Roboplasmid: A remotely programmable plasmid for rapid viral inactivation

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ABSTRACT

For eons, humankind has been plagued by viral infections that have decimated populations; we lack a system that can simply inactivate any virus at will. Here we describe a model for a non-nucleotide-based, robotic plasmid that could act as a sentry and lead a swift antiviral response using CRISPR-Cas9 technology. This "Roboplasmid" would be delivered via lipid nanoparticles to various cells in the body and would contain a programmable sequence locus that can customize crRNA sequences by a novel charge-based mechanism. When a viral infection is detected, the programmable locus can be coded to induce complementarity with the viral target sequence, after which the sequences can be transcribed by host RNA polymerases and viral inactivation is possible. Creating Roboplasmids with nanoscale 3D printers, conducting *in vitro* tests, and routinely having intravascular injections of plasmids into the body can provide timely intervention that can eventually eradicate all human viruses.

Keywords: plasmid, nanomedicine, CRISPR-Cas9, programmable nucleotide

1. MOTIVATION AND APPROACH

CRISPR-Cas9 systems are bacterial immune systems that are designed to actively interfere with and cleave nucleic acid strands of invading viruses. Generally, they utilize a guide RNA (gRNA), which is composed of a trans-activating CRISPR RNA (tracrRNA) for scaffolding, and a CRISPR RNA (crRNA), which contains a spacer sequence that is complementary to a particular target sequence on an invading virus [1]. This, along with the Cas9 enzyme, binds to the gRNA and has the ability to incur double-strand breaks (DSBs) onto the strand of choice, which can inactivate the virus [2]. Apart from this, we truly only need a spacer that can be used for the crRNA, which is generally achieved by bacterial spacer acquisition [3].

The appealing nature of CRISPR-Cas9 as a gene-editing tool has attracted many to pursue its options within human beings; most of the current work is being done with animal embryos in general and proposing mechanisms that could emulate this in humans [4]. However, a key long-term goal that exists thus far is to have CRISPR as a go-to interventional response within humans [5]. The premise for this is relatively simple: upon viral infection, use a certain gene vector that contains sequences for tracrRNA, a spacer, and Cas9, along with necessary coenzymes, and deliver it to human cells, from where the systems can be transcribed, translated, and induce the desired viral cleavage.

Although the bounds are limitless for exploiting these systems to aid in battling human infections, one of the most significant limitations thus far has been optimizing it in a way that is not only relatively fast, but also effective in targeting multiple viruses without (a) having to acquire new spacers after every infection, (b) having to inject a new gene vector after every infection due to gene degradation, or (c) limiting its effects such that it is active only when we want it to–that is, only when an infection exists that mandates the gene vector's presence.

An optimal solution is the Roboplasmid–a fully automated gene delivery system that can be remotely coded using a piezoelectric ZnO nanogenerator [6]. With each plasmid encased in a lipid nanoparticle, these devices can be intravascularly injected with long time intervals between each injection to allow for a swift response against any form of a viral infection, given that its genomic sequence is known. Instead of containing nucleotides, each locus on the plasmid will have a series of electrodes connected to a carbon nanotube capacitor. If the electrode is on, then there is a charge at that point on the locus; if it is off, there is no charge. Coding for a particular sequence of ons-and-offs can allow for a specific sequence of free nucleotides to bind to the plasmid during transcription, and thus code for a specific strand of mRNA.

The step-by-step process once the plasmids have been injected would be as follows:

1. Each individual nanoparticle flows through the bloodstream, and randomly enters a nearby cell through the membrane, and then through a nuclear pore complex within the nuclear membrane, where the plasmid is able to exit the nanoparticle.

2. Upon viral infection and a positive viral test, (and given that the viral genome is known), a spacer sequence is added to the plasmid code, which is sent via wireless transmission to the ZnO nanogenerator within each plasmid. This code is a binary sequence that determines whether or not each electrode is on (and what charge it has), and thus what the nucleotide sequence is. Although most of the plasmid's sequence (i.e., the promoter, tracrRNA, Cas9 sequence, etc.) remain constant, the crRNA sequence depends on which virus has infected the body.

