



Drug delivery with living cells[☆]



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ARTICLE INFO

Article history:

Received 1 January 2016

Received in revised form 18 April 2016

Accepted 19 April 2016

Available online 27 April 2016

Keywords:

Nanomedicine
Synthetic biology
Cell engineering
Biopharmaceutics
Cell therapy

ABSTRACT

The field of drug delivery has grown tremendously in the past few decades by developing a wide range of advanced drug delivery systems. An interesting category is cell-based drug delivery, which includes encapsulation of drugs inside cells or attached to the surface and subsequent transportation through the body. Another approach involves genetic engineering of cells to secrete therapeutic molecules in a controlled way. The next-generation systems integrate expertise from synthetic biology to generate therapeutic gene networks for highly advanced sensory and output devices. These developments are very exciting for the drug delivery field and could radically change the way we administer biological medicines to chronically ill patients. This review is covering the use of living cells, either as transport system or production-unit, to deliver therapeutic molecules and bioactive proteins inside the body. It describes a wide range of approaches in cell-based drug delivery and highlights exceptional examples.

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[☆] This review is part of the *Advanced Drug Delivery Reviews* theme issue on “Biologically-inspired drug delivery systems”.

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1. Introduction

The field of drug delivery has grown tremendously in the past few decades by the development of a wide range of nano-sized advanced drug delivery systems for systemic drug delivery. Lipid nanoparticles, such as liposomes, micelles and extracellular vesicles along with virus-inspired vectors and polymeric particles have been studied to deliver bioactive compounds into targeted tissues. However, such nanomedicines have several limitations. Mainly due to their size, they have difficulties in passing the endothelial lining, which restricts targeting to tissues with a naturally enhanced vascular permeability, such as the liver and spleen, or tissues with an inflammation-mediated induced permeability [1]. In addition, nanoparticles can be cleared rapidly from the circulation by phagocytic uptake and hepatic filtration which shorten their blood circulation half-lives. Moreover, when reaching the target cells, intracellular delivery may be limited due to inefficient endosomal escape of the particle and its cargo [2,3]. Finally, also toxicity may play a role in the failure of nanomedicine therapy. To address these challenges, cell-based drug delivery could offer a number of advantages. Encapsulation inside cells could improve pharmacokinetic profiles of drugs or significantly increase drug targeting toward pathological conditions [4]. Microorganisms and immune cells are known to home toward sites of inflammation, including cancer and are therefore considered to be perfect candidates to function as transporters for cytostatic agents or enzymes that can locally convert a prodrug into its cytotoxic counterpart [5,6]. Another approach involves a cell-based system that can sense the environment and if necessary respond to changes in an appropriate way. This system would have a huge potential in treating metabolic disorders by restoring homeostasis and the first proof-of-concepts have already been successfully demonstrated in animal models [7,8]. These developments are very exciting for the drug delivery field and could have an enormous impact on pharmaceutical technology. This review is covering the use of living cells, either as transport system or production-unit, to deliver therapeutic molecules and bioactive proteins inside the body.

2. Cell-based drug carriers

One application of the use of living cells for drug delivery is by utilizing the circulation capacity of blood cells to prolong the circulation time of drugs or the capacity of certain immune cells to home into inflamed tissues for targeted drug delivery. To this end, drugs can be loaded inside the cells or attached to the cell surface and in this way the therapeutic cargo is transported through the circulation.

2.1. Red blood cells

The idea of using red blood cells (RBCs) as drug delivery systems was first described in the 1970s [9] and recently re-discussed as an alternative for polymer-based drug vehicles. RBCs have various characteristics, such as long circulation-time, high drug-loading capacity and good biocompatibility, which make them interesting for the delivery of drugs in the circulation [4]. Furthermore, several techniques allow easy processing of RBCs and because of their large volume a reasonable amount of drugs can be loaded [10]. Dependent on the type of drug and the desired effect (e.g. slow delivery or stabilization of the drug in the circulation) different approaches can be applied which are discussed below and a schematic representation is presented in Fig. 1.

2.1.1. Prodrug approach

Encapsulation of drugs into RBCs can be used to develop a slow-releasing depot of active compounds in the circulation [11]. Therefore, a prodrug is loaded inside RBCs where internal enzymes convert the drug to its active form followed by release in the circulation. The non-diffusible prodrug is loaded into autologous RBCs by temporary opening the cell membrane pores under hypotonic conditions. The pores are then re-sealed once normal osmosis had been restored [12]. The release process is usually based on phosphorylation, whereby dephosphorylation of the non-diffusible prodrug results in a diffusible active molecule [4]. However, not every drug is suitable for this approach since the dephosphorylated compound has to be able to pass the RBC membrane by passive diffusion or by transporter-mediated mechanisms [13]. Furthermore, the prodrug needs to contain a certain degree of hydrophilic properties to enable encapsulation. Several types of prodrugs, including anti-inflammatory, antiviral and anti-cancer drugs have been used for this purpose [4,10,11]. Magnani et al. showed that dexamethasone 21-phosphate could be efficiently loaded into RBCs in a wide concentration range [10]. Subsequently, dexamethasone is released in the circulation by passive diffusion (Fig. 1A). This advanced dexamethasone drug delivery system has been further developed to find its application in the clinic for the treatment of chronic inflammatory diseases (e.g. cystic fibrosis, inflammatory bowel disease and ataxia telangiectasia). In a recent finished trial in ataxia telangiectasia patients, treatment with the EryDex system (i.e. autologous loaded RBCs) resulted in significant neurological improvement, as well as the system proved to be safe and well tolerated [14].

2.1.2. Loading of active substances

In contrast to the prodrug approach, active pharmaceuticals can also be directly encapsulated into RBCs to increase the circulation time [15]. In addition, protein-based drugs are protected by clearance in different organs and from circulating proteases and antibodies leading to a more stable pharmaceutical product [4,10]. This approach finds its best implementation in therapies using enzymes. Enzymes are rapidly cleared by the liver and after repeated administration antibodies are produced that can inactivate the protein [16]. However, when encapsulated into RBCs the enzyme benefits from the above described properties without impairing its catalytic function. The (toxic) substrate can enter the RBC where it is converted into a (nontoxic) product which is subsequently released in the circulation (Fig. 1B) [4,17]. The most advanced example is the encapsulation of asparaginase in RBCs, known as ERY-ASP, for the treatment of acute lymphoblastic leukemia and acute myeloid leukemia [18]. Phase IIb/III clinical studies are currently ongoing using this product.

