exocyclic amino groups of the nucleic bases A and C need to be protected with acyl protecting groups. G does not have an exocyclic amino group and does not often perform undesired side reactions but is nonetheless protected by an acyl group to increase solubility to facilitate oligonucleotide synthesis. T (and U) also do not have exocyclic amino groups in their structure, and typically do not require protection. In order to allow the preparation of the phosphoramidite, the nucleoside is left with a single free

unprotected hydroxy group at the 3'-position and treated with phosphorodiamidite, a class of phosphorous compounds, in a weak acid that increases catalytic action, causing the two substances to bind and creating the nucleoside phosphoramidite. The phosphorodiamidite successor, which is now a phosphite group, then also has one of its functional groups protected with 2-cyanoethyl, a commonly used protective group (Nielsen et al., 1986). Still, even after the phosphoramidites have been appropriately protected, the oligonucleotide synthesis process in its entirety remains rather error prone, setting the practical limit of each oligonucleotide at about 100 bases in length.

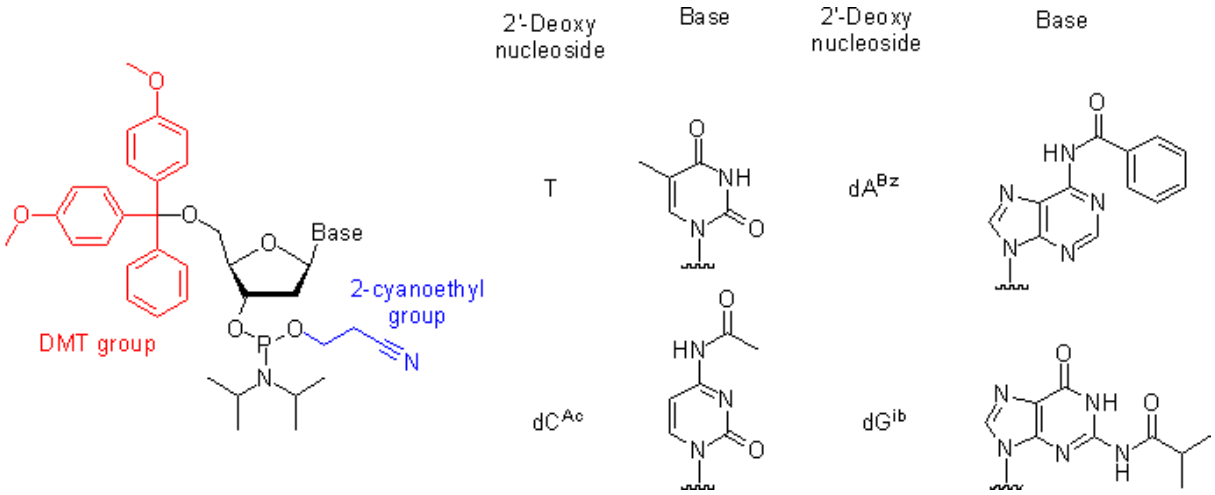

Figure 3: ***Protected nucleoside phosphoramidite and DNA bases.*** *Respectively, DMT and 2-cyanoethyl are the standard 5'-end and 3'-end protective groups of phosphoramidites. Adenine is typically protected with a benzoyl group (Bz), guanine with an isobutyryl group (ib), and cytosine with a recently popularized acetyl group (Ac), while thymine typically does not require protection. The wavy lines connected to the bases show where the base connects with the ribose. ("Oligonucleotide synthesis", n.d.)*

The procedure of oligonucleotide synthesis is carried out as solid-phase synthesis, a method that covalently binds building blocks to a solid support material while the product is being formed. The oligonucleotide that is being assembled is bound to the solid support material via the first phosphoramidite for the entire duration of the chain assembly, after which the entire completed oligonucleotide is detached from the solid support material in one go (Guzaev & Manoharan, 2003). This specific and more recent method for binding materials with a single attachment is called universal solid support. In the case of oligonucleotide synthesis, the two most widely used solid-phase materials are controlled pore glass and macroporous polystyrene (Guzaev, 2013, Pon, 1993).

Oligonucleotide synthesis is executed step by step by adding phosphoramidites to the 5'-end of the growing phosphoramidite chain until the desired sequence is constructed. Contrarily to natural DNA synthesis, artificial DNA synthesis is not limited to a single direction, although it is almost always performed in the unnatural upstream 3' to 5' direction. The addition of each single phosphoramidite to the chain is called a "synthesis cycle", which consists of four different chemical reactions, or steps: de-blocking, coupling, capping, and oxidation (Figure 4). For the first step, de-blocking, also called detritylation or deprotection, the protective group DMT, which protects the 5'-end hydroxy group, is removed by treating it with a solution of acid in a chemically inactive solvent. There is now an "oligonucleotide precursor" with a free 5'-end hydroxy group (Hughes & Ellington, 2017). The second step, coupling, involves activating a solution of phosphoramidites in acetonitrile with an acidic catalyst. If the activated phosphoramidite is the first of its chain, it is presented to the solid support material, which it then binds to (first coupling). If it is instead a following part of the chain, it is presented to the growing end of the incomplete oligonucleotide (following couplings). The incoming phosphoramidite's 3'-end phosphite group will react with the receiving phosphoramidite's free 5'-end hydroxy group and form a phosphite triester link, thus lengthening the developing oligonucleotide by one phosphoramidite unit (Roy & Caruthers, 2013). The third step is capping. In it, the solution containing the support-bound incomplete oligonucleotide is treated with a mixture of acetic anhydride, which is a common reagent in organic synthesis, and some catalytic substances. This serves two purposes important to the entire synthesis cycle. First, after the coupling step, a small part of the 5'-end hydroxy groups will not have undergone any reaction and will still have the capacity to cause unwanted reactions. The capping procedure renders the vast majority of these hydroxy groups inactive. Secondly, phosphoramidites that have been activated with a certain chemical, 1*H*-tetrazole, can react with the O^6 position of the nucleic base guanine (G) and give rise to a side product that upon oxidation during the fourth and final step undergoes depurination, i.e., cleavage of the bond between ribose and base, effectively removing the base from the phosphoramidite. However, as long as the capping step is executed before the oxidation step, the O^6 modification will be removed and rendered harmless (Pon et al., 1986). The fourth and last step consists of oxidation. The tricoordinated phosphite triester link between the phosphoramidites is not natural and not very stable under the conditions needed for further synthesis cycles. Treating the oligonucleotide precursor with water and iodine while in the presence of a weak base results in the oxidation of the phosphite triester into a tetracoordinated phosphate triester, which is more similar to the naturally occurring phosphate diester links and connects each phosphoramidite with greater stability (Hughes & Ellington, 2017).