3. The transcribed sequences enter the cytoplasm, where ribosomes can translate the necessary sequences and cause

the formation of the CRISPR-Cas9 complex, which can cleave the viral nucleic acid sequence and inactivate it.
4. Upon a subsequent negative viral test, wireless transmission is used to turn all the electrodes on the plasmids off so it remains inactivated until another infection occurs.

2. LOGISTICAL ORGANIZATION

Here we outline three key features of this Roboplasmid that we believe can facilitate its implementation—analogousness to DNA, programmability, and sustainability—and describe its feasibility in the context of current advances in the field.

The Roboplasmid itself would act as a DNA analogue by having a nearly identical backbone to that of DNA; however, instead of having nucleobases attached to the phosphodiester backbone, there will be a carbon nanotube-based capacitor linked to multiple electrodes that can be charged a complementary amount to another nucleotide that can facilitate base pairing. As an example, if the plasmid needed to code for thymine, there must be two active electrodes, each exactly charged to induce complementarity with an adenine nucleotide.

This nanotube must have a diameter between 11 and 13 Å since this is the average width of a nucleotide–if the diameter of the nanotube was larger than this, then RNA Polymerase II (RNAP II) would likely not have a binding affinity for the plasmid. Ie et al. [7] have already demonstrated that oligothiophenes can be used for "electron hopping" and acting as nanoelectrodes; incorporating some oligothiophene moieties and having an intercalated carbon nanotube can allow for it to act as a capacitor with electrodes to fulfill our purposes.

The Cas9 gene locus and the gRNA must both be expressed by the plasmid for a functional response. It has been accepted that gRNA is a limiting factor to the efficiency of the CRISPR/Cas9 system; therefore, usage of a mono-promoter system may alleviate expression issues and will decrease the genome size of the Roboplasmid [8]. The vector design for our Roboplasmid will represent one designed already [9]. Modifications would include the removal of BsmBI restriction sites (for gene insertion) as well as the AmpR gene, which is unnecessary because of no selection.

In the event that a person becomes infected and tests positive for a certain virus, the goal would be to (1) determine a target locus that should be cleaved to inactivate the virus, and (2) select a short crRNA sequence within this locus that is roughly 20 nucleotides upstream of a PAM site for the Cas9 enzyme, to aid in specificity. A target locus should be chosen such that it is conserved and does not often change due to mutations; additionally, cleaving it should result in loss of vital viral activity (such as sequences encoding capsid or outer viral glycoproteins needed for entry), and thus stop the infection in its track. Since most viruses that commonly cause infection have been sequenced, we can determine a target sequence to encode the crRNA relatively easily. To actually translate this into real sequences, we must use a signaling mechanism to relay information to each Roboplasmid, hence the presence of piezoelectric ZnO nanogenerators to accept wireless signals [6].

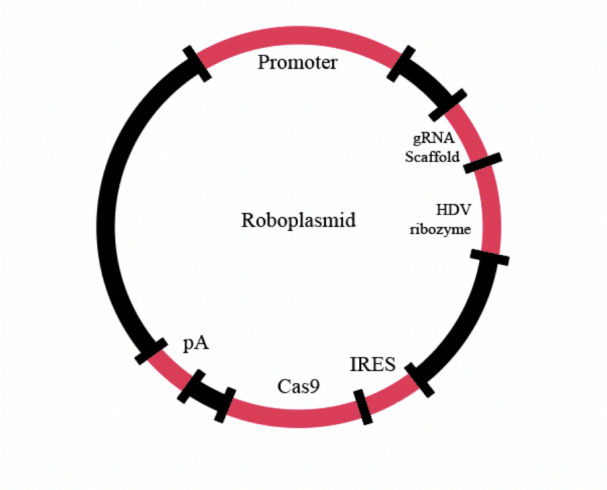

Figure 1: Plasmid sequence. The plasmid includes the sequences for the promoter, gRNA scaffold (tracrRNA and crRNA), HDV ribozyme, IRES, Cas9, and pA. The crRNA will be encoded by wireless transmission to the ZnO nano generator, which is located at the center of the plasmid and attached to the backbone via a conductive silicon "tube."