2.1.3. Drug-binding proteins or protein domains

Encapsulation of drugs that diffuse rapidly through the RBC membrane is of no use, because no advantages in pharmacokinetic properties are obtained compared to conventional delivery. In order to retain the diffusible drug inside the RBC, Biagiotti et al. proposed a new strategy in which specific drug-binding substances can be used to bind the drug in a reversible way inside the RBC [10]. It was demonstrated that encapsulated phenytoin in human RBCs with bovine serum albumin showed an 8-times higher drug loading compared with normal RBCs [19]. Other researchers have focused on using recombinant immunophilins, which are proteins that can bind immunosuppressive drugs [12]. Tacrolimus possesses a poor pharmacokinetic profile (i.e. low oral bioavailability, narrow therapeutic window), which can be improved when encapsulated in RBCs with the corresponding

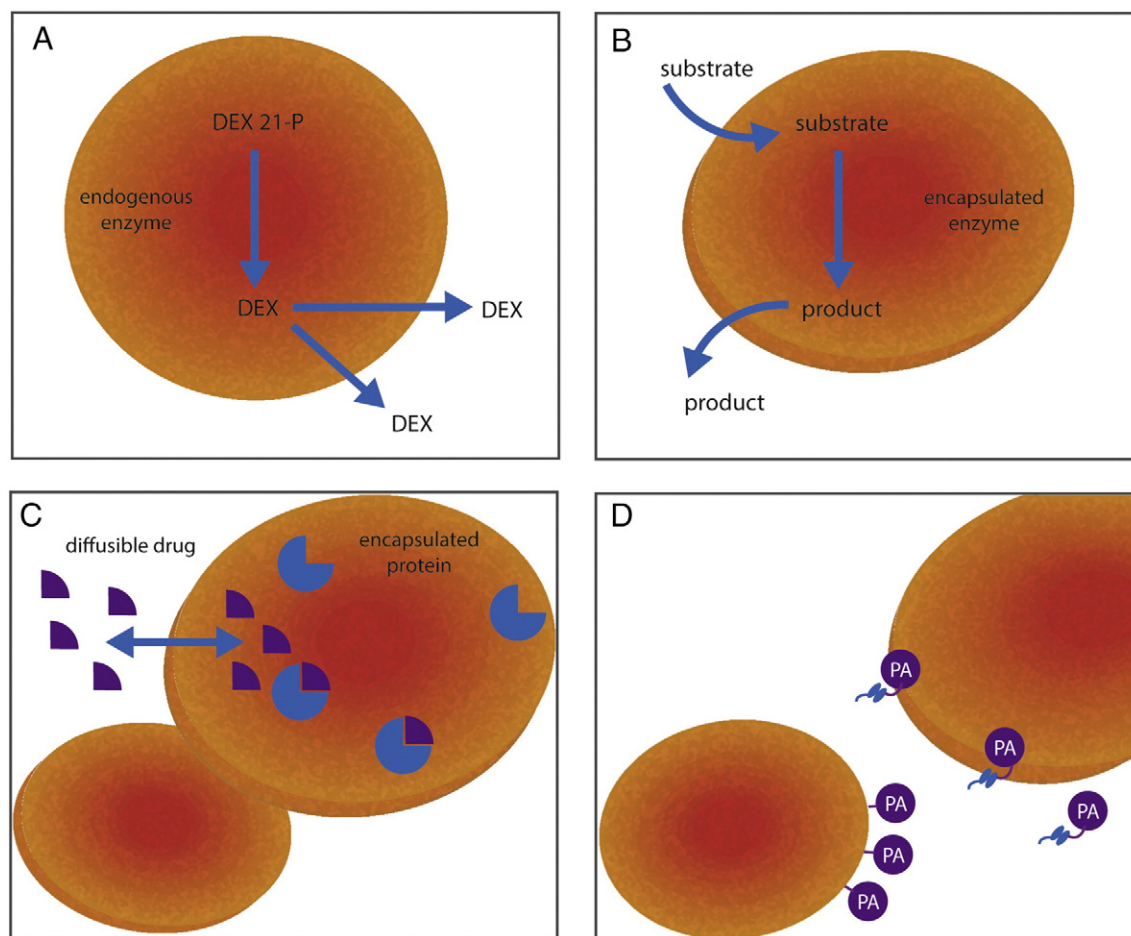


Fig. 1. Strategies for RBC-based drug delivery systems. A) Prodrug strategy. Dexamethasone 21-phosphate (DEX 21-P) is loaded inside RBCs and by endogenous enzymes converted into dexamethasone (DEX), which can diffuse through the cell membrane. B) Enzyme therapy. The (toxic) substrate can enter the RBCs where it is converted into a (nontoxic) product, which is subsequently released in the circulation. C) Drug-binding proteins. RBCs are loaded with proteins and the diffusible drug enters the RBCs, where it reversibly binds to the protein. D) Surface coupling. Plasminogen activator (PA) is either directly covalently coupled to the surface of RBCs or via an antibody fragment directed against a surface RBC antigen.

immunophilin (Fig. 1C). By administration through RBCs, premature hepatic metabolism of the drug is circumvented. Researchers showed that RBCs could be loaded with such sufficient amounts of tacrolimus that a single dose of loaded RBCs could ensure a drug concentration within the therapeutic window [12]. In this way, low and constant doses of the drug could be reached and because no plasma peak levels are created, the side effects will be reduced.