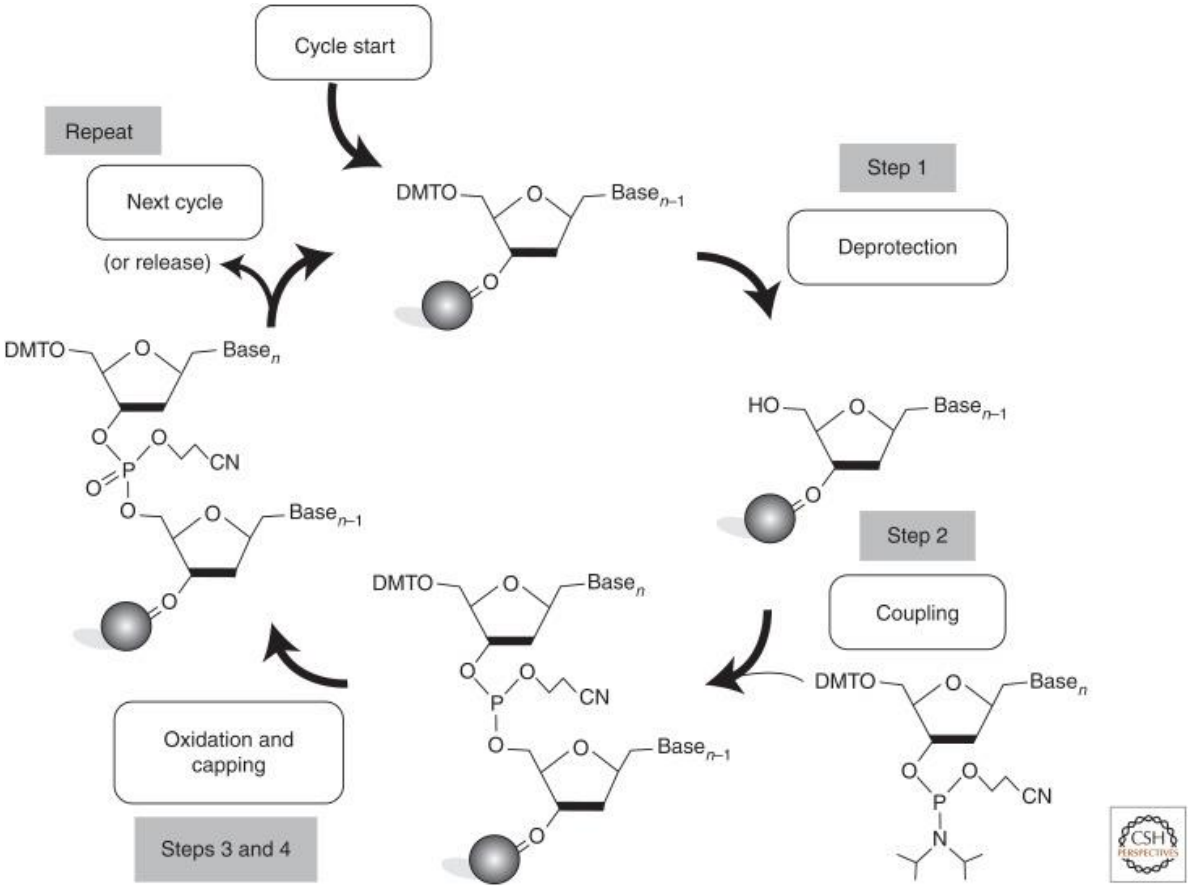

Figure 4: ***Synthesis cycle of oligonucleotides.*** *After preparation of the nucleoside phosphoramidites, the oligonucleotide synthesis cycle is responsible for assembling phosphoramidites into short genetic fragments called oligonucleotides by adding one phosphoramidite at a time to the end of a growing chain. This process occurs in four steps: deblocking (here deprotection), coupling, capping, and oxidation. The shaded circular shapes represent the solid support, or, in this case, the preceding phosphoramidite. (Hughes & Ellington, 2017)*

After the oligonucleotide has been assembled, all the functional groups that are still protected need to be deprotected in order for the oligonucleotide to be usable. As the DMT group has already been removed as part of the first step of the synthesis cycle, only the protective groups of the phosphate and of the bases are left. The phosphate group's 2-cyanoethyl protection and the bases' acyl protection are typically removed at the same time by treating the oligonucleotide with inorganic bases or amines, such as ammonium hydroxide, a solution of ammonia in water often used for this purpose. (Reddy et al., 2006)

In the past, oligonucleotide synthesis was conducted manually. Currently, the entire procedure can be carried out automatically using computer-controlled instruments, greatly increasing oligonucleotide production rate for use in studies. Although it is still the most common method, the phosphoramidite synthesis process is far from free of error, and since oligonucleotides serve many other purposes in synthetic biology and medicine other than the creation of synthetic genomes (e.g., primers for PCR, genes for introduction of mutations, etc.), several other technologies, such as enzymatic synthesis, are being developed that could potentially produce

longer oligonucleotide strands more reliably, with a higher yield, and for a lower cost. (Luo et al., 2018)

Oligonucleotide synthesis cannot produce usable DNA sequences longer than about a hundred bases in a reliable way, so, DNA assembly methods must be used to assemble the oligonucleotides into functional genetic structures. These structures can range in size from genes that encode instructions for a single protein to entire chromosomes and even genomes. As of 2015, 14 standardized DNA assembly methods have been developed, all of which can be categorized into three main categories: endonuclease-mediated assembly, site-specific recombination, and long-overlap-based assembly (Casini et al., 2015). The first class, endonuclease-mediated assembly, makes use of endonucleases, enzymes capable of separating segments of DNA in the middle of a strand from surrounding nucleotides. Endonuclease also plays a role in DNA repair, which consists of removing stretches of nucleotides at damaged sites and then preparing the ends of the two backbone ends that remain around the removed segment for connection with the repaired DNA segment which will be introduced. Through various technologies, all of which fall under the endonuclease-mediated assembly category, this function of endonucleases can be exploited to assemble large chains of DNA (Knight, 2003). Site-specific recombination utilizes recombinases instead of nucleases. Recombinases are enzymes that in certain ways function similarly to endonucleases but that are specialized to rearrange two segments of nucleic acids. They first bind to a spot on the DNA sequence, the recombination site, cleave the DNA backbone made of phosphate groups and sugars, exchange the two involved DNA segments, and finally re-join the cleaved strands into a single functional one, effectively replacing the two involved segments with one another (Kolb, 2004). In nature, recombinases aid in homologous recombination, DNA repair, transportation of mobile genetic elements, and in pathogenesis (Nash, 1999). A popularly used method for long-overlap-based assembly (and DNA assembly in general) is the Gibson assembly, since it needs only a few components and little manipulation (Figure 5). The process requires three enzymes: exonuclease, which is an enzyme similar to endonuclease, but that only removes nucleotides from the 5’-end of the chain rather than in the middle of it; DNA polymerase, which synthesizes complementary strands from a parental strand; and DNA ligase, whose function is to join DNA strands together. First, two DNA segments that are to be joined are needed and they require an overlap of 20-40 base pairs, which can be achieved with PCR. Then, the process continues by having the exonuclease cleave phosphodiester bonds and remove nucleotides from the 5’-end of both DNA fragments, creating stretches of unpaired nucleotides at the end of each chain, called overhangs, on both fragments. Since the ends of the fragments were overlapping, the overhangs are complementary to each other, and due to complementary base pairing, they now anneal. After annealing they often display nucleotide gaps where the 3’-end and 5’-end of the two fragments would meet. These gaps are quickly filled in by the

polymerase, which attaches nucleotides as needed. Lasty, the DNA ligase joins the fragments covalently and removes any DNA nicks, i.e., positions in the DNA molecule that lack phosphodiester bonds (Gibson et al., 2009).

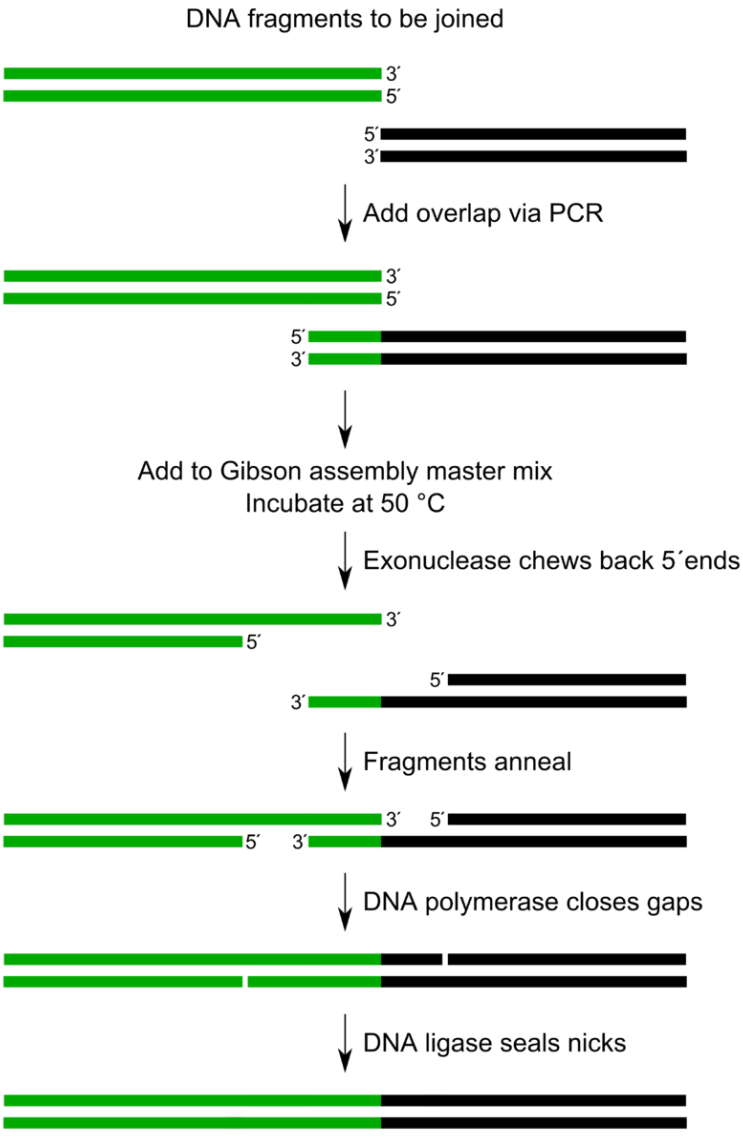

Figure 5: ***The Gibson assembly method.*** *This popular DNA assembly method makes use of only a few components to join DNA fragments together reliably and swiftly. ("Gibson assembly", n.d.)*

It is also interesting to note that although minimal cell studies certainly have much to contribute to our knowledge of cellular processes, they also make apparent how our understanding of cells' genomes is far from complete. In 2016, great attention was given to a minimal bacterial cell derived via whole genome synthesis named JCVI-Syn3.0, which was capable of survival and efficient reproduction. JCVI-Syn3.0 exhibits a genome of only 531'490 base pairs, 438 protein-coding genes, and 35 RNA-coding genes (totaling 473 genes), compared to the

genetically simplest known natural self-replicating organism *M. genitalium*'s 580'070 base pairs, 482 protein-coding genes, and 43 RNA-coding genes (totaling 525 genes) (Hutchison et al., 2016). As can be expected, the vast majority of JCVI-Syn3.0's remaining genes, all of which essential by definition, were responsible for vital tasks such as genome expression and preservation, metabolism, membrane related functions, and so on. However, it was also discovered that this minimal genome incorporated 55 genes whose functions were entirely unknown and 94 who could not be assigned to a specific task or set of tasks. The fact that the precise function of 149 of 473 essential genes in a minimal cell escapes us spells out how far form completion our understanding of cellular organisms and their components really is (Hutchison et al., 2016, Glass et al., 2017).

