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Hraber, Peter Thomas
Bradfute, Steven
Clarke, Elizabeth
Ye, Chunyan
Pitard, Bruno

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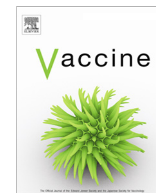

# Amphiphilic block copolymer delivery of a DNA vaccine against Zika virus

Peter Hraber [a,*,2], Steven Bradfute [b,2], Elizabeth Clarke [b], Chunyan Ye [b], Bruno Pitard [c,1]

[a] Theoretical Biology and Biophysics, MS K710, Los Alamos National Laboratory, Los Alamos, NM 87545, USA
[b] Center for Global Health, MSC10 5550, Division of Infectious Diseases, Department of Internal Medicine, University of New Mexico, Albuquerque, NM 87131, USA
[c] In-Cell-Art, 21 rue La Noue Bras de Fer, Nantes F-44200, France

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A B S T R A C T

Zika virus (ZIKV) is a mosquito-borne flavivirus that was first discovered in 1947. Since then, outbreaks have been reported in tropical Africa, Southeast Asia, the Pacific Islands, and, in 2015, in the Americas. Since 2013, many countries have reported cases of microcephaly and other central nervous system malformation associated with ZIKV. Because the initial target population for a ZIKV vaccine is expected to be women of child-bearing age, including those who may be pregnant, it is necessary to develop safe, easily administered, and non-viral vaccines. Here, we show that a single tetrafunctional Amphiphilic Block Copolymer (ABC) delivers DNA that encodes the full natural sequence of prM-E, among other antigen designs tested, induces the highest antibody titer and neutralization activity against three divergent ZIKV isolates. Vaccination with a single tetrafunctional block copolymer delivering low dose (10 μg) DNA plasmid rapidly induces protection from detectable viremia during acute infection in mice challenged by ZIKV more than 7 months after their first vaccination and boosted 2 weeks before challenge. This use of tetrafunctional ABCs is a new approach to deliver DNA antigens against flaviviruses. The data demonstrate that DNA formulated by a tetrafunctional block copolymer rapidly elicits protective responses against multiple diverse ZIKV isolates. This represents potential for an easy-to-administer and simple to manufacture vaccine candidate against ZIKV and possibly other emerging threats to global health.

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## 1. Introduction

Flaviviruses are diverse, numerous, and serious causes of concern for public health. Currently, high incidence of Asian-clade Zika virus (ZIKV) globally illustrates that vaccines to confer immunity against flaviviruses are a high priority [1]. West Nile virus (WNV) is another recent example of rapid flavivirus expansion into naive host populations [2–4]. Dengue virus (DENV) afflicts between 50 and 100 million people and causes roughly 500,000 hospitalizations each year [5,6]. DENV occurs in tropical regions populated by a total of about 2.5 billion people and places a tremendous economic burden on global health [6]. Infection with ZIKV is largely asymptomatic and rapidly cleared by host immune responses. However, its sequelae (fetal microcephaly and Guillain Barré syndrome) motivated the WHO to declare a public-health emergency in February 2016 [7–10]. ZIKV shares genomic and structural features with other flaviviruses [11–13], which include DENV, WNV, and St. Louis Encephalitis viruses. Vaccines against these are greatly needed [14,15]. However, vaccine candidates against ZIKV have very recently been demonstrated effective in mice [16,17] and macaques [18,19], with a Phase I clinical trial conducted in humans [20], and several tetravalent DENV vaccines are now being evaluated in clinical trials, with one being registered for use in certain age groups in endemic areas [14,21].

Among the different vaccine candidates against ZIKV under development, nucleic-acid vaccine immunogens are the most highly investigated. Two different mRNA vaccines encoding engineered versions of the pre-membrane and envelope (prM-E) were reported, both using non-natural nucleoside-modified mRNA molecules formulated with the same lipid nanoparticles [22,23], which were used originally to deliver siRNA but shown to induce some toxicity in human clinical trials. Formulations with

* Corresponding author.
*E-mail address:* pth@lanl.gov (P. Hraber).
[1] Present address: CRCINA, INSERM, Université d'Angers, Université de Nantes, Nantes, France.
[2] Authors contributed equally to the work.