2.1.4. Coupling of drugs to the surface of RBCs

Advantages of coupling drugs to the RBC surface include circumvention of possible harmful loading techniques, bypassing release issues of the drugs and possibility of coupling drugs to RBCs without the need for extraction and reinfusion. In addition, coupled drugs no longer encounter diffusional restrictions. For example, enzymes have been shown to be more catalytically active when bound to the surface than within the RBC [15,20]. Three different approaches can be used for surface-coupling, including direct coupling (either covalent or noncovalent), receptor-mediated coupling and coupling via conjugated affinity ligands (such as antibodies or their fragments) [15]. The coupling of several biotherapeutics to RBCs has been investigated, including antibodies and antigens for stimulating an immune response [21,22]. In addition, drug-loaded nanoparticles (NP) could also be attached to RBCs to increase the circulation time of the nanoparticles. It was shown that polystyrene nanoparticles adhere to the surfaces of RBCs and detach passively because of shear forces and cell–cell interactions [23,24]. This resulted in 2–3 times higher blood levels of RBC–NP compared to free NPs and the accumulation in the lungs was increased 5-fold [24].

Muzykantov and coworkers have developed strategies for the treatment and prevention of thrombotic events by using tissue plasminogen activator (tPA) coupled to RBCs (Fig. 1D). They have examined the effect of RBC–tPA complexes in animal models of cerebrovascular thromboembolism and ischemia and the results demonstrated that after intravenous injection nascent thrombi were rapidly lysed, leading to an increased perfusion and protection of the brain tissue [20].

2.2. Macrophages

Macrophages are differentiated cells derived from monocytes and are part of the innate immune system. The most characteristic feature of these cells is their ability to phagocytose microorganisms and other particles. Moreover, monocytes have the ability to migrate toward pathological sites, such as infections, inflammation and tumors, and after tissue infiltration they differentiate into macrophages [25]. When monocytes from the bone marrow or blood are cultured with the human macrophage colony stimulating factor, macrophages are generated which have been shown to migrate to sites of infection [25–29]. Consequently, this has resulted in the concept of using macrophages as carriers for therapeutic agents.

The best example is shown in antiretroviral therapy, where macrophages are used to transport antiretroviral drugs to sites of active viral replication [26–28,30,31]. HIV-1 challenged mice were injected with drug-carrying macrophages and antiretroviral therapeutic responses were evaluated. The amount of drug was elevated in targeted disease sites (lymph nodes, spleen, liver, lung and kidney) when loaded

into macrophages compared with intravenous administration of the drug alone [27]. In addition, this effect was observed up to two weeks. Additional studies showed that indinavir-loaded macrophages could play a significant role in the treatment of HIV-associated neurocognitive disorders [26]. Another possible therapeutic application of macrophage-based delivery is in cancer treatment. Tumors have the natural ability to recruit monocytes and macrophages, thus the delivery of therapeutic agents within these cells was investigated in this context. Choi and coworkers generated Au nanoshells and allowed macrophage to phagocytize the nanoparticles in vitro [32]. Afterwards, infiltration of nanoparticle-loaded macrophages into tumor spheroids was observed and upon near infrared radiation they successfully demonstrated tumor cell death [32]. A challenging issue in macrophage-based drug delivery approach includes a high loading capacity of drugs inside the macrophages. Because macrophages are known to phagocytose particles, the idea of creating nanoparticles which facilitates this internalization arose. Therefore, drugs can be incorporated into lipidic or polymeric nanocarriers, such as liposomes, micelles, nanogels, nanosuspensions, nanofibers or nanotubes [30,31]. Recognition and subsequently internalization of the nanocarriers by macrophages are initiated through receptors (e.g. mannose, complement and Fc receptors) localized on the cell membrane [30]. The charge, size and shape of the nanoparticles play a role in cellular uptake as described by Champion and colleagues [33]. Control over the intracellular degradation rate of internalized nanoparticles is important to enable timely drug release from these macrophages.

2.3. Dendritic cells

Dendritic cells (DCs) are antigen-presenting cells from the immune system and known to interact with T and B lymphocytes and natural killer cells resulting in stimulation of the immune system [34].

Antigen-specific active immunotherapy (i.e. vaccination) using dendritic cells has been investigated for the development of new cancer treatments. DCs can be directly isolated from circulating blood or derived from CD34 + progenitor cells or CD14 + monocytes, and all types of DCs have been studied [35]. Subsequently, DCs have been loaded with tumor-associated antigens *ex vivo* to induce a therapeutic and protective anti-tumor effect [35]. Numerous immunogenic peptides associated with tumors have been discovered, such as antigenic peptides derived from telomerase, tyrosinase, p53, Her-2 and several other antigens [35,36]. In 2010, the FDA approved the first therapeutic vaccine for prostate cancer called PROVENGE® (sipuleucel-T). Peripheral-blood mononuclear cells are obtained from patients and *ex vivo* co-cultured with a recombinant fusion protein to reprogram the cells to attack prostate cancer cells [37]. The fusion protein consists of prostate acid phosphatase as tumor-associated antigen and granulocyte-macrophage colony-stimulating factor for the activation of the immune cells [37]. The IMPACT study showed that treatment with sipuleucel-T prolonged overall survival of men with metastatic castration-resistant prostate cancer [37]. Currently, more DC-based therapies are under evaluation in clinical trials for the treatment of several types of cancer, such as melanoma, prostate cancer, breast cancer and leukemia [35,36,38,39]. Overall, data has shown that DCs can be safely administered without any serious side effects [38]. However, a limited rate of tumor regression is observed during these studies questioning the efficacy of therapeutic vaccination against cancer using DCs [35,39]. This could be due to the complex interactions associated with DCs and additional studies unraveling these processes might lead to new insight and opportunities to manipulate DCs for improving their therapeutic immunity [36].

2.4. Challenges and limitations

Drug delivery with living cells raises certain questions related to issues as immunogenicity and safety. Regarding the source of eukaryotic

cell types, two approaches can generally be distinguished including the use of autologous and allogeneic cells [40]. The advantage of autologous therapies is that minimal immunogenic responses are expected, since the immune system of the host recognizes the engineered cells as 'self' [41]. As seen for RBC-based drug delivery, systems currently under clinical evaluation are following the autologous strategy [14,18]. However, maintenance of autologous cells is usually difficult and regulation issues hamper the development. Alternatively, when allogeneic cell types are used to engineer advanced drug delivery systems concerns about immune responses from the host immune system are raised. Repeated administration in clinical setting could be complicated, due to the development of neutralizing antibodies or severe infusion reactions. In macrophage- and dendritic-based therapies the ability of the cells to migrate toward pathological tissues is exploited. However, the main challenge remains control over these homing properties to ensure efficacy of these therapies.