2.2 Bottom-up Approach

Although bottom-up creation of an organism that can be considered as living is much more challenging than the previously described top-down approach, the top-down approach does not necessarily uncover in a satisfying way how the cell parts that remain interact and link with one another to sustain life. This research-oriented shortcoming can be relieved by the bottom-up approach. Furthermore, synthesizing a cell from scratch would also overcome some of the cumbersome practical limitations and drawbacks of the top-down method. For example, by modifying a living organism in order to produce certain chemicals, such as medicinal drugs, unexpected impairment of the organism's metabolism or generation of unwanted side-products that might be harmful towards the organism could result, leading to low productive efficiency. Cells synthesized by the bottom-up approach, with only the minimal number of components needed for efficiency, are simpler and more controllable than their top-down counterparts, and as such would be more favorable. (C. Xu et al., 2016)

For construction of a single living entity, three fundamental elements must be considered: information-carrying molecules, a membrane boundary system, and metabolism. In addition, if one also wishes to consider continued survival in dynamic environments and propagation of the species, responsiveness to the environment and cell division would also be essential. However, the latter two elements go beyond the scope of this Matura paper, and as such will not be included (Buddingh & van Hest, 2017). The information-carrying molecules, DNA and RNA, define the inner workings of the organism. The membrane system separates the inside of the cell from its environment while allowing exchange of waste and nutrients with the outside, and, in more complex cells, provides inner compartments which separate regions of the cell with different functions and chemical reactions. Finally, metabolism is responsible for the generation of the energy required for the cell's activities (C. Xu et al., 2016).

2.2.1 Genetic Information

Genetic information systems have been studied in depth and artificial DNA synthesis, which has already been described, is a wide-spread technology. However, even those organisms with the smallest known genomes contain hundreds of carefully regulated genes, with structures and functions that have been determined through ceaseless evolutionary processes over the course of time. This renders bottom-up creation of genetic systems with the same level of complexity as those of the simplest prokaryotic organisms unattainable as of now. Still, synthetic gene circuits (SGCs) which can be used to “program” cells and that display a certain level of intricacy have been developed (Buddingh & van Hest, 2017). Gene circuits are systems of biological parts inside of a cell, mainly consisting of genes, proteins, promoters and so forth, designed to produce a certain protein or perform a certain function when prompted. Since gene circuits make use of systems analogous to logical operations, as they require certain input signals to start generating output, they can be compared to logical functions in electronic circuits. Useful examples for this include biological AND gates, which generate a gene output when all inputs signals, such as the presence of transcription factors or activated promoters, are activate simultaneously, or OR gates, which do not require all input signals for output generation (Figure 6) (Kobayashi et al., 2004). Although incorporation into bottom-up created cells is limited and not yet widely researched, SGCs have been implemented into natural cells and transcription-translation (TX-TL) cell extracts to produce many kinds of proteins with success. Cell extracts are solutions that contain the agents responsible for cellular chemical activities of cells, such as ribosomes, enzymes and other proteins, and the relevant energy sources. They are obtained by first breaking down a cell’s membranes and then centrifuging the cell in order to separate the cell wall, genetic material, and other debris from the rest of the cell, the remains of which consist mainly of ribosomes and many proteins. These cell extracts have the same biochemical properties and capabilities as a cell, while being simpler to handle and manipulate (Garcia et al., 2018). TX-TL extracts are cell extracts made specifically for transcription of DNA and translation of RNA. SGC systems capable of complex behavior such as pattern generation and gene oscillations, i.e., periodic gene expression, have been developed in such environments. However, behavior as intricate as this necessitates precise control of reagent concentrations, and, due to the random and unpredictable nature of many common vesicle formation methods which result in considerable heterogeneity, fine-tuned control of reagents is difficult when compartments are present, thus greatly hindering, and even almost prohibiting SGC efficiency at more complex levels in bottom-up created cells (Buddingh & van Hest, 2017, Nishimura et al., 2015).

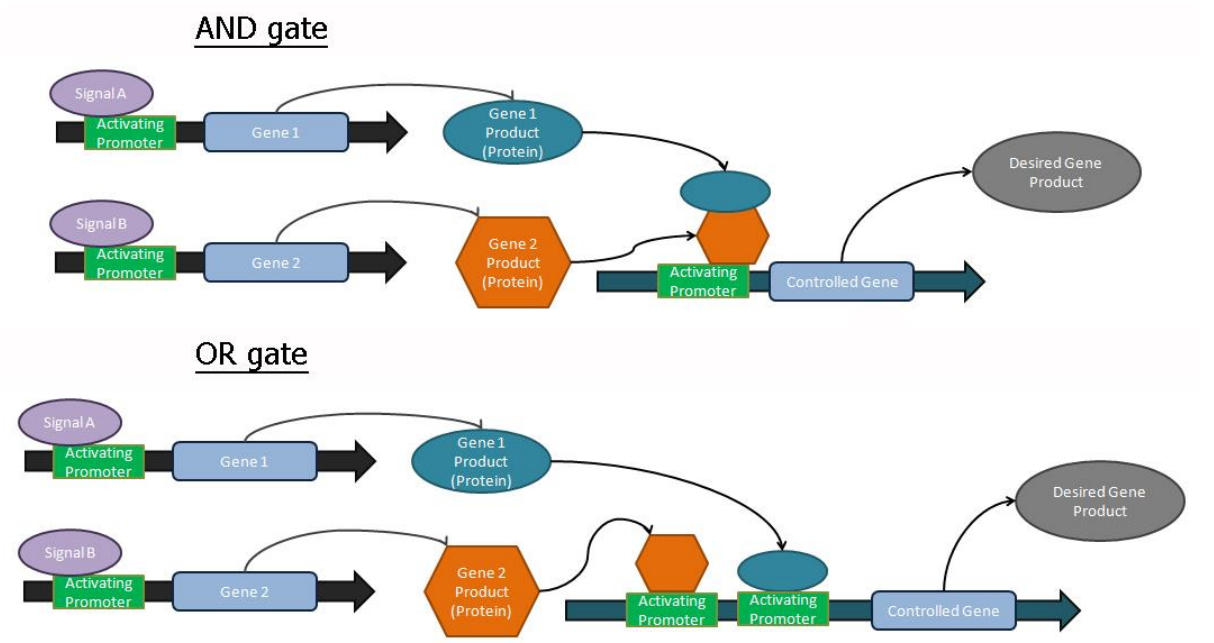

Figure 6: ***AND and OR gene circuit gates.*** *These two simple gates illustrate the functionality of gene circuits. The AND gate requires the product of both gene 1 and gene 2 to activate a promoter and produce the desired gene end product, i.e., it requires signals A and B. The proteins expressed by genes 1 and 2 can activate the promoter either by forming a complex, as shown in the figure, or by functioning separately. The OR gate requires the product of either gene 1 or gene 2 to activate the gene end product's promoter, i.e., either signal is capable of inducing expression. ("Synthetic biological circuits", n.d.)*