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nucleoside-modified mRNA were prepared following a complex, small-scale and long process involving the mixture of four different lipids (cationic lipid, phosphatidylcholine, cholesterol, PEG-lipid) at precise molar ratios dissolved in ethanol, then associated with mRNA in aqueous solution by a microfluidic mixer, followed by dialysis, diafiltration to concentrate particles, and filtration to ensure sterilization. DNA vaccines reported have also used an engineered version of prM-E antigen fused with exogenous Japanese Encephalitis virus leader sequence, which were delivered either naked [16] or by such physical methods as applying electrical pulses (electroporation) after injecting several milligrams of DNA in humans [24] or DNA injection under pressure with a needle-free device [19]. Even with efficacy in mice and non-human primates to induce neutralizing antibodies tested against only one ZIKV isolate and protection against viral challenge, translation of those mRNA and DNA-based vaccine candidates for safe, effective, easily prepared and administered human vaccination against ZIKV remains to be demonstrated.

Therefore, there is a need to improve the way nucleic-acid vaccines are prepared and delivered, in order to develop simple, easily administered (without advanced delivery device technology), and effective vaccines containing low doses of nucleic-acid molecules that encode the viral antigen. With this goal in mind, we investigated preparation of a much simpler DNA vaccine using a full prM-E sequence from the virus encoded by natural DNA delivered by a simple, non-toxic delivery system consisting of a single molecule, and tested it against three different ZIKV isolates.

To this end, we investigated plasmid DNA-encoding ZIKV antigen formulated with tetrafunctional ABC, made up of four amphiphilic diblocks centered on an ethylenediamine moiety. In preclinical models, ABC formulation has demonstrated a dramatic enhancement of target protein production. It works by an intracellular delivery mechanism that maximizes access of the vaccine to activate antiviral innate immunity pathways in the cell. Ability of ABCs to deliver DNA into cells and subsequent expression have been shown consistently greater than "naked" plasmid DNA over a wide range of tissue/cell types (e.g. muscle, lung, heart) and genes [25–30]. For example, ABC-formulated DNA transgene expression has been demonstrated in an animal model of hepatocellular carcinoma, observing elimination of tumors expressing the target protein [31], in a mouse model of allergic asthma by using plasmid DNA encoding Derf1 [30], and against the Cystic Fibrosis pathogen *Mycobacterium abscessus* using plasmid DNA encoding phospholipase C [32] or Mgtc [33]. Success in this earlier work led us to explore, for the first time, the use of ABCs to deliver flavivirus antigens for DNA vaccination against an emerging viral threat to global health.

The potential of this tetrafunctional ABC/DNA vaccine prompt us to explore the immunogenicity and functionality of antibodies against ZIKV proteins. Flavivirus genomes encode one polypeptide, comprised of three structural proteins (capsid, membrane, and envelope, or E) and seven non-structural proteins (designated NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) [13,17]. Because it coats the virion and enables cell fusion, the E protein is a primary target for neutralizing antibodies. We investigated the use of candidate DNA vaccine formulations with a single tetrafunctional ABC and plasmid that encodes E, together with additional regions of the viral genome necessary for localization to the cell membrane and cell-surface antigen presentation, namely the pre-membrane (prM) and membrane protein, which are cleaved by furin and host protease. Our results show high immunogenicity cross reacting against divergent ZIKV isolates including Asian PRVABC59, West African IbH 30656 and East African MR766, and protection in mice vaccinated with DNA encoding full natural sequence of prM-E delivered by a single tetrafunctional ABC.