3. Transduced cells: constant therapeutic producers

In addition to the previously described strategies for cell-based drug delivery where cells are used as drug carriers by actively loading the therapeutic compounds into the cells or attach them to the cells, an alternative approach involves the transduction of cells with genes to achieve a constant production of therapeutic products. Different cell types are investigated for this purpose, including bacteria, stem cells, tumor cells and T lymphocytes, which are discussed in the following sections.

3.1. Bacteria

The use of bacteria as therapeutic agents is most clear in cancer treatment. Currently, anti-cancer therapeutics face certain problems which are related to an incomplete tumor targeting, an inadequate tissue penetration and numerous therapeutics possess a limited toxicity to all cancer cells [42]. Certain bacteria have unique characteristics which can be beneficial to overcome these issues and become a new strategy in the treatment of cancer [5,42,43]. The anti-cancer agents delivered by bacteria can be divided into four categories, including cytotoxic agents [44], cytokines [45], tumor antigens [46] or antibodies [47] and prodrug converting proteins [48]. The major advance of using bacteria is their ability to migrate toward tumor cells and this mechanism is partially dependent on the oxygen level [42]. Obligate anaerobic bacteria (e.g. *Clostridium*) cannot survive in the presence of oxygen, and therefore they will preferentially grow in hypoxic areas [42,49]. Typically, rapidly growing solid tumors often contain hypoxic regions making these sites attractive for such bacteria to accumulate in. This was described by Malmgren and Flanagan who showed that after injection of *Clostridium* spores in tumor-bearing mice only the mice with tumors died from this bacterial infection [50]. More complex mechanisms to target tumor cells are used by facultative anaerobic bacteria, such as *Salmonella* or *Escherichia*. These mechanisms include entrapment of bacteria in the chaotic vasculature of tumors, flooding into tumors following inflammation, chemotaxis toward compounds produced by tumors, preferential growth in tumor-specific environments, and protection from clearance by a defective or depressed immune system within the tumor microenvironment [5,42,49,51].

Another advantage of bacterial therapy is their motility which enables penetration into the tumor tissue after arriving at the specific site [42]. Nanoparticle-based drug delivery is often hampered by a poor tissue penetration after extravasation where most nanoparticles are retained in the perivascular regions of a tumor with the vast majority of tumor tissue being out of reach [5,52]. Bacteria are often motile allowing them to migrate through the tumor tissue away from the vasculature [42]. It has been shown that *Salmonella* possess receptors detecting small molecules such as serine, aspartate and ribose, initiating chemotaxis toward tumor cells [53].

Bacteria have a natural cytotoxicity which can be used to reduce tumor size. The mechanisms behind this phenomenon are tumor cell kill by attracting and activating immune cells and direct tumor cell kill. After systemic administration, *Salmonella choleraesuis* accumulates in tumors and induces the infiltration of neutrophils and triggers other anti-tumor immune responses [5]. In addition to their natural cytotoxicity, bacteria can also be genetically modified to deliver drugs to tumor cells and increase their therapeutic efficacy. Therefore, proteins with anti-tumor effects can be directly expressed by bacteria or bacteria can transfect cancer cells with eukaryotic expression vectors [42]. The last approach might result in a more permanent and stable expression of proteins, however some obstacles have to be dealt with. For example, poor transfer efficiency can limit the therapy efficacy. Furthermore, control over gene expression might be difficult and genes could be unequally distributed through tissues. In addition, unwanted effects might occur by transfecting tissues other than the intended target tissue.

Although the proof-of-principle of bacteria-mediated cancer treatment was shown by the use of pathogenic *Clostridium* strain, clinical translation is limited due to safety issues and immune responses [49, 54]. Therefore, non-pathogenic species are investigated, such as the attenuated strain of *Clostridium novyi* (*C. novyi*-NT). Researchers have shown in preclinical settings that *C. novyi*-NT has good tumor colonizing properties and can induce tumor necrosis after intratumoral injection [55]. Local administration increases the colonization efficiency and might minimize immune responses. However, this might not always be practicably possible and limits the applicability to only reachable solid tumors. In addition, dissemination in other organs could still occur [42,56]. Attempts to enhance the homing capacity of bacteria to tumor sites regulated via environmental triggers could provide new strategies to evade immune responses, and in addition increase the targeted delivery of drugs.

3.2. Stem cells

Research on stem cell-based therapy is a fast growing field and mostly focusing on the use of stem cells in regenerative medicine and tissue engineering. In this review the focus is on using adult stem cells and induced pluripotent stem cells as drug carriers to make use of their homing capacity to deliver biological drugs into specific tissues or organs. Several types of stem cells have been studied for this purpose, including mesenchymal stem cells (MSCs) [57], neural stem cells (NSCs) [58–61] and hematopoietic stem cells [62]. The use of MSCs to deliver therapeutic agents is widely investigated for cancer treatment. Unmodified MSCs have been shown to release factors that have anti-tumor properties inhibiting the proliferation of several tumor types [63]. In addition, MSCs have been modified to introduce therapeutic genes for the delivery of anti-cancer drugs, including immunomodulatory factors [64,65], which can also be used to treat multiple inflammatory diseases. The prodrug strategy, as discussed in the previous sections, could also be applied when using stem cells. The most illustrative example is the expression of cytosine deaminase, which can convert the non-toxic 5-fluorocytosine (5-FC) to the active drug 5-fluorouracil (5-FU) [63,66]. Results from preclinical studies showed migration of engineered MSCs to prostate cancer cells and after co-administration with 5-FC tumor growth was inhibited [67]. A relatively new type of anti-cancer agents transported by MSCs is the delivery of pro-apoptotic proteins, such as tumor necrosis factor-related apoptosis induced ligand (TRAIL) [62]. TRAIL is a member of the TNF family and has been shown to induce apoptosis via activation of caspases in many types of cancer cells while sparing normal cells. However, recombinant TRAIL proteins were found to have safety issues and concerns regarding species specificity [62]. In addition, the existence of TRAIL-resistant tumor cells is also reported. Preclinical data demonstrated that expression of TRAIL by MSCs can overcome TRAIL-resistance resulting in genotoxic damage of colorectal carcinoma