2.2.2 Membrane

Although there are many types of plasma membranes suitable for use in an artificial cell, the phospholipid membrane seems the most natural candidate, as its presence as the outer membrane of almost all living organisms makes phospholipids stand out among other possible components of artificial cell membranes. The selective permeability of natural phospholipid membranes is a precisely calibrated system that allows expulsion of waste and absorption of nutrients via various types of proteins lining the membrane and functioning as channels, receptors, enzymes, and more, while conserving vital cell components. Traditionally, research on gene expression and metabolism efficiency in an artificial compartmentalized environment has been conducted with the use of small unilamellar liposomes, artificial vesicles with a single phospholipid bilayer, which are very similar to natural cells, albeit on the smaller bacterial size range (Buddingh & van Hest, 2017). Synthesis of long RNA strands, synthesis of proteins, and PCR, among other procedures, have been successfully carried out inside of unilamellar liposomes (Chakrabarti et al., 1994, Oberholzer et al., 1999, Oberholzer et al., 1995). In recent years, however, giant unilamellar vesicles (GUVs) have gained much attention in this field, as their size of up to 200µm is much greater than that of regular liposomes (typically up to 1µm), which has several advantages (Rideau et al., 2018). For example, GUVs are large and stable enough

to be punctured by a microneedle without causing damage, thus allowing microinjections with precise control of concentrations, they display more advanced membrane permeability, and, also due to their size, respond to internal and external factors in ways that can be easy to observe and measure. Still, since even the largest eukaryotic cells typically do not reach diameters larger than 100µm, most research is conducted with GUVs of that approximate size (C. Xu et al., 2016, Dimova, 2019). As with most of the laboratory procedures that have been described, there exist several methods to create GUVs, a common one being the lipid film hydration method. Lipid film hydration first requires several phospholipid bilayers organized as sheets, or films, on a glass surface. To ensure the phospholipid layers are organized in flat, smooth, and uniform films, they are first dried by treatment with nitrogen gas. Then, they are hydrated first by water-saturated nitrogen, and then by addition of an aqueous solution. The temperature of the hydrating medium must be high enough to allow the phospholipids to be in their disordered state for giant vesicles to form, otherwise the process will not yield vesicles of the desired size (Walde et al., 2010). The addition of the aqueous solution first results in hydration of the hydrophilic phospholipid heads, and then to swelling of the bilayers as more and more water molecules pass through the films and into the space between each bilayer film, resulting in a very large vesicle, the GUV, which with enough swelling becomes its own compartment separate from the original sheet. By also adding certain monosaccharides to the lipid film before hydration, it is possible to affect the osmotic pressure, thus also affecting the extent of the water movement that causes the swelling, and, ultimately, the size of the finished vesicle product. The procedure in its entirety needs to be tightly controlled, as there are several delicate factors that can lead to heterogenous vesicle products of various sizes, and to the formation of small undesired multilamellar vesicles. For example, multilamellar vesicles will form if the hydration procedure is disturbed by even very slight shaking, or if too many phospholipid bilayers are present. Since artificial unilamellar vesicles are typically not in a true thermodynamic equilibrium, but rather in a trapped kinetic state, it is not possible to fully rely on the self-assembling properties of phospholipids to build stable vesicles of such large sizes. The transition from film to vesicle must be controlled and very stable conditions kept to ensure high yield (Walde et al., 2010). This specific method is called the gentle hydration method (“gentle” due to the fact that the swelling is caused spontaneously without further intervention). In addition to gentle hydration, there exists another, newer, and recently much more utilized variation of the lipid film hydration method which utilizes electric fields, called the electroformation method (Figure 7). The phospholipids are placed on glass coated with a conductive material, and, after hydration, external electric fields are applied that promote more reliable formation of GUV populations with higher homogeneity than would typically result from gentle hydration. GUVs produced by electroformation often exhibit thin phospholipid protrusions that keep each GUV attached to the residue of the film they originated from. This attachment can be very

advantageous if the vesicles are intended to be punctured by microneedles, as the connection holds the GUV in place during microneedle insertion. If the connection is undesired, it can be disposed of with ease (Pereno et al., 2017, Walde et al., 2010).

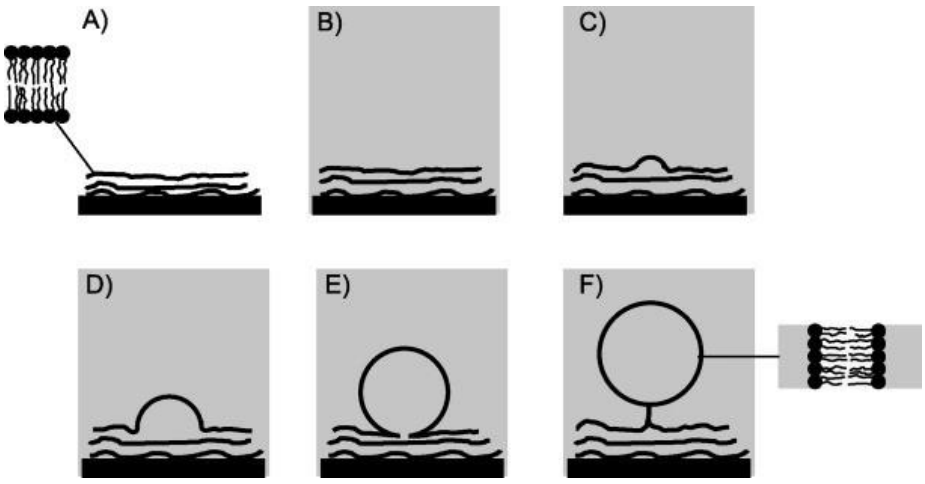

Figure 7: ***Electroformation of a GUV in six stages.*** *After hydration of the phospholipid bilayer sheets, swelling is mediated by usage of electric fields to increase homogeneity and reliability of GUV formation. Stage F shows the thin protrusion connecting the GUV to the bilayer sheet, a characteristic reminiscent of electroformation-formed GUVs. (Walde et al., 2010)*

However, neither of the described lipid film hydration methods allows efficient encapsulation of large water-soluble molecules like enzymes. Such molecules would need to be transferred to the lower layers of the lipid films before hydration or in the early stages of hydration, but this is difficult due to the very slow movement of molecules between layers. Naturally, it is possible to microinject molecules into the GUV after completed formation by using a microneedle, but the GUV is already filled with water, and so only a comparatively small, but not totally insignificant, amount of volume can be added without causing the vesicle to burst. (Walde et al., 2010)

Of the other various methods to form GUVs, the lipid-stabilized water/oil/water (w/o/w) double emulsion method allows much easier encapsulation of vast amounts of large molecules than lipid film hydration, albeit at the cost of a rather unreliable formation process. A w/o/w double emulsion consists of an innermost water droplet phase inside of a middle oil droplet phase, which in turn is inside of an outermost water phase (Figure 8). The innermost water droplet is coated with phospholipids whose hydrophilic heads naturally face inwards towards the water, while the lipophilic tails face outwards towards the oil droplet. The oil droplet also has a coating, but the phospholipids' direction is reversed: the hydrophilic heads face outwards towards the outermost water phase, while the lipophilic tails face inwards towards the oil phase. Upon removal of the oil through evaporation, the two coatings join as the phospholipids that covered the inner water droplet become the inner monolayer of the GUVs bilayer, while the oil droplet's

coating phospholipids come to constitute the outer monolayer (Sato & Takinoue, 2019). Since the size of the inner water droplet is smaller than that of the oil droplet, more phospholipids are present on the oil droplet than there are on the water droplet. This causes considerable phospholipid rearrangements that often result in undesired groupings of phospholipids inside the GUV membrane. Furthermore, completely removing the oil might not be easy. Despite the unreliability of lipid-stabilized w/o/w double emulsion GUV formation, this method allows very easy and efficient encapsulation of large amounts of water-soluble molecules, such as enzymes, as encapsulation only requires for the enzymes to be placed inside of the w/o/w emulsion prior to introduction of the phospholipids (Walde et al., 2010).