## 2. Materials and methods

### 2.1. Plasmid

The various cDNA sequences designed from Asian-clade variant (KU321639) isolated on March 2015 in Brazil) were codon-optimized for mammalian expression, synthesized (Thermo-Fisher Scientific), and cloned through 5′ HindIII/3′ XhoI into pVAX1 plasmids (Thermo-Fisher) under the control of a CMV promoter and containing a Kozak sequence [34]. All plasmid DNA was purified using EndoFree plasmid purification columns (Qiagen).

### 2.2. Formulation

We formulated ZIKV ABC-DNA vaccine by mixing equal volumes of ABC stock solution in water and plasmid DNA solution at the desired concentration in 2× buffer solution, immediately prior to intramuscular injection. The mixing of ABC and plasmid DNA is a self-assembly process that results from hydrogen bonding, hydrophobic, and electrostatic interactions between ABC and DNA. Physicochemical properties of tetrafunctional amphiphilic block copolymer/DNA formulation have been described previously, in a study that showed the formation of small, negatively charged particles about 50 nm in diameter, and lacking any particular inner structure [26].

### 2.3. Mouse vaccination

Animal experiments were performed according to institutional and national ethical guidelines. Plasmids were formulated with ABC (In-Cell-Art, Nantes, France) immediately before vaccination. Groups of 5 six-week old female C57BL/6C mice were vaccinated with a prime and boosts at 24 and 42 days after the prime, with a final boost on day 199. Each group was administered one of the candidate constructs C1–C4. Intra-muscular of tetrafunctional ABC/DNA formulations were injected bilaterally into both tibial anterior muscles using an 8-mm, 30-gauge syringe. Mice were anesthetized by isoflurane before injection of ABC/DNA solution at 1 mg/ml. A group of five unvaccinated mice, housed alongside the treated mice, were used as controls. Samples of 50 μL serum were taken before each boost, while anesthetized. Serum prepared after centrifugation was stored at −80 °C until assayed.

### 2.4. Mouse challenge

The second set of experiments was conducted using different doses of construct C1 to vaccinate mice and analyze long-term antibody responses and protection from ZIKV challenge. Two weeks after final vaccination, mice were infected intravenously with 10,000 plaque-forming units of Asian ZIKV PRVABC59. Mice were injected intraperitoneally with 1 mg of anti-IFNAR antibody (Leica Biosystems) on days −1 and + 1, and were bled via the submandibular route on days 2, 3, and 5.

### 2.5. ELISA and neutralization assays

For measurement of anti-ZIKV envelope IgG antibody titers in serum of vaccinated mice, a commercial kit was used according to the manufacturer's instructions (Alpha Diagnostics). For plaque reduction neutralization titer (PRNT) 80 assays, Vero-E6 cells were seeded onto a 6-well plate and incubated at 37 °C for 12–24 h to 90% confluency. Two-fold serial dilutions of serum, beginning at 1:40, were mixed with equal volumes of ZIKV (Asian isolate PRVABC59, West African isolate IbH 30656, or East African isolate MR766) for 1 h at 37C, then added to cells for 2 h at 37 °C. Virus/

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serum mix was then aspirated, and the cells were washed with PBS and overlayed with 1% agarose in media with 2.5% fetal calf serum and antibiotics. Cells were incubated at 37 °C for 5 days. The cells were fixed with 4% formaldehyde for at least 2 h, then fixative was aspirated and the agarose overlay gently removed. The cell monolayer was gently washed, and 0.5% Crystal Violet was added to wells for 1–2 min, the wells dried, and plaques counted.