cells in vitro [66]. Similar to the use of MSCs, NSCs could also be considered, in particular for the treatment of neurological-associated diseases such as gliomas and neuroblastoma [58,59,68,69]. Hematopoietic stem cells are best known for their application in bone marrow transplantation in the treatment of cancer, in which they are modified to express tumor-specific antigens and subsequently re-injected into patients [62]. However, only a few articles describe the use of hematopoietic stem cells transduced with therapeutic gene constructs but none of them for the purpose of drug delivery.

3.3. Tumor cells

Since the discovery of gene transfection by retroviral vectors, this technique was also applied to tumor cells to increase their recognition and subsequent elimination by immune cells. Furthermore, introduction of particular genes could enhance the susceptibility to toxic drugs. Retroviral vector-mediated transfection of herpes simplex virus thymidine kinase (HSV-TK) gene has been applied to create susceptibility against antiviral drugs, such as acyclovir and ganciclovir [70]. The ex vivo modified tumor cells were re-injected intraperitoneal in mice and showed indeed tumor regression of treated subjects after co-administration with ganciclovir. However, clinical translation is restricted because gene transfection to non-targeted cells is uncontrollable and due to recombination unknown viral constructs could be generated possessing serious safety issues [30]. Another strategy is the development of cancer vaccines, which includes the transfection of tumor cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) to elicit strong anti-cancer immune responses [71]. After genetic modification, tumor cells are irradiated and administrated intradermal as a vaccine for the treatment of cancer. First, the recruitment of immune cells is stimulated by the secretion of GM-CSF and afterwards cancer cells are phagocytized and processed by dendritic cells or macrophages [72,73]. Subsequently, these cells present tumor-specific antigens to T-cells to provoke anti-cancer immune responses [72,73]. Several clinical trials were initiated to evaluate this vaccine program and both patient-specific (autologous) and non-patient-specific (allogeneic) approaches were used although the last few years they have been focusing on allogeneic-based vaccines [71–75].

3.4. T lymphocytes

The potential of T lymphocytes in the treatment of cancer is well described in literature [41,76,77]. Autologous immune T lymphocytes are ex vivo modified with tumor-specific antigens and re-infused in patients for the treatment of malignant diseases [78]. Limitations of these therapies include the requirement of simultaneous use of adjuvant drugs which generally target multiple cell populations leading to dose-limiting toxicities [79]. To optimize these cell therapies, a new strategy was proposed to attach synthetic drug carriers to the surface of the transferred cells [80]. Based on the fact that many cells have free thiol groups on their surfaces, nanoparticles containing a drug-loaded core can be covalently coupled via a two-step process, including coupling of maleimide-thiol followed by PEGylation to cover resident reactive groups of the nanoparticles [80]. Studies demonstrated no impairment of essential T-cell functions after binding of nanoparticles and additional studies further evaluated T-cell functions and drug release from the system [80]. A combination of two interleukins (IL-5 and IL-21) was loaded inside the nanoparticle and after intravenous administration of the modified T-cells, drugs were continuously released affecting their carrier cells in an autocrine fashion, resulting in extensive T-cell expansion and complete clearance of tumors [80, 81]. These low drug levels have no effect when administered by traditional systemic routes, indicating the advantage of this drug-delivery system. The same technique was applied to modify hematopoietic stem cells with nanoparticles containing glycogen synthase kinase-3 β inhibitor to stimulate the engraftment and growth of these cells after

transplantation [82]. Preclinical data supported the idea of implementation of adoptive T cell therapy in the clinic and currently this approach is under evaluation in different stages of clinical trials [41,76–78]. Although approval of this technology for use in the clinic is still in the future, the above demonstrated results clearly indicate the main benefits of this technique.

3.5. Challenges and limitations

As with all drugs, specific dosing regimens regarding amount and interval of dosing are necessary to reach therapeutic concentrations without inducing toxicity. It is highly important to gain control over the administered cell population, since cell proliferation or cell death both influence the number of drug producers and subsequently drug concentrations. In addition, the capacity of cells to migrate toward specific tissues should be controlled, dependent on the desired therapeutic effect. Cell-based drug delivery aiming for systemic drug concentrations does not necessarily involve the distribution of the producing cells. In contrast, engineered cells that are targeted to specific tissues (e.g. cancer cells) should have accurate migration properties. Cells could be modulated with specific genes or proteins to optimize these homing features as described for bacteria and stem cells. However, their efficacy and safety in a clinical setting should be carefully evaluated. Regardless of the therapeutic strategy, it is important that these migration properties are fully understood for each cell type and, if necessary, accurately controlled to reduce unwanted side effects. For this purpose, it would be ideal to design highly predictable and controllable synthetic networks, which are discussed in the next section.

4. Transduced cells: highly advanced sensory and output devices using synthetic circuits

Synthetic biology is an emerging research field, in which scientists aim to design novel biological systems through the engineering of genetic devices [83,84]. The field has significantly advanced over the past few years in designing complex genetic networks which can reprogram metabolic activities in mammalian cells [85,86]. These engineering approaches could provide innovative treatment strategies including cell-based therapies using engineered cells to “sense” the patient’s need for a drug and to provide this drug at will.

4.1. External trigger-induced genetic devices

As discussed in the previous section, transduction of cells with genetic constructs has been extensively investigated for the delivery of therapeutic agents. However, control over the expression rate is preferable for conducting clinical trials in humans. Therefore, artificial gene regulation systems using different kinds of triggers and strategies have been evaluated and reported [87]. The inducers have to be carefully chosen to achieve high specificity, maximum efficacy and low adverse effects. The ideal system should have none or low basal expression and a high induction ratio. Most of the designed control devices are responsive to peptides and small molecules, including approved drug substances [87,88]. However, the latest generation of transgene control systems shows a shift toward the use of trigger molecules with negligible possible side effects, such as physiologically inert molecules [89,90].