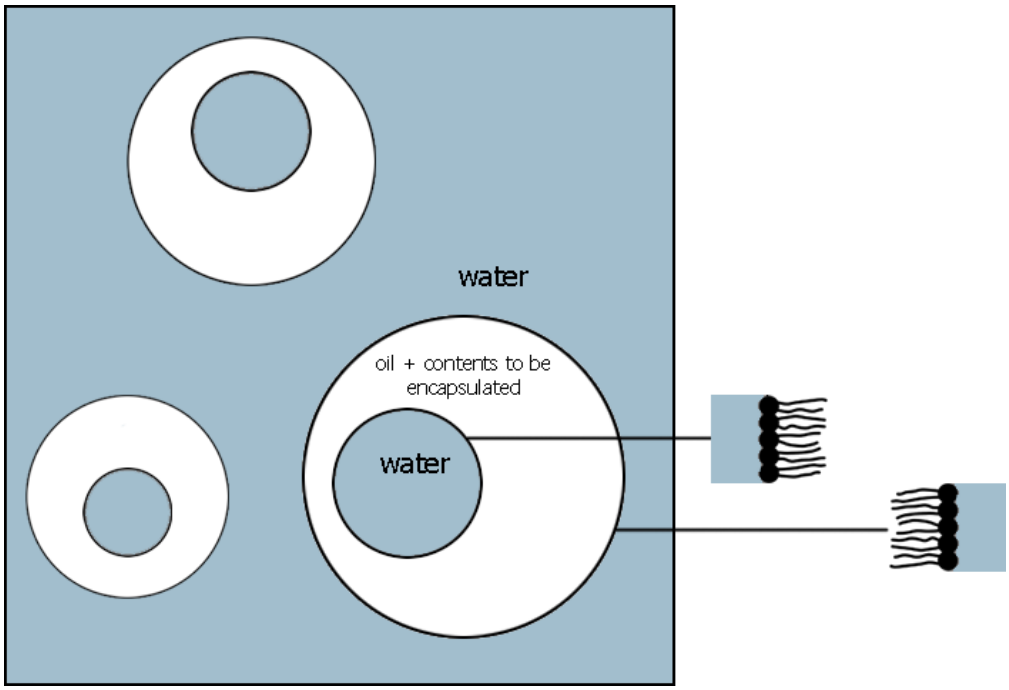

Figure 8: ***A lipid-stabilized w/o/w double emulsion.*** *The amphiphilic phospholipid monolayers are oriented according to their nature along the boundaries between the water and oil phases: The hydrophilic heads are inside of the water phase, while the lipophilic tails are inside the oil phases. (Walde et al., 2010)*

The selective semi-permeability of a cell's plasma membrane strongly depends on protein channels, pores, and various types of active transporters. These proteins allow the movement of molecules vital or toxic to the cell, such as water and ions, from one side of the phospholipid bilayer to the other as needed. For example, the transport of water is vastly facilitated by the presence of the membrane protein aquaporin, which allows passage of water with high efficiency, while also almost perfectly rejecting ions, protons, and any other molecule. Although their essential purpose is simple, natural aquaporins are often highly complex molecular structures comprising several α-helices (a common secondary structure of proteins) which span across the entire membrane bilayer multiple times (Agre, 2006). Typically, for the construction

of artificial water channels (AWCs) similar to aquaporin, more simple molecular arrangements are used, which can fall under one of two main categories: Single molecular channels, which consist of a single molecular component reaching the inner and outer ends of the membrane, or supramolecular channels, assembled from multiple molecular components. Single molecular channels often consist of several arms of hydrazide, an organic compound, held together by H-bonds that are technically intramolecular, but that also confer enough sturdiness that the entire complex behaves as a single molecule. As these single molecular channels have very similar interactions with water molecules as the fundamental mechanisms of aquaporin, they facilitate water transport by just about the same magnitude as natural aquaporin. However, many of the most effective single molecular channels do not have very efficient ion selectivity, as their large pore size allows the passage of cations with sufficient hydration energy (Kocsis et al., 2018). Supramolecular channels are formed by self-assembly. Self-assembly of a popular supramolecular channel occurs by allowing several instances of the organic compound alkylureido-imidazoles to form into pillar shaped channels that provide each other with mutual stabilization via strings of bonded water molecules. Supramolecular channels feature a transport rate that is lower than that of their single molecular counterparts, but they possess much better selectivity, as they entirely reject all ions except for protons (Kocsis et al., 2018, Le Duc et al., 2011). A brief explanation of the concepts of AWC formation has been provided to present an idea of how synthetic passive channel molecules are constructed. However, the full scope of the subject, which would include much more complicated ion channels, active transport systems, and polypeptide alternatives, among other things, is too complicated for inclusion in this Matura paper in a way that would be satisfactory.

Although over the recent years our understanding and control over the size and permeability of artificial membranes has greatly improved and still is, what has been achieved can still be considered to be only a basic replication of the framework of natural cells. Eukaryotic cells in particular feature a highly complex membrane system that imparts the cell with separated inner compartments, each comprising its own chemical environment for a specified function or set of functions, for higher efficiency and better managing of the many, sometimes incompatible chemical processes that take place inside of a eukaryote, without even mentioning the full extent of the endomembrane system’s functions. As such, the creation of cell-sized vesicles with functional compartments would represent a vital step toward the development of a higher order of artificial cell. To this end, synthetic vesicles with inner multicompartments called vesosomes can be produced by various means, one of the more intuitive of these methods involves spontaneous or induced GUV endobudding. However, this specific vesosome creation process is difficult to control, as the quantity of the inner compartments, the composition of the membranes, and even the contents of the compartments can go through unexpected changes during creation or be of insufficient quality after vesosome formation (Okumura et al., 2011). Even

so, by using microfluidic techniques, methods have been elaborated that can overcome these restrictions, allowing for vesosome formation processes with significant control over the quantity, size, and content of compartments. One of the most efficient of these microfluidic methods involves, briefly explained, preformed unilamellar vesicles dispersed inside of a water phase that are injected into an oil phase, creating one or more vesicles inside of a water droplet inside of an oil droplet, which in turn is yet again injected into a water phase (Figure 9). The end product of these injections is one or more vesicles inside of a water droplet inside of an oil droplet inside of a water phase, i.e., a number of vesicles inside of a w/o/w double emulsion. Identically to the w/o/w double emulsion GUV formation method, phospholipids are assembled around the boundaries of the inner water droplet and the oil droplet, and, once the oil is removed, they are allowed to form into a lipid bilayer, thus resulting into smaller inner vesicles inside of a larger outer vesicle, i.e., a vesosome. However, unlike the previously described w/o/w double emulsion GUV formation, the oil is not removed via evaporation but by dewetting, a method that causes the oil to physically separate from the remaining complex. Dewetting is generally more effective than evaporation, as more oil is removed and excess phospholipids are also separated from the resulting vesosome (Deng et al., 2016). Still, inner compartments of artificial cells almost exclusively exhibit simple spherical shapes, which cannot truly be compared to eukaryotic organelles and their exceedingly intricate and versatile shapes and inner folds.

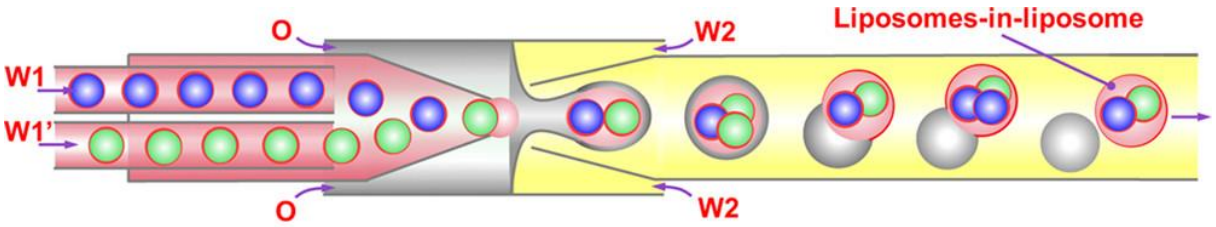

Figure 9: ***Microfluidic assembly of vesosomes.*** *With microfluidic techniques, reliable formation of vesosomes with simple spherical inner vesicles can be achieved. W1/W2: water phase 1/water phase 2, O: oil phase.*

2.2.3 Metabolism and Energy

As with any organism, no cell can survive without adequate energy production. In natural cells, much of this energy is provided through the set of catabolic processes of cellular respiration, whose product is the universally utilized adenosine triphosphate (ATP). When energy is needed, the ATP molecule is then consumed for use in a vast variety of energy requiring cellular activities by breaking the bonds between the phosphate groups via hydrolysis, thus releasing energy, and resulting in the byproduct adenosine diphosphate (ADP). ADP could in

turn also be used for energy, finally resulting in adenosine monophosphate (AMP), although most cells typically prefer not to hydrolyze ADP. ADP (and, if present, AMP) can then be recycled back into ATP by forming bonds with new phosphate groups (Bonora et al., 2012). Cellular respiration is a highly complex process that consists of several stages, each including its own set of chemical reactions and processes, which, in turn, also each include their own sets of enzymes, coenzymes, cofactors, and more. However, the phosphorylation stage of cellular respiration, during which a phosphate group is attached to ADP, is responsible for an estimated 34 of the 38 ATP molecules a cell can produce with ideal conditions from a single glucose (Rich, 2003). Attention will thus focus on alternatives to natural phosphorylation that are more viable for application in artificial cells. Still, this does not mean that the stages other than phosphorylation are in any way insignificant to the cell.