### 2.6. Measure of viral replication in mice

For assessment of viral replication in infected mice, viral RNA was extracted from mouse serum using the Purelink Viral RNA/DNA Mini kit (Invitrogen) in a volume of 50 μL and stored at -80 °C before use. Synthesis of cDNA was achieved using the iScript cDNA synthesis kit (BioRad). Viral cDNA was quantitated using the SYBR green system. MicroAmp EnduraPlate Optical 96-well Fast Clear reaction plates (Applied Biosystems) were used for Q-PCR, with StepOnePlus thermocycler RT-PCR machine (Applied Biosystems). Cycle settings were: denature at 95 °C (15 sec) and Anneal/Extend at 60 °C (1 min). Quantity of cDNA was assessed by Ct values of samples. Primers used bind to the prM-E region of PRVABC59, forward: 5′–TGAGGCATCAATATCAGACATG–3′ and reverse: 5′–GTTCTTTTGCAGACATATTGAGTG–3′.

### 2.7. Statistical inference

We used one-sided Fisher's exact tests in R (version 3.5.1) to test for significant differences in protective effects of vaccination between animal groups.

## 3. Results

To evaluate the feasibility of ABC delivery of antigen DNA to induce immune responses, we designed four ZIKV cDNA inserts and formulated them for vaccine delivery. Fig. 1 illustrates four candidate constructs made from genome features of an Asian-clade variant (KU321639, isolated March 2015 in Brazil). Three candidates (C1-C3) are excerpts from the polyprotein that encode immunogenic and accessory proteins. Expressing them in a DNA delivery vector is hypothesized to enable synthesis and presentation of envelope (E) in a relatively natural conformation as dimers, facilitated by pre-membrane (prM) and membrane (M) accessory proteins. ZIKV Envelope structure closely resembles Dengue and other flavivirus Envelopes [35,36]. As described in Poggianella et al. [37], a DENV E protein domain III-based DNA immunization induces strong antibody responses to all four DENV serotypes. We designed a ZIKV Envelope domain III construct resembling the DENV DIII antigen, with secretion signal and IgG Fc dimerization domain CH3, as a fourth construct (C4). Fig. 1 depicts the candidate antigens among relevant features near the N-terminus of the ZIKV polyprotein, which encodes structural proteins.

We tested sera for ZIKV-specific antibodies with commercially manufactured mouse IgG ZIKV MR766 (East African isolate) Envelope-specific ELISA kits. Results are expressed as anti-Envelope specific antibodies in units based on an internal assay standard. Results (Fig. 2) show moderate IgG responses in the C1 group after the priming vaccination (day 24, left), which increase with subsequent boosts (days 42 and 56). Results also show homogenous immune responses over time among mice vaccinated with the C1 construct. Weaker responses emerge in the C4 group, sporadic responses in C2, and none in C3. These findings suggested that including prM with the Envelope facilitates antigen expression and increases immunogenicity over constructs that lack it. The C1 construct, which corresponds to plasmid DNA encoding premembrane and envelope protein (prM-E), was selected for the further characterization of the efficiency of ABC/prM-E ZIKV vaccine.

We tested for serum neutralization activity by plaque-reduction neutralization titer (PRNT) assays, using three ZIKV isolates. Plaque reduction neutralization testing was conducted using standard methods. ZIKV MR766 (East African clade) [38], IbH 30656 (West African clade) [39], or PRVABC59 (Asian clade, from Puerto Rico