4.1.1. Drugs and biochemical substances

In the development of inducible mammalian genetic expression systems, the focus has been on using small-molecule triggers, such as steroid hormones [91], antibiotics [92], and immunosuppressive drugs [88]. In addition, L-arginine [93] and biotin (vitamin H) [94] have also been investigated to function as gene inducers. It has been demonstrated that ex vivo transduced fibroblasts expressing IFN- α migrate toward sites of ovarian cancer and constitutively produce the cytokine to elicit anti-tumor responses. In order to control the gene expression of IFN- α ,

the fibroblasts were transduced with a rapamycin-inducible retroviral vector encoding IFN- α [88]. This system is previously described and considered to be useful for clinical integration because rapamycin is found to have anti-tumor properties through specific inhibition of the mTOR signaling pathway which is activated in several tumor types [88]. It is important to control the interaction between bacteria and mammalian cells, which can be regulated via environmental triggers [95]. Although bacteria have a native tendency to home to certain pathological sites, it is preferable to enhance the targeted delivery especially in the case of tumor cells. Therefore, the migration of *Escherichia coli* to cancer cells under the control of different triggers has been investigated. *E. coli* was transfected with genes encoding for invasion, a protein known to bind to cell surface β 1-integrins leading to internalization [96]. The expression of this gene can be induced by chemical signals, as was shown by the introduction of an arabinose-responsive promoter [97]. Addition of arabinose resulted in an increased invasion of modified *E. coli* strains to HeLa cells [95,97]. Furthermore, the hypoxia microenvironment of tumors could be exploited by designing a hypoxia-inducible system [98]. To further restrict the invasion of bacteria to healthy tissue, the use of quorum sensing genetic circuits has been investigated [95,99]. This system can distinguish between high and low cell densities and when expression of invasion was put under quorum control, only at high bacteria concentrations protein invasion levels were detectable [95].

4.1.2. Light and γ -irradiation

The use of light to induce gene expression was first studied in bacteria, which can be engineered with the light-responsive receptors phytochromes [100]. Several strategies to generate such light-dependent gene expression systems have been developed, as previously reviewed by Bacchus and Fussenegger [100]. Recently, Kim et al. reported an erectile optogenetic stimulator (EROS) device, which enabled penile erection associated with ejaculation in EROS-transfected male rats upon illumination with blue-light [101]. Weber and colleagues demonstrated that gene expression of IFN- β transduced in CHO cells could be controlled by acetaldehyde-inducible regulation elements [102]. These results provide another technology for the development of gene regulation systems. However, the field of optogenetics is still in its early stage and potential problems or side effects should be carefully evaluated [100]. Another approach is the use of a radiation-inducible system for the delivery of therapeutic anti-cancer agents. Engineered *Salmonella* bacteria were systemically injected into mice carrying breast tumors and after two days the mice received γ -irradiation. Subsequently, TRAIL expression was activated and results showed a delay of 19 days for tumor growth to 1000 mm³ and an increase of 37.5% in a 30-day survival of the treated mice compared to controls [103]. In this approach, the combination with radiation therapy is important to obtain a full response of the gene expression.

4.1.3. Physiologically inert molecules

The previously discussed triggers for induction of gene expression meet certain issues related to their side effects, especially when administered for a long period of time [89,90]. To overcome these problems, molecules that do not elicit physiological effects could be used to control the transcription of therapeutic genes. Gitzinger and colleagues engineered a synthetic vanillic acid-responsive mammalian expression system [90]. This strategy was tested in vivo in mice by implanting CHO cells showing SEAP production in response to vanillic acid [90]. SEAP, human placental secreted alkaline phosphatase, was used as model protein to demonstrate the proof-of-principle [90]. The previously described gene inducers need to be taken orally or systemically injected and in order to improve therapy convenience and patient’s compliance alternative routes of inducer administration could be explored. In this regard, a trigger-inducible transcription system for mammalian cells was developed, which was subcutaneously implanted and sensitive to

dermally administrated phloretin [89]. Phloretin has been naturally found in the roots of apple trees where it functions as an antimicrobial agent [104]. However, *Pseudomonas putida* was found to hold resistance against such plant-derived antimicrobials and this was induced by phloretin binding to a specific promoter [104]. Based on these findings, a synthetic mammalian phloretin-adjustable control element (PEACE) was designed [89]. Subsequently, PEACE-transfected CHO cells were microencapsulated and subcutaneously implanted in mice. In addition, mice were treated with a skin lotion containing different concentrations of phloretin and SEAP levels measured in the treated mice showed phloretin-dependent dose–response profiles [89]. Furthermore, the DREADD (designed receptors exclusively activated by designer drug) technology was developed to generate receptors that are solely activated by synthetic ligands with no biologic activity [105,106].

4.2. Advanced therapeutic and prosthetic networks

The next-generation cell-based drug delivery systems take advantage of endogenous triggers for inducing therapeutic genes. By combining different potent triggers with the unlimited possibilities in designing genetic switches, various advanced therapeutic strategies have evolved. Among these synthetic devices are the so called prosthetic networks, which can act in an automatic and self-sufficient way [83, 107]. Prosthetic networks can sense their environment and, if necessary, response to it in an appropriate way [84].