A biological pathway is a series of interactions between molecules inside of a cell that can create a certain product. The arginine breakdown pathway validates itself as a strong candidate for energy production in artificial cells, as it is a particularly simple version of phosphorylation that can reliably sustain ATP production for extended periods of time by creating energy needed for ATP formation through breakdown of the amino acid arginine (Arg) (Figure 10) (Sikkema et al., 2019). After absorption of arginine, the molecule is processed by a deiminase enzyme that removes the arginine's amino group in a reaction called deamination, giving rise to the related, but non-coded, amino acid citrulline and the waste product NH_4^+. Citrulline subsequently undergoes phosphorolysis, i.e., cleavage of citrulline caused by an attacking phosphate group that splits the molecule and then also binds to one of the resulting molecules, yielding a carbamoyl phosphate ion and an ornithine amino acid through the involvement of a transferase enzyme, which is an enzyme that transfers functional groups from a donor to an acceptor molecule. In this case, the specific relevant enzyme is ornithine carbamoyltransferase. Ornithine, also a non-coded amino acid, is technically a waste product, but it aids the arginine breakdown pathway as it mutually regulates absorption of arginine into the cell. The structural similarities of the two molecules can be taken advantage of by an antiport mechanism, which is a single active transport membrane protein that steers arginine into the cell as it expels ornithine. Finally, ATP can be formed. Through an enzyme that catalyzes the transfer of phosphate groups, called a kinase (in this case a carbamate kinase), carbamoyl phosphate and ADP can be converted into ATP plus the waste byproducts NH_4^+ and CO_2, thus conserving a large part of the energy used for the initial breakdown of arginine (Sikkema et al., 2019, Pols et al., 2020). Although when condensed into such a tight description arginine breakdown might seem complicated, its comparative simplicity is attested by the fact that the entire pathway only requires three enzymes: a deiminase, a transferase, and a kinase. Furthermore, since absorption of arginine and expulsion of ornithine are linked to one another through the antiporter protein, and since the waste products NH_4^+ and CO_2 can simply diffuse through the membrane

and out of the cell, the arginine breakdown pathway could theoretically enable long-term ATP synthesis. Still, if ATP is not consumed by the cell at a fast enough rate, the arginine breakdown pathway will continue producing ATP from ADP until no more ADP is present. Once this happens, arginine absorption does not halt and the cell continues to break down arginine into citrulline and NH_4^+ via deamination, the first stage of the arginine breakdown pathway. This leads to accumulation of waste, acidification, und, ultimately, unsustainability of the cell. This demonstrates that even with an efficient energy supply, careful balancing of resources and cellular activity is essential for long-term functionality of an artificial cell with its own metabolism (Pols et al., 2020).

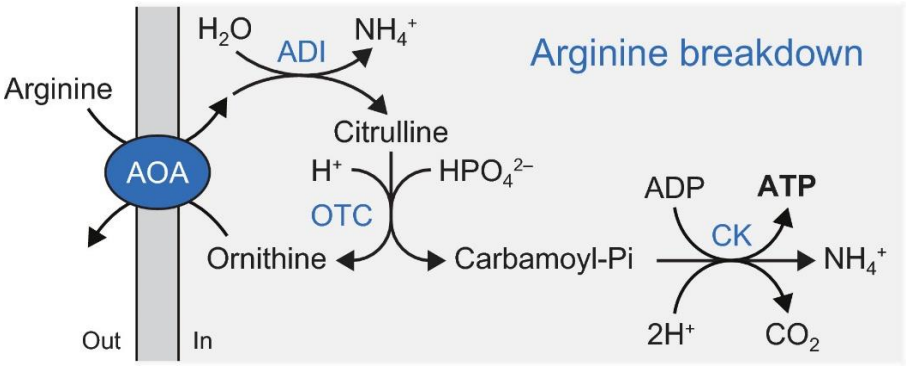

Figure 10: **The arginine breakdown pathway**. *Breakdown of the amino acid arginine provides a simple and reasonably reliable pathway for ATP synthesis. AOA: arginine/ornithine antiporter, ADI: arginine deiminase, OTC: ornithine carbamoyltransferase, CK: carbamate kinase. (Pols et al., 2020)*

Artificial systems that mimic photosynthesis have also been created. These consist of small vesicles that establish a chemiosmotic potential inside of the membrane to drive synthesis of ATP just outside of the membrane via membrane-bound enzymes (Figure 11). The difference between such an artificial photosynthetic pathway and a typical natural one is that the artificial pathway does not utilize light to form starch or sugar, but rather to power ATP synthesis via phosphorylation (Sikkema et al., 2019). The proteins needed for this include an ATP synthase for formation of ATP from ADP and a phosphate group, a photosystem II protein complex to create a chemiosmotic potential, and a proteorhodopsin that counteracts the chemiosmotic potential when activated by a different wavelength of light than the photosystem II complex. Both the photosystem II and the proteorhodopsin are light dependent, which means they are only activated when exposed to certain wavelengths of light. In this case, the photosystem II requires red light and the proteorhodopsin green light. The first step of the procedure is exposure of the photosystem II to red light, which triggers it to oxidize water into O_2 and two protons, which causes the proton number inside of the vesicle to rise, and thus establishes a proton gradient that can be used for energy (Gogarten & Taiz, 1992). The energy from the

chemiosmotic potential derived from the proton gradient then drives phosphorylation of ADP into ATP via ATP synthase, which necessarily requires a proton gradient to function. ATP synthase is a membrane bound enzyme that utilizes ADP and phosphate present outside of the artificial cell to form ATP, while using the chemiosmotic potential present inside of the cell for energy. Thus, after synthesis, ATP is only present outside of the membrane. It consequently follows that such a photosynthetic system would be best utilized as a rudimentary organelle inside of a larger vesicle that would constitute the actual cell. ATP would then also be readily available for transport to ATP entailing processes throughout this cell. Once sufficient ATP has been produced, the proteorhodopsin protein can be exposed to green light, causing it to pump protons from the inside to the outside of the organelle, thus counteracting the chemiosmotic potential initially established by the photosystem II. Although this proton pump mechanism has not yet been mentioned and will not be explained in further detail, it is worth noting, for the sake of completion, that it is a fundamental mechanism used universally by all groups of organisms to provide energy for cellular respiration, although it typically has the purpose of building a chemiosmotic potential, rather than curbing it. Proteorhodopsin will neutralize the chemiosmotic potential, or even reverse its polarity, thereby cutting off the ATP synthase's energy source and halting ATP formation (Lee et al., 2018). Although it may seem useless to provide an artificial cell with a device that discontinues its own energy production, regulation and balancing of resources is a vital task of every living being, as has already been illustrated by describing the eventual fate of an arginine breakdown pathway without a termination mechanism.

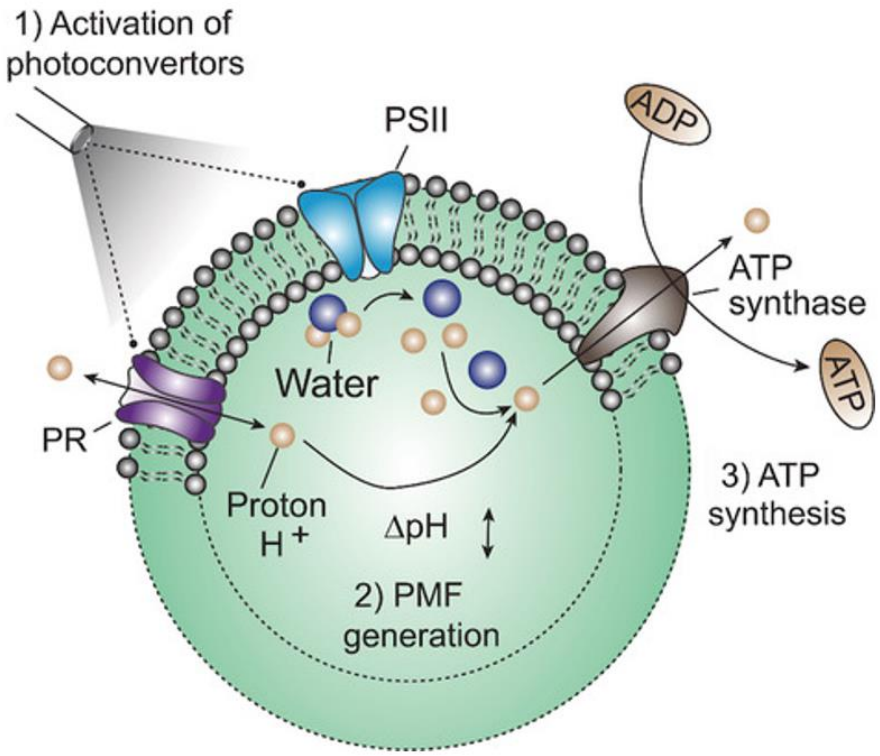

Figure 11: ***Artificial photosynthesis.*** *Via the protein complex photosystem II (PSII), a chemiosmotic potential is established inside of the vesicle that allows ATP synthase to synthesize ATP from ADP and phosphate. Once sufficient ATP has been produced and the process must be halted, the protein proteorhodopsin (PR) can be activated and protons will be pumped out of the vesicle, neutralizing the chemiosmotic potential and ATP synthesis. PMF: proton motive force (another term for chemiosmotic potential) (Sikkema et al., 2019)*

In practice, artificial cells do not possess the means necessary for autonomous, or often any, balancing of resources, and consequently their metabolism typically paralyses itself quickly due to inefficient recycling of nutrients and accumulation of toxic waste, even if the cell is continuously supplied with fresh nutrients (Buddingh & van Hest, 2017, Sikkema et al., 2019). In addition, inner compartments specialized in ATP production like the eukaryotic mitochondria (or the described artificial light-driven organelle) would be highly beneficial to an artificial cell, since it is thought that the complexity of simpler organisms like prokaryotes may be limited by the energy constriction caused by their unfavorable surface to volume ratio for ATP production compared to eukaryotes. Thus, elaboration of systems for long-term maintenance of a functional metabolism and efficient inclusion of artificial energy-producing organelles represents a crucial and necessary next step that would allow development of artificial cells of a much more advanced level of complexity (Göpfrich et al., 2018).