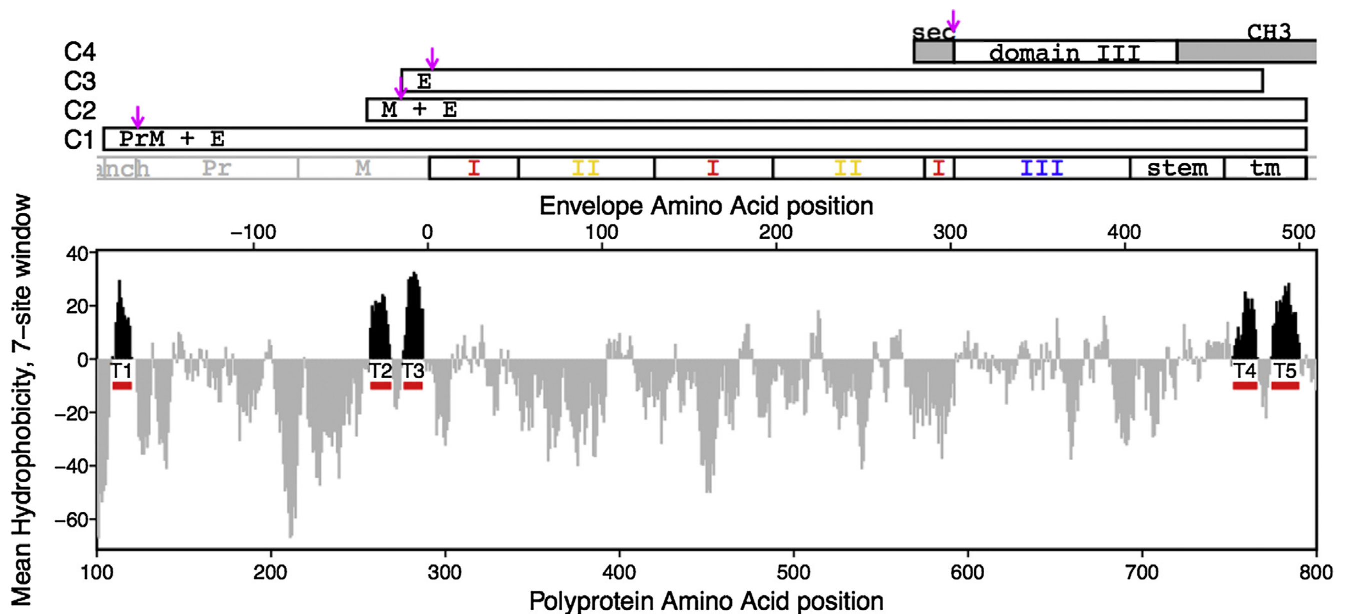


**Fig. 1.** Design of four candidate cDNA constructs for ZIKV immunogenicity testing. Genetic map from an Asian-clade variant (KU321639 isolated March on 2015 in Brazil) of ZIKV N-terminal polyprotein shows hydrophobic transmembrane domains (T1-T5, red bands), host-restricted signal peptide cleavage sites (arrows), and locations of corresponding candidate DNA antigen constructs (C1–C4). C1–C3 are intended to be expressed as native-like structural protein on host cells. C4 is engineered with an immunoglobulin heavy-chain dimerization domain (CH3) to present dimeric Envelope domain-III protein. N-term Capsid protein and the 5′ UTR are not shown. Other abbreviations: pr, precursor; M, membrane; E, Envelope; anch, anchor peptide; tm, transmembrane domain; sec, secretory domain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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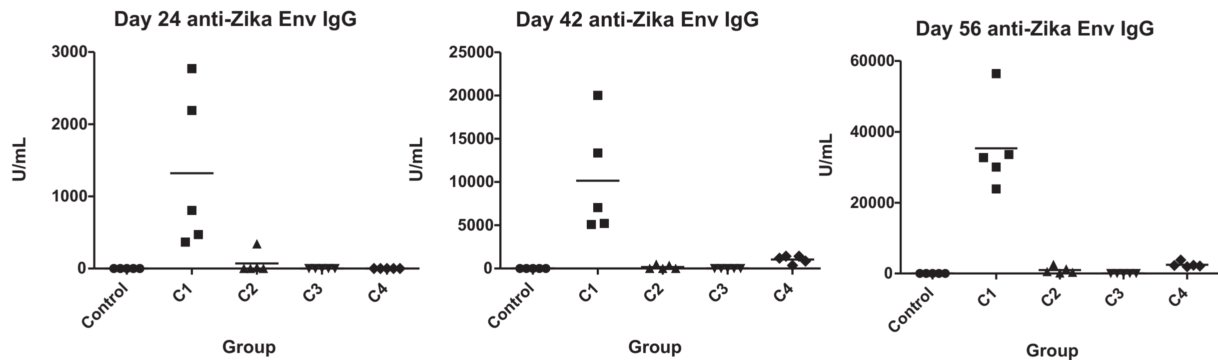


