Journal of Controlled Release 193 (2014) 316–323

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

"Programmed packaging" for gene delivery

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ARTICLE INFO

Article history:
Received 14 February 2014
Accepted 10 April 2014
Available online 26 April 2014

Keywords:
Gene therapy
Drug delivery system
MEND
pDNA
siRNA
Active targeting

ABSTRACT

We report on the development of a multifunctional envelope-type nano device (MEND) based on our packaging concept "Programmed packaging" to control not only intracellular trafficking but also the biodistribution of encapsulated compounds such as nucleic acids/proteins/peptides. Our strategy for achieving this is based on molecular mechanisms of cell biology such as endocytosis, vesicular trafficking, *etc.* In this review, we summarize the concept of programmed packaging and discuss some of our recent successful examples of using MENDs. Systematic evolution of ligands by exponential enrichment (SELEX) was applied as a new methodology for identifying a new ligand toward cell or mitochondria. The delivery of siRNA to tumors and the tumor vasculature was achieved using pH sensitive lipid (YSK05), which was newly designed and optimized under *in vivo* conditions. The efficient delivery of pDNA to immune cells such as dendritic cells has also been developed using the KALA ligand, which can be a breakthrough technology for DNA vaccine. Finally, ss-cleavable and pH-activated lipid-like surfactant (ssPalm) which is a lipid like material with pH-activatable and SS-cleavable properties is also introduced as a proof of our concept.

1. Introduction

In the 21st century, a paradigm-shift emerged in the field of drug discovery and development and the field of nanomedicine is anticipated to have a major impact on addressing unmet medical needs such as cancer, central nervous system diseases and immunological diseases, where the currently used low molecular weight molecules do not function. Antibody based therapy is a rapidly emerging area and promises to have a significant impact as a bio-medicine, mainly because it is so selective. However, antibodies typically function in the blood circulation and extracellular space. They cannot enter into cells to treat diseases. Nanomedicines are expected to be a breakthrough technology for delivering new medicines such as nucleic acids as well as proteins to the site of action inside a cell. Therefore it is important to control not only the biodistribution of new drugs but also the intracellular trafficking of these compounds, such as endosomal escape (in the case of siRNA, protein), transport in the cytosol, nuclear entry (in the case of pDNA). To achieve this goal, sophisticated strategies will be needed to successfully develop such systems.

We have developed a multifunctional envelope-type nano device (MEND) to control intracellular trafficking as well as the biodistribution of nucleic acids/proteins/peptides based on a strategy called "Programmed packaging" where 1) a program is designed to deliver drugs based on molecular mechanisms of transport in our body, 2) nano devices are designed to overcome each barrier, 3) these materials are assembled into nano-sized 3 dimensional structures so that they can exert their ability according to the program [1]. The MEND was developed originally to deliver pDNA into target cells such as tumor cells and immune cells by mimicking an envelope-type virus and its purpose was extended not only to pDNA but also siRNA where the site of action is the cytosol. While it is difficult to achieve the efficiency of viral vectors even for the system used in *in vitro* conditions, we have been able to achieve a level that is equivalent to that of a viral vector when we transfect pDNA into immune cells such as a dendritic cells to develop a DNA vaccine. It has also been possible to develop a system which can deliver siRNA to tumor cells or tumor endothelial cells via an intravenous administration which requires stable blood circulation and efficient intracellular trafficking [2]. Recently developed breakthrough technologies in the area of tumor therapy based on active targeting using selective ligands are the focus of this review.

2. Original ligands for drug delivery system via active targeting strategies

2.1. Benefits of active targeting on drug delivery system (DDS)

Development of novel drug delivery system which can control and release of drugs are important issues on the field of medicine. A good drug delivery system can increase the efficacy of drugs and also reduce side effects. For this purpose, researchers tried to use nanoparticles as DDS and many findings were explored and nanoparticulate drug delivery

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http://dx.doi.org/10.1016/j.jconrel.2014.04.023
0168-3659/

systems were developed. In this situation, the most important discovery was the discovery of the enhanced permeability and retention (EPR) effect [3]. Tumor vasculature is leaky due to angiogenesis on tumor tissue therefore nanoparticles, with sizes of around 100 nm can pass through tumor blood vessels. Generally, the duration of nanoparticles in the circulation is increased, the accumulation of nanoparticles is also increased. This approach takes advantage of the phenomenon of a tumor in a clever manner and is referred to as passive targeting. On the other hand, active targeting involves sending nanoparticles via specific devices that can bind to the targeted receptor. Small molecules, peptides, proteins and antibodies were used as the targeting ligands and can include folate, transferin, *etc.* and have succeeded in enhancing the efficacy of DDS. Some nanoparticles using active targeting strategy have proceeded to phase I/II clinical trials and have shown positive results [4]. The active targeting strategy can increase the accumulation of drugs in the desired tissue and reduce side effects. Moreover, active targeted nanoparticles can escape multidrug resistance because efflux pumps are not able to remove nanoparticles that enter via receptor mediated endocytosis and such benefits have prompted researchers to investigate active targeting [5].