3 Applications

Simple structures that can be classified as artificial cells have been developed and put to use in a variety of medical applications. Although many of these structures are so simple that they may well be described as membranous sacks carrying pharmaceuticals rather than artificial cells, they still represent one of many steps towards real-world usage of artificially created living beings for medical purposes. As the focus of this Matura paper is the research-oriented development of artificial cells rather than the application-oriented one, only brief summaries of the main current applications will follow.

Structures resembling artificial cells are widely used as drug delivery systems. Put simply, artificial vesicles that encapsulate pharmaceuticals are delivered to the body. Once inside the body, these cells typically have one of three fates: Their contents can be directly taken up by the patient, the cells can be made to last inside of the body while slowly releasing their contents through their membrane, or the contents may not be meant for release at all, but rather for processing of materials that permeate through the membrane. In any case, after completed delivery, the membrane biodegrades in a way that is harmless to the host. The encapsulated materials can include hormones, vaccines, enzymes and other proteins, etc. The delivery of genetic material and enzymes makes up a large category of drug delivery mediated by artificial cells that will be summarized separately further on. Often, the membrane of drug delivery systems is polymer-based instead of lipid-based, as the wide variety of harmless polymers that can be chosen from to constitute the membrane of the polymersome (a vesicle with a polymer membrane) allows greater control of the cell's expected durability until biodegradation by the patient's body. Furthermore, thickness and molecular weight of the polymer components can be specified to determine the rate of drug release (Chang, 2019). However, for those types of drug delivery that do not require slow release of the drug, but for which one-time release may be sufficient (such as vaccines), nanoparticles made of lipids are also often used. Furthermore, patients that exhibit defective specialized cells can be treated by introducing encapsulated cells. Natural cells that have been encapsulated inside of an artificial protective and semipermeable membrane and that have the purpose of fulfilling the function of the defective cells while being protected by the membrane. Encapsulated cells typically do not last long, as after a short time they start to build clusters that greatly inhibit nutrient and waste exchange (Ponce et al., 2005). In 2016, Bowerman and colleagues reported increased efficiency of the delivery of a chemotherapeutical drug when encapsulated inside of small polymersomes (Bowerman et al., 2016). Delivery of encapsulated mRNA has recently become a widely used and successful vaccination technique (Park et al., 2021).

Enzyme replacement therapy is a form of drug delivery that utilizes artificial cells as carriers to introduce enzymes that replace under-expressed or defective enzymes in a patient's body. Conditions that cause over-expression of enzymes can also be treated with this concept by administering non-functional enzymes that modify the relevant molecules into inactive products, thus competing with the over-expressed enzyme and counteracting its adverse effects (Datta et al., 2020). Encapsulation protects the enzymes from degradation or other immunological attacks by the body, but also only allows processing of molecules that can permeate through the artificial cell's membrane. On the other hand, free floating enzymes only last for a very short time inside the body compared to encapsulated ones. Enzyme replacement therapy with artificial encapsulation had already been studied and proven successful in 1971 by treating mice affected with lymphosarcoma, a type of lymphocyte tumor. The pharmaceutical enzyme asparaginase was encapsulated and delivered to the mice, who experienced delayed tumor onset and growth (Chang, 1971). Enzyme encapsulation has also been put through clinical trials with patients suffering from cancer as a tool for tumor site accurate activation of drugs with a certain level of toxicity. The drug is injected in an inactivated state into the patient, then, artificial cells containing the activator enzymes can be implanted close to the tumor site or be made to accumulate there, thus activating the drug in much higher concentrations on the tumor site than in the rest of the body and preventing toxic consequences (Löhr et al., 2002). In 2018, Abed and colleagues reported efficient oral delivery of polymer nanocapsules packed with DNA nuclease and lysozyme, an antimicrobial enzyme of the animal immune system (Abed et al., 2018).

Another similar application artificial cells can be related to is gene therapy. The aim of gene therapy is to introduce, alter, or remove genetic material in order to repair a cell's defective genome. Artificial cells can be used to introduce DNA or RNA into genetically faulty cells with inadequate protein production that would ideally take up the introduced material and integrate it into their genome permanently. Genetic material can be transported into the cell with vectors, which, in the context of molecular biology, are transport vehicles used to carry foreign genetic material into a host cell. In gene therapy, viral and non-viral vectors can be used. Viral vectors make use of a natural virus's ability to inject its nucleic acids into an invaded cell and then trick the cell into replicating and translating the virus's own genes. The desired therapeutical genetic material is simply inserted into a virus whose genome has been made harmless, and the viral vector is then introduced to the targeted cells. Challenges in the application of viral vectors, without including the engineering of the therapeutical genetic material, are mainly brought forth by immune responses of the patient's body that can sometimes have detrimental effects on the patient. There are only a finite number of distinct viral vectors that can be used, and, if the body recognizes the viral vector as a foreign invader, the same viral vector cannot efficiently be used a second time as it will be immediately recognized by the immune system.

Furthermore, a patient may exhibit pre-existing immunity. Although methods and techniques to evade immune responses exist, these can be very cumbersome and impractical (Nayak & Herzog, 2010). Clinical trials using viral vectors in the early 2000s have led to fatal immune responses and to patients developing leukemia. Other patients benefitted from the treatment (Carmen, 2001, Raper et al., 2003, Cavazzano-Calvo et al., 2000, Hacein-Bei-Abina et al., 2003). Non-viral vectors have the advantage of causing much lower host immune responses and being considerably easier to produce and administer repeatedly. However, they exhibit significantly lower transfection rates and thus lower therapeutic efficacy. Complexes of DNA or RNA molecules encapsulated by either lipids or polymers, called respectively lipoplexes and polyplexes, can be made to introduce the genetic material into the cell. Lipoplexes and polyplexes make use of the same set of concepts for their injection methods, although lipoplexes are generally simpler (Figure 12). The lipid membrane of lipoplexes has a positive charge that allows them to interact with the cell membrane and enter the cell via endocytosis, forming into an endosome that is fundamentally identical to naturally occurring ones. Once inside the cell, the therapeutical genetic code is still encapsulated by the lipid membrane, and if it is not released, it will be targeted and destroyed by a lysosome. Therefore, fusogenic lipids are added to the lipoplex. Fusogenic lipids are lipids capable of disrupting endosomal membranes, thus allowing the DNA or RNA to escape the endosome and enter the cytoplasm. Then, if it is DNA that is inserted, several natural transport processes take place, and the DNA can enter the nucleus either during its disassembly during mitosis or through the nuclear envelope. In the case of RNA, its purpose can be fulfilled in the cytoplasm. RNA gene therapy can for example be meant to produce certain proteins short-term, in which case mRNA will be used, or silence certain genes, in which case an RNA-induced silencing complex (RISC) will be used that is capable of interfering with the cell's natural mRNA before it can be translated. Furthermore, certain studies and trials have shown that cationic lipids can have adverse toxic effects on the patient (Junquera & Aicart, 2015, Bai et al., 2017, MacRae, 2009). Polyplexes cannot simply release their cargo into the cytoplasm the way lipoplexes do after entry into the cell. Rather, they often receive help from certain agents that facilitate breakdown of the membrane during the endosome stage, such as inactivated adenoviruses. Understanding of the mechanisms by which endosome cargo can break free from endosomes has been broadened in recent years, and new techniques that do not involve supplementary agents, such as the inactivated viruses, have been developed. One of these techniques incorporates the usage of the "proton sponge effect", which, by using weakly basic molecules, may swell the endosome until it bursts. Furthermore, unlike their lipoplex counterparts, polyplexes generally exhibit no toxicity on patients (Junquera & Aicart, 2015, Freeman et al., 2012).