**Fig. 2.** ZIKV envelope DNA/Amphiphilic Block Copolymer vaccine testing in mice. C57Bl6 mice (n = 5 per group) were immunized by the i.m. route at day 0, 24 and 42 with 50 μg plasmid DNA encoding the various antigens presented in Fig. 1, formulated with ABC. Serum samples were drawn before administration of boost doses. Uninjected mice were also included and used as control (n = 5 per group). Humoral IgG immune response was assessed at days 24, 42 and 56 after the initial, priming vaccination by ENV-specific ELISA. Horizontal bars reflect mean of anti ENV antibody concentration expressed as U/ml.

in 2015) [40] was mixed with sera from vaccinated or control mice and incubated with Vero E6 cells. After incubation, cells were fixed and stained to visualize plaques. PRNT80 was determined by the lowest dilution of serum that reduced plaque number by 80%. Overall, results (Fig. 3) show that ZIKV neutralization by serum of mice immunized by ABC/prM-E ZIKV vaccine increased between days 42 and 56, which suggests that the boost at day 42 led to an increased amount of functional antibodies cross-neutralizing all three ZIKV isolates tested.

To test the durability of antibody neutralization responses against all three divergent ZIKV isolates, we monitored titers of ABC/prM-E-vaccinated mice longitudinally over several months, using the same groups of animals depicted in Fig. 3. As shown in Fig. 4, neutralization titers rapidly dropped by day 85 and remained low through day 139, but remained higher than the background level.

Based on these data, we performed additional experiments with this prM-E DNA. Mice were vaccinated with 5, 10, or 50 μg of prM-E DNA formulated with ABC, on days 0, 24, and 42, and sera displayed neutralizing activity against all three divergent ZIKV isolates over time (Supplemental Fig. 1). However, both neutralization and ELISA titers were greatly diminished by day 199. To address this, mice were boosted again, and neutralization titers increased two weeks later (Fig. 5).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.10.022.

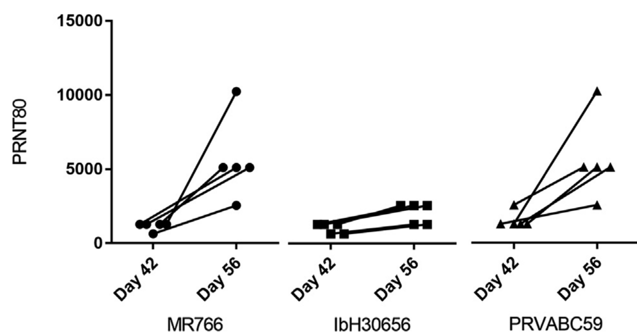


**Fig. 3.** Neutralizing antibody responses of ABC/prM-E DNA vaccine. Serum of mice immunized with ABC/prM-E DNA were collected at day 42 and 56 after vaccination at day 0, 24 and 42, and analyzed for neutralization of ZIKV by plaque reduction neutralization 80 titers (PRNT80) assay. Each point represents an individual animal in the C1 group (Fig. 2), and a line connects paired observations from the same animal at different timepoints. PRNT80 assay was performed using three different ZIKV isolates, MR766, IbH30656, and PRVABC59.