2.2. Nucleic acid aptamers and the choice of target molecules

Aptamers are molecules recently focused on in the fields of biosensors, diagnoses and ligands. Nucleic acid aptamers are small ssDNA and RNA molecules, having specific 3-dimensional structures. They can bind to the target molecule via an induced-fit mechanism and show strong affinities and high selectivity. Aptamers can be considered to be a type of antibody but aptamers have some advantages compared to antibodies such as ease of chemical modification to enhance some functions, such as conferring stability toward nucleases, relatively low immunogenicity, small size and heat stability. Under such circumstances, many researchers are now attempting to identify such types of aptamers [6]. The procedure of the systematic evolution of ligands by exponential enrichment (SELEX) was independently investigated by two groups in 1990 [7,8]. Nucleic acids having random sequences were mixed with the target molecules and the bound nucleic acids were then collected. These nucleic acids were amplified and used for the next selection. After several rounds of selection, the nucleic acids are enriched and aptamers can be isolated. The choice of the target molecule is important in identifying and isolating an acceptable aptamer. When we attempt to use aptamers as ligands, we need to choose a suitable target molecule that is specifically expressed in large amounts in the target cells. For DDS purposes, the target molecule is located on the surface of cells or organelles and, therefore, can be a membrane associated protein. Sometimes it is difficult to mimic the higher order structure of a target protein facing only the outer membrane using a recombinant protein and apply regular SELEX. In contrast, when target cells are used as the target, these problems can be overcome. Actually, cell-based SELEX was explored in 2006 by Tan's group [9]. Cell-based SELEX have already shown several benefits such as the isolation of a new ligand along with a new receptor which is guaranteed to exist on the surface of a membrane as the natural higher order structure. Therefore we attempted to isolate a ligand that is specific for tumor endothelial cells and certain types of organelles.

2.3. Selection of aptamers for tumor endothelial cells

Angiogenesis-dependent tumor growth was first reported by Folkman in 1971 [10]. Tumor blood vessels provide nutrients and oxygen, and remove waste from the tumor tissue, resulting in tumor progression and are different from their normal counterparts in that they show leakiness and the thickness of the basement membrane is uneven. This suggests that tumor endothelial cells (TECs) may well express surface markers that are different from those found in normal cells. Tumor blood vessels contain tumor endothelial cells that might be genetically normal and stable, even though these endothelial cells are structurally and functionally abnormal. Preventing or inhibiting angiogenesis, which is associated with tumor progression and metastasis, is a challenging issue in combating cancer. Since tumor growth is dependent, to some extent, on the development of a neo-vascular supply, inhibiting angiogenesis by targeting tumor endothelial cells represents the ultimate goal in cancer therapy. Therefore a ligand for targeting tumor endothelial cells would represent an ideal candidate for anti-angiogenesis therapy (Fig. 1A). First, a library for the selection was chemically synthesized via the phosphoramidite method. The resulting library had a 40mer random sequence flanked by 21mer sequences for forward and reverse primers which can be amplified by PCR to regenerate the recovered library. The sequence was (5′-CGTAGAATTCATGAGGACGTT-N40-AGCTAAGCTTACCAGTGCGAT-3′). This ssDNA library was mixed with primary cultured mouse TECs, the unbound sequences removed and the bound ssDNAs collected. Trypsin which can be used to detach cells, can be used to remove the surface proteins on TEC and this was not desirable in the case of cell-based SELEX. We introduced temperature-sensitive cell culture dishes, referred to as RepCells, in order to overcome this problem. This permits cells to be detached on cooling and the surface proteins can be kept intact and this method allows for greater recovered DNA libraries to be produced. The library was amplified by conventional PCR and the next round of selection proceeded. Amplifying the random sequence by PCR was sometimes a problem, because the random sequence functions as a primer and an unexpected sequence can be produced. It is known that too much template can cause unexpected amplification, therefore the amount of produced library in each PCR cycle must be checked. The PCR amplified library was double stranded DNA but single stranded DNA is needed for the selection and the asymmetric PCR method was employed to generate ssDNA. Asymmetric PCR (also called Linear After The Exponential PCR (LATE PCR)) was conceived by Gyllensten [11]. This method contains two sequential PCR procedures. The first PCR was regular PCR and the second PCR was carried out without a reverse primer to produce only the desired strand. Counter selection was also applied with Skin-EC as the normal endothelial cell model and OS-RC-II as the tumor parenchymal cell model. The affinity of each cycle of the library was checked in FACS experiments and the entire SELEX procedure was finished when the binding affinity of the library reached a sufficient level.

2.4. Identification of aptamers for tumor endothelial cells

After 12 rounds of selection, a significant shift in fluorescent intensity was found on FACS. This indicates that the DNA library was enriched to a sufficient level. The DNA library was cloned and sequenced to determine the DNA sequence and 48 sequences were identified as aptamer candidates. The binding affinities of these aptamers were investigated by flow cytometry and an aptamer that can bind strongly to mTEC but not Skin-EC and OS-RC-II cells was identified. We refer to this aptamer as AraHH001. Mean fluorescent intensities were measured for various concentrations of the aptamer and the dissociation constant of this aptamer was determined to be 43.8 ± 13.7 nM (Fig. 1B). The findings indicated that this aptamer had a strong binding affinity and acceptable selectivity [12].

2.5. Organelle-based SELEX for mitochondria

After mitochondria were incorporated into eukaryotic cells, eukaryotic cells became dependent on the many functions of mitochondria and a symbiotic relationship eventually developed. Mitochondria host the citric acid cycle and oxidative phosphorylation and can supply energy to eukaryotic cells. Mitochondria have their own DNA called mitochondrial DNA (mtDNA) and prepare some proteins by transcribing and translating their DNA, thus mitochondria have unique functions and machinery for sustaining life. Therefore, the mutation of mtDNA or the down-regulation of protein expression can result in a loss of mitochondrial function, thus resulting in the development of illnesses called