To code for a particular nucleotide sequence, a two-stage binary protocol will be used: since A-T base pairs have two hydrogen bonds but C-G pairs have three, the first binary sequence will indicate whether two or three electrodes are active for a certain base pair locus, with 1 indicating three active electrodes, and 2 indicating two. Then, the second binary sequence would use an if-then protocol to indicate which nucleotide is actually being coded for: in sites that had a value of "1" for the first sequence, three "1" values would execute a code that already incorporated the partial charges for guanine, while three "0" values would cause the partial charges for cytosine to be executed onto the electrode. Similarly, in sites that had a value of "0" for the first sequence, two "1" values would execute a program charging the electrodes to have partial charges matching those of adenine, and two "0" values would cause two electrodes to be charged like thymine. Translating this binary protocol and then sending a wireless signal to the nanogenerator could provide a simple way of transmitting codes for plasmid sequences [6].

3. RISK ANALYSIS

Now we describe some issues that may be associated with Roboplasmid implementation and some counterplans designed to prevent them. Namely, these risks are (1) how human RNAP II can maintain binding specificity and accuracy when transcribing the Roboplasmid sequences, (2) dosage size and distribution of Roboplasmids throughout

the body, and (3) what happens to the plasmids after a host cell undergoes apoptosis.

Transcription initiation through an RNAP II-dependent method requires multiple factors such as those of the TATA-box binding protein (TBP), transcription factors, and GTFs among other necessary factors naturally available within the nucleus [9]. Some factors require dsDNA so the promoter region will be double-stranded [10]. The TBP affinity (and other proteins) may rely on chromatin structure [11], but the relative flexibility of the Roboplasmid will likely subsidize any steric issues. The promoter needs not to be programmed specifically, so a consensus sequence can be used. Concerning RNAP II, the cleft is about 25 Å wide [12], which leaves a conservative estimate of 11-12 Å left for the width of the nanotube capacitor. The single-stranded template strand of the Roboplasmid allows for relative ease for backbone structural conformation changes to occur during initiation.

Solid lipid nanoparticles containing the Roboplasmid will be administered intravenously similar to modern gene therapy methods [13]. Biodistribution is a major factor to consider to maximize effectiveness [14], and accumulation within the liver and spleen is common [15]. Physicochemical properties of nanoparticles such as size, shape, surface charge, surface chemistry will have to be taken into account for future design as they influence biodistribution [16]. Similar applications use lipid nanoparticle concentration at about 2.0 x 1011 particles/mL [17]; however, this varies, so the most effective dosage should be considered for the future.

Given that up to 10 billion human cells undergo apoptosis every day [18], it is a reasonable concern that many of the plasmids delivered may render themselves useless within a very short period of time once their host cells die. To circumvent this, we propose that each plasmid store 2-3 lipid nanoparticle "templates" in addition to a mechanically connected charge indicator; if a cell undergoes apoptosis, then the charge the indicator is exposed to will change, inducing a conformational change that can cause a release of one of the nanoparticle templates. This template can then encase the plasmid, which will then be able to enter a nearby cell's nuclear membrane and remain there for further activities. Once all of the nanoparticle templates run out, the bare Roboplasmid, which has already been described as mimicking actual DNA structure to a certain extent, can be degraded by Caspase-activated DNase, which normally degrades an apoptotic cell's DNA. Thus, we can prolong the lifetime of a Roboplasmid.

4. CONCLUSIONS

If this model is successfully implemented, it is possible that there may be a massive paradigm shift in the way viral outbreaks and individual infections are handled. If this technology had been available to us in the past, it is quite possible that some of the dire impacts of the COVID-19 pandemic itself could have been prevented or at the very least significantly lessened immediately after the viral genome was sequenced. We go forth with this project with the high hopes that we and future generations may be able to grow up in a world that lacks any of the catastrophic losses and tragedies that have come in 2020-2021, or deaths due to viral infections of any kind.

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