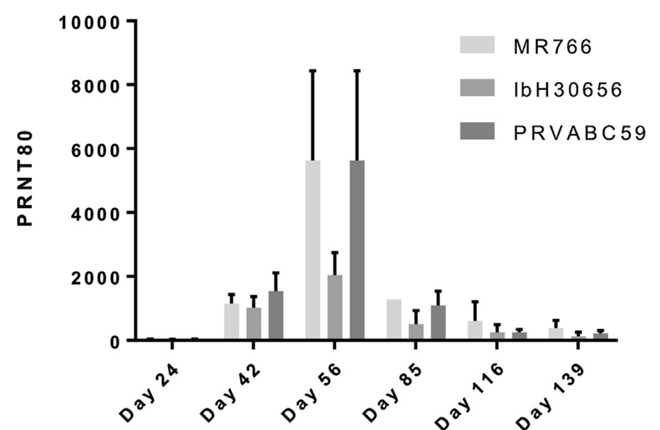


**Fig. 4.** Immunization of ABC/prM-E DNA vaccine provides longevity of neutralizing antibody response against various ZIKV isolates. Sera (from the same animals shown in Fig. 3) of mice immunized with ABC/prM-E DNA were collected at different time points up to day 139 after the priming vaccination. Neutralization of three different ZIKV isolates including MR766, IbH30656, and PRVABC59 was assayed by PRNT80. Each bar represents the mean and standard deviation of a group of 5 animals, sampled longitudinally.

To test efficacy of the vaccine, mice were challenged with live virus. After injection of an anti-IFNAR blocking antibody, mice were infected with ZIKV PRVABC59 (Asian isolate). Mice vaccinated with 10 or 50 μg of prM-E DNA formulated with ABC were protected from ZIKV viremia (one-sided Fisher's exact $p = 0.003663$), whereas mice vaccinated with 5 μg of ABC/prM-E had transient viremia at day 2 that was eliminated by day 3 (one-sided Fisher's exact $p = 0.02381$). Control unvaccinated mice were not protected (Fig. 6).

## 4. Discussion

Prevention of sequelae from ZIKV infection, including microcephaly and Guillain Barré syndrome, is a public-health priority. Specific immunotherapy based on DNA vaccination seems to be a promising approach. High levels of binding and neutralizing antibodies and T cell responses have been reported among 100% of participants in a phase-1 clinical trial of an electroporated DNA ZIKV vaccine [20]. However, since the initial target population for a ZIKV vaccine is expected to be women of child-bearing age, it would be preferable to develop a low-dose DNA vaccine that is simple, easily administered by conventional injection methods, and deliverable without apprehension from patients.

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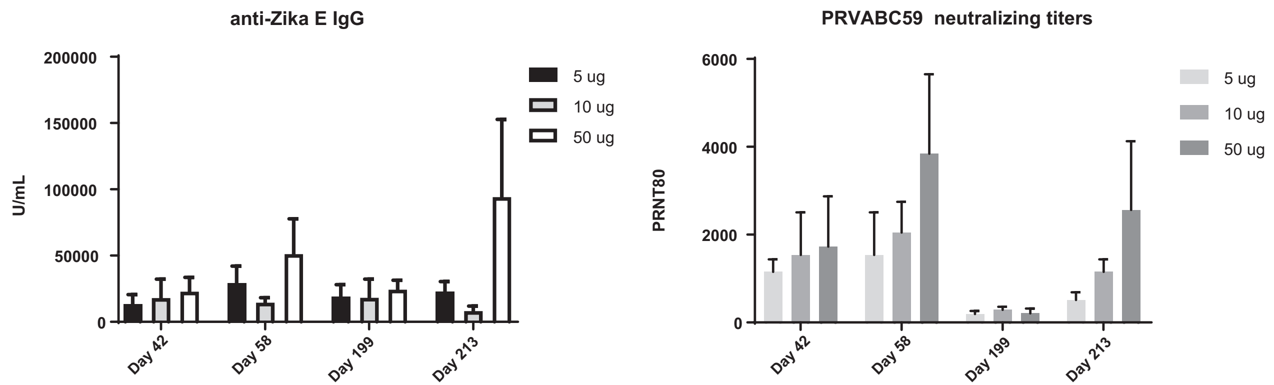


**Fig. 5.** Booster vaccinations increase anti-ENV antibody and anti-PRVABC59 neutralization titers. C57Bl6 mice were immunized by the i.m. route at day 0, 21, 42 and 199 with doses of prM-E DNA ranging from 5 to 50 μg formulated with ABC. Sera were collected at different time points up to 213 days after the first vaccination. Humoral response (left) was assayed by ENV-specific ELISA and expressed as anti ENV antibody concentration in U/ml. Neutralizing activity (right) of ZIKA PRVABC59 was assayed by PRNT80. PRNT80 titers for other ZIKV isolates are shown in Supplemental Fig. 1. Each bar represents the mean and standard deviation in 3 dosage groups (5 animals per group), sampled longitudinally.