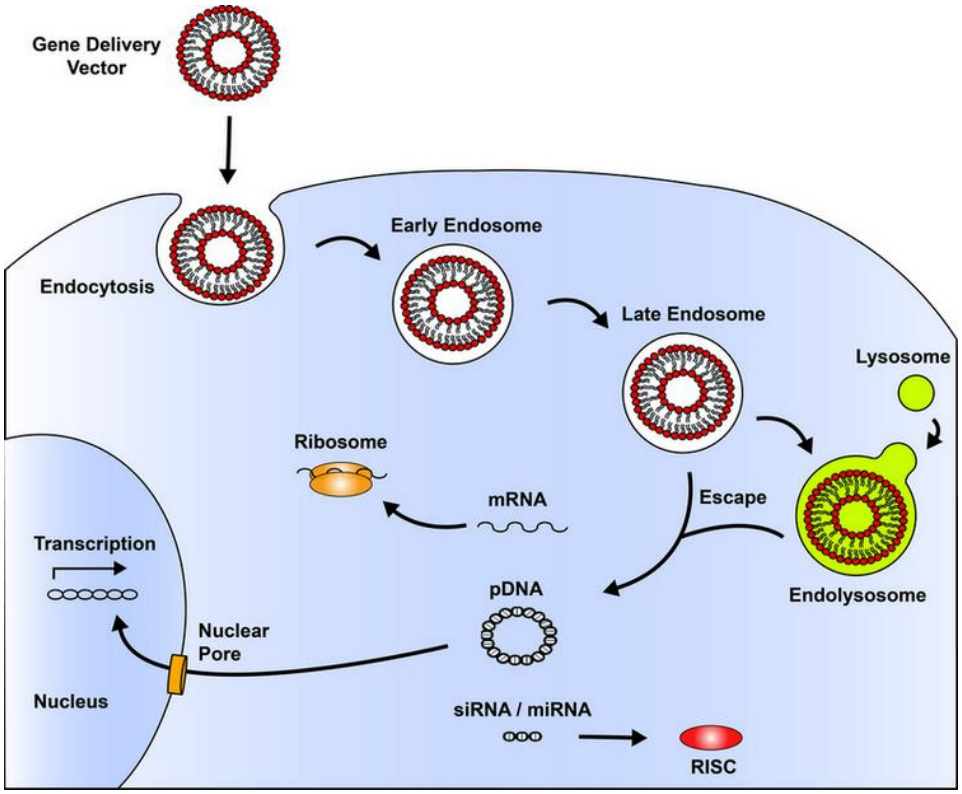

Figure 12: ***Non-viral vector in gene therapy.*** *When a vector enters a cell, it first forms an endsome that must set ist contents free before being targeted and destroyed by a lysosome. If the purpose of the gene therapy is to allow the cell to produce new functional proteins, the vector's cargo will consist of either only mRNA for short-term expression or plasmid DNA (pDNA) made to enter the nucleus and be incorporated into the genome long-term. In the first case, the contents of the vector will remain in the cytoplasm, in the second, they will attempt to enter the nucleus. This figure also includes an RNA-induced silencing complex (RISC), which usually incorporates double stranded small interfering RNA (siRNA) or single stranded microRNA (miRNA), whose purpose is to silence specific genes by disrupting the relevant mRNA before it reaches the ribosomes for translation of the gene product. (Jones et al., 2015, Pratt & MacRae, 2009)*

Hemoperfusion is a treatment for intoxicated blood that involves pumping blood outside of the patient's body and into a machine, filtering and detoxifying it extracorporeally as it passes through the machine, and then pumping it back into the patient. Artificial cells with sorbent properties (capable of trapping materials) serve as the filtering mechanism. These cells are filled with a microporous material, a material that is lined with microscopic pores, in the case of hemoperfusion often activated carbon, that effectively traps toxic molecules. As the intoxicated blood passes through the artificial cells, the toxins that are found within the blood serum (the extracellular fluid component of blood not responsible for clotting) pass through the artificial cells' membranes and become trapped by the microporous activated carbon, while blood cells and other blood components simply brush past the artificial cells (Chang, 2019). Hemoperfusion was first developed in the 1940s, and by the 1970s the process had been refined and popularized after successful trials had demonstrated its efficiency. However, as hemoperfusion also features certain complications that were made evident by the development

of other, safer, and more complicated blood detoxification methods, the technique became increasingly unpopular, although it is still widely used in developing countries (Ghannoum et al., 2014). Complications after treatment include low concentrations of white blood cells, blood sugar, platelets, and clotting factors, for which the patient typically requires 1-2 days of time to recover from (Ahmad, 2009, p. 231).

Artificial cells have also received attention as potential red blood cell (RBC) substitutes. RBCs are comparatively very simple cells. They lack most organelles including the mitochondria, and even the nucleus and all the genetic material therein is removed from the cell shortly after it is birthed in the bone merrow in order to spare as much space as possible for hemoglobin, the oxygen carrying molecule of red blood cells. Even so, although the RBC is so strictly specialized for a specific function that it does not require further production of proteins, it remains metabolically active while maintaining membrane and structure integrity, fulfilling its functions, and still being capable of interacting with its environment for its entire life span of approximately 120 days (Hillyer et al., 2006). Unlike natural RBCs from a blood donor, artificial RBC substitutes can be easily sterilized, exhibit no blood groups, and can be stored at room temperature for about a year, compared to donated RBCs' maximum refrigerated storage time of 42 days (Chang, 2019). Still, it is not an easy feat to design RBC substitutes from artificial cells that efficiently perform RBCs' main functions, i.e., transport of oxygen and of CO_2. To fulfil the first function, hemoglobin-based oxygen carriers with the above-mentioned advantages have been developed, put through clinical trials, and had even been approved for routine use in South Africa to avoid using blood from an H.I.V. contaminated donor (Mer et al., 2016). Still, hemoglobin-based oxygen carriers remain challenging to design, as unmodified hemoglobin cannot be utilized due to its highly toxic properties. Toxicity can be overcome through encapsulation, genetic engineering, polymerization, cross-linking, or typically a mixture of these. The challenge lies in synthesizing hemoglobin or a hemoglobin complex that is non-toxic, while remaining capable of interacting with oxygen in the desired manner. Transport of CO_2 is less problematic. CO_2 is transported across the body via the blood vessels in three ways: dissolved in the blood plasma, attached to hemoglobin, or in the form of carbonic acid inside of an RBC. Only the last two transport routes include RBCs. Hemoglobin capable of binding to oxygen is almost always also automatically capable of binding to CO_2, while the synthesis of carbonic acid from CO_2 and water can be accelerated without difficulties by simply introducing carbonic anhydrase enzymes to the RBC (Alayash, 2017, Arthurs & Sudhakar, 2005).

4 Conclusion

Although often referred to as the most basic and simplest unit of life, the cell is anything but simple. Replicating any single cellular component, process, or organelle is already difficult, and more troublesome complications arise when trying to couple an artificial component with another component or when attempting to set up the artificial cell in a way that would enable it of independent resource managing, typically resulting in structures that are inefficient and only viable for a short time. This is not entirely surprising, as it would be very daring to believe that within a few decades it could be possible to successfully recreate something that is the product of billions of years of nature's biological sculpting (Cavalazzi et al., 2021). Even so, the way to a goal as daunting as this is taken one step at a time, and each step could not occur without the one before it. Through a variety of methods by the top-down approach that alters cells' genomes and by the bottom-up approach that constructs cells from scratch, the gap between artificial construct and living being seems to be getting more and more narrow as time passes. Many of the key characteristics that the major components of cells, such as genetic material, membranes, and metabolism, encompass have been elucidated, and man-made creations that exhibit some of these characteristics in a reasonably efficient manner have been designed. However, there is still much that needs to be resolved: How can artificial cells divide, replicate, and evolve effectively? How can artificial cells communicate with each other and with the environment? How can artificial cells with different specializations work together and build artificial cell networks? How can artificial cells respond to changes in their environment? Attempting to find solutions to these and many more such questions will certainly challenge our technology and understanding enough to drive us to greater progress and growth. Indeed, the harnessing of the basic unit of life may be one of the turning points that every advanced civilization needs to go through.

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6 List of Figures

7 Declaration

Erklärung

„Ich erkläre, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Alle wörtlichen und sinngemässen Übernahmen aus andern Werken habe ich als solche kenntlich gemacht.

Ich nehme ausserdem zur Kenntnis, dass meine Arbeit zur Überprüfung der korrekten und vollständigen Angabe der Quellen mit Hilfe einer Software (Plagiaterkennungstool) geprüft wird.“

Datum Unterschrift