4.2.1. Identifying cancer cells

In addition to designing expression systems which are responsive to a single trigger, Nissim and Bar-Ziv proposed a novel strategy to engineer dual-promoter integrators (DPIs) to ensure a more precise regulation of gene expression [108]. This principle has been studied for the delivery of the oncolytic gene coding for herpes simplex virus thymidine kinase (HSV-TK). In the presence of nucleotide analogs, such as ganciclovir, HSV-TK is cytotoxic and induces cell death. It is important to ensure HSV-TK expression only in the target cells and therefore cell cycle-specific and cancer cell-specific promoters were combined. Activation of each promoter leads to the production of one protein subunit, which can form a transcription factor after heterodimerization (Fig. 2). Consequently, the transcription factor induces gene expression of HSV-TK and in the co-presence of ganciclovir the cancer cells are killed [108]. Three types of promoters, which are

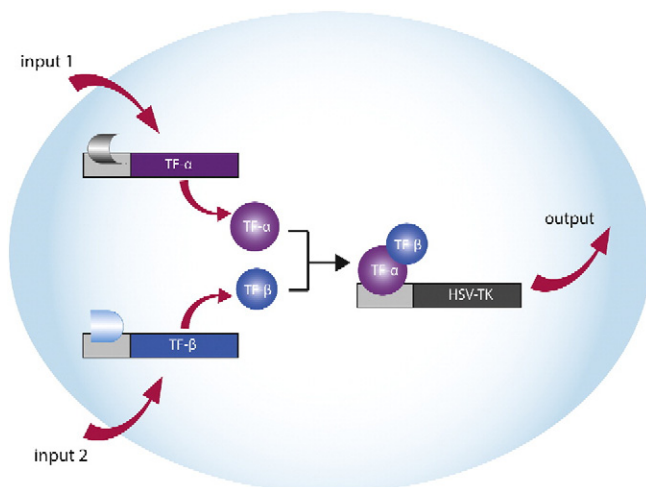


Fig. 2. Schematic overview of dual-promoter integrator (DPI). Activation of two different promoters leads to the production of the transcription factor (TF) subunits α and β , respectively. After heterodimerization of the two subunits α and β , the active TF is formed, which induces the expression of the output gene, which is in this case the herpes simplex virus thymidine kinase (HSV-TK) gene. In the co-presence with ganciclovir, HSV-TK is cytotoxic and induces cell death.

all regulated by endogenous transcription factors, were integrated and the activity and specificity of the combination of these constructs were demonstrated in four different cancer cell lines [108]. These data show that a more regulated expression system could be developed, which provides flexibility regarding the choice of promoter inputs and effector gene outputs.

4.2.2. Blood glucose homeostasis

As already mentioned in the previous section, light could be used to trigger gene expression in living systems. An interesting development is the use of genetic light switches in blood glucose homeostasis. Glucagon-like peptide 1 (GLP1) is a promising drug candidate for the treatment of patients with type II diabetes because of its glucose homeostasis-modulating properties [109]. HEK-293 cells were engineered to express this peptide under the control of a light-induced genetic device [110]. Therefore, melanopsin-mediated G protein-coupled receptor signaling was rewired together with the control circuit of the nuclear factor of activated T cells (NFAT), utilizing their shared second-messenger cascade system [110]. Blue light activates melanopsin and subsequently turns on a signaling pathway, involving activation of phospholipase C, phosphokinase C, calmodulin and eventually NFAT. Inside the nucleus, NFAT binds to specific promoters and induces the gene transcription of GLP1 [110]. This mechanism was studied in a mice model with type 2 diabetes by subcutaneous implantation of microencapsulated engineered HEK-293 cells [110]. Upon light induction, the system was able to control glucose homeostasis, and thus considered to be promising for the treatment of type II diabetes patients [110].

4.2.3. Synthetic interfaces between brain activities and peripheral circulation

Dopamine transmission in the brain is involved in the reward system and serum dopamine has been known to control respiration and glucose homeostasis [111]. It is considered that a correlation between central and peripheral dopamine levels exists, and to demonstrate this principle a dopamine-responsive device was used which is controlled by stimuli triggering the reward system such as food, addictive drugs and sexual arousal [111,112]. HEK-293 cells were engineered to express the dopamine receptor D1 on their surfaces and the signaling cascade was rewired to trigger transcription of genes containing synthetic CREB1-specific cAMP response elements. Engineered cells were implanted in mice and after activating the central reward system via sex, drugs and food the peripheral levels of the model protein SEAP were increased [112]. This indicates that the produced expression response is related to dopamine-specific brain activities. In addition, the authors studied the dopamine-sensor device in a more therapeutic context by introducing the gene encoding atrial natriuretic peptide (ANP) for the treatment of hypertension [112]. Microencapsulated cells were implanted in hypertensive mice and after reward system was triggered by sexual arousal ANP levels were increased and systolic blood pressure returned to normal [112]. In the future, it might even be possible to use human brain waves as the input signal to control gene transcription. Proof-of-principle was shown by Folcher and colleagues, who connected mind-triggered electrophysiological signals to a synthetic NIR light-triggered optogenetic device [113]. The new method could be used for the treatment of epilepsy, in which characteristic brain waves at the onset of a seizure trigger the release of a therapeutic drug from a brain implant.

4.2.4. The metabolic syndrome

The metabolic syndrome shows a complex pathophysiology and involves different disorders and risk factors, such as hypertension, hyperglycemia and obesity [107,114]. Current therapies are focusing on one single target, which results in polypharmacy [114]. A new approach combines the therapeutic effect of guanabenz, an antihypertensive drug, with the drug-induced effect of GLP-1 and leptin [107]. Engineered cells containing genes encoding GLP-1 and leptin and

expressing guanabenz-sensitive receptors were encapsulated and implanted in mice. Upon administration of guanabenz, blood pressure and glucose and lipid homeostasis were restored simultaneously [107]. With this approach, multiple targets are tackled while administering only 1 drug.

4.2.5. Prosthetic networks

The first confirmed proof-of-principle of a prosthetic network was successfully designed by Kemmer et al. for the regulation of uric acid homeostasis [7]. Accumulation of uric acid leads to pathological events, such as crystal formation in the joints, kidneys and subcutaneous tissues [115,116]. The synthetic circuit consists of the bacterial transcriptional repressor (HucR) that can bind to the operator hucO only in the absence of uric acid [7]. Dissociation of HucR from DNA occurs when uric acid is present and this allows transcription of the output gene, uricase, which converts uric acid into an easier secretable molecule [7]. This system is referred to as the uric acid-responsive expression network (UREX) and the intelligence of this genetic device is the integrated feedback-loop. When uric acid levels are elevated, expression of uricase is induced resulting in a decrease of uric acid concentration and subsequently silencing of the output gene by binding of the transcription repressor HucR. The functioning of UREX-based control devices was studied in urate oxidase-deficient mice by implanting HeLa cells with these synthetic circuits [7]. In addition, the engineered cells were transduced with the human uric acid-anion transporter, URAT1, to improve uric acid sensitivity. Results showed indeed a decrease of uric acid levels and hyperuricemia symptoms disappeared [7]. In summary, this control system is able to sense uric acid levels, automatically induce the expression of uricase to maintain physiologic concentrations of uric acid and when appropriate levels are reached, is able to stop gene expression. A second closed-loop genetic gene circuit monitoring blood fatty acid levels was developed for the treatment of diet-induced obesity [131]. Therefore, a lipid-sensing receptor was designed and connected with pramlintide expression, a clinically approved peptide hormone which suppresses appetite, stimulates satiety and slows gastric emptying (FDA). The device was implanted into diet-induced obese mice and results showed a significant decrease in food intake, blood fat levels and body weight. In addition, at normal blood fat levels the device is automatically switched off. Interestingly, the designers have built in an extra safety because the genetic circuit can be switched off using a phloretin-containing skin lotion, irrespective of fatty acid levels. Another example focused on the treatment of diabetic ketoacidosis, which involves the excessive production of acidic ketone bodies in alternative energy production due to low insulin-mediated glucose uptake [117, 118].

4.3. Challenges and limitations

The above discussed prosthetic gene networks are great examples of how synthetic biology has inspired to design sophisticated devices that reprogram the dynamic behavior, metabolism and physiology of mammalian cells in an automatic and self-sufficient way leading to new treatment concepts. However, the major part of cell-based drug delivery strategies uses genetic modification to eventually achieve the release of therapeutic agents. The main concern about those approaches is the safety of the genetic devices, in particular regarding their stability inside cells [119,120]. It is highly undesired that genetic constructs are transferred to surrounding cells and alter their normal function. As seen with virus-inspired drug delivery systems, they carry the risk to induce mutagenesis of oncogenesis [121]. Moreover, the engineered therapeutic cells should not interfere with the human metabolism of other essential processes [119]. Suicide functions could be integrated in the genetic switches, which can be activated in case of emergency. Besides the effects of the introduced cells on the body, it is also necessary to protect the cell implants from the environment, especially from immunological active molecules and immune cells. The

simultaneous use of immunosuppressing drugs, as seen in organ transplantations, is unfavorable due to severe side effects associated with long-term administration [122]. A promising approach is the cell microencapsulation technology, in which engineered cells are enclosed into a semipermeable membrane capsule [123]. The semipermeable membrane is necessary to allow the diffusion of important nutrients and metabolites, involved in cellular processes but prevent influx of immune cells or antibodies [122,124]. In addition to protect the engineered cells from host influences, microencapsulation has become a promising strategy to achieve long-term drug delivery for week or months [125]. For example, encapsulated PC12 cells, engineered with tyrosine hydroxylase-dependent dopamine secretion systems, were implanted in rats and upon ligand addition increased dopamine levels were observed [126]. As mentioned above, both prosthetic networks as well as the glucose homeostasis-modulating HEK-293 cells were implanted as encapsulated cells. Several studies showed indeed successful delivery of therapeutic agents by producer cells when encapsulated in hydrogels without eliciting immune responses [7,8,89,90]. However, for clinical translation special attention should be given to critical parameters of the biomaterials, including toxicity, permeability properties and functioning of encapsulated devices after implantation. Finally, synthetic biology can also give rise to ethical debates. As in the case of several scientific fields, the introduction of synthetic biology is dual: developments have been groundbreaking for future biomedical therapies, however when the knowledge is not properly applied or reaches people with despicable intents it can turn into harmful behavior [127]. But probably the most commonly raised ethical issue is the influence of releasing synthetic entities into the environment regarding mutations, adaptation and eventually evolution [128]. These reasons should motivate researchers to design techniques to create fully predictable and controllable genetic circuits. However, this is the main challenge nowadays and particularly difficult to achieve.

5. Conclusions

Cell-based drug delivery has great potential to become implemented in the clinics for the treatment of several diseases. As discussed in the previous sections, there are numerous opportunities and dependent on the desired therapeutic effect a certain strategy or approach can be selected. Cell-based carriers found their best application in improving pharmacokinetic profiles or for generating slow delivery depots as seen with drug-loaded RBCs which are already in clinical evaluation [4,11]. Bacteria have the ability to home toward hypoxic areas and this effect could be further optimized through modification of the microorganisms [42]. However, since the discovery of their engraftment at hypoxic areas over 50 years ago [50], no therapeutic treatments have been approved for application in the clinic. Researchers have to ensure that the administered bacteria are non-pathogenic and only transfect targeted disease sites and these issues are still challenging. As for stem cell-based drug delivery systems, the same limitations regarding the migration efficiency as observed with bacteria apply [57], which might also be improved with intelligent and advanced genetic switches. Besides the function of transporting pharmaceuticals inside the body, cell-based drug delivery could also be approached by creating “cell-factories” producing therapeutic compounds for release in the circulation. Several approaches in designing genetic circuits with various triggers for inducing gene expression have been investigated [87,129,130], but it is in essence a universal principle. Dependent on the disease the gene of interest is chosen and placed downstream of a specific promoter element. Upon addition of the trigger, gene expression is induced and the therapeutic protein can be released in the bloodstream. This could lead to a significant improvement of current therapies, in which patients are dependent on injections of therapeutic proteins. In addition, by linking the output gene to different triggers, a more patient friendly therapy could be generated [89,90].

There are still limitations in cell-based drug delivery approaches, which have to be overcome before it can be applied as standard practice in clinics. However, given the short period of time, researchers from the synthetic biology field have shown proof-of-concept for a wide range of treatment strategies. It is no longer unthinkable before we can safely modify the patient's own cells and convert them into apothecary cells that produce biological drugs at will and in a highly controlled fashion. This will without doubt have a dramatic impact on the way we treat chronically ill patients and will lead us to a new era of drug delivery.

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