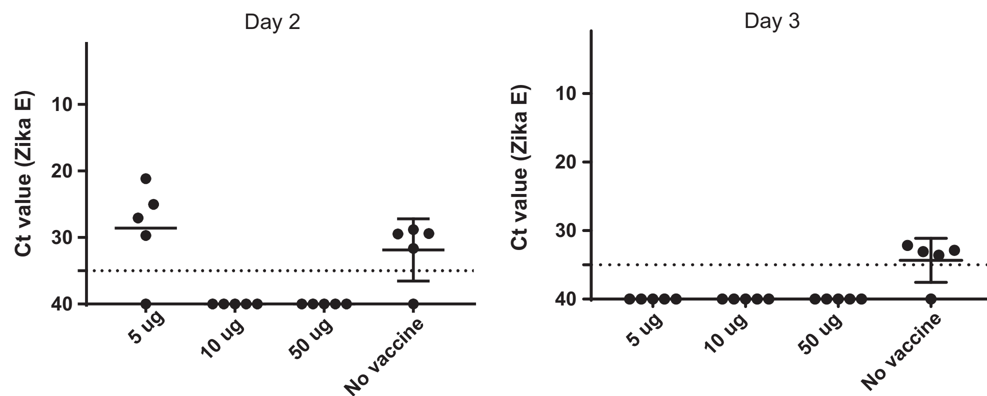


**Fig. 6.** Protection of vaccinated mice against ZIKV infection. Mice in Fig. 5 were challenged i.v. with 10,000 PFU PRVABC59. On days 2 and 3, sera were obtained and tested for viremia by qRT-PCR for ZIKV E RNA. Unvaccinated mice were included in the experiment and were used as control. Each point represents individual animals and horizontal bar reflects mean of viral loads expressed as Ct value of ZIKV Envelope RNA. Limit of detection is Ct value ≥35. Any value ≥Ct 35 is listed as 40. Error bars represent standard deviation for each group of 5 animals.

We studied a synthetic delivery system, based on a single molecule of tetrafunctional ABC, and formulated by self-assembly with low-dose plasmid DNA encoding pre-membrane and envelope protein from an Asian-clade variant of ZIKV. Such an approach to vaccine formulation against flaviviruses has not been reported previously. The block copolymer acts as a delivery vehicle. A recent regulatory-enabling Good Laboratory Practice safety evaluation indicates that the ABC does not act as adjuvant, because no inflammation in injected muscles could be detected (unpublished results). Those observations were confirmed by another experiment, which showed that only muscles injected with both DNA and ABC produced inflammatory cytokines, and not with DNA or ABC alone (unpublished results). ABC delivery of DNA appears to activate cellular molecular sensors responsible for cytokine and interferon synthesis.

We found the induction of significant antibody titers against ZIKV Envelope protein and evidence for antibody neutralization against at least three different ZIKV isolates, chosen to represent West African, East African, and Asian clades [40,41]. Our findings agree with a recent study, which showed the East African and Asian ZIKV are one serotype, yielding similar neutralization assay outcomes from human or mouse immune sera across strains [42]. In our experiments, we also included a West African isolate (IbH 30656), which in some assays seems to have lower sensitivity to neutralizing antibodies than East African or Asian isolates, though results from the IbH 30656 isolate have not been reported in other ZIKV vaccine protection studies [16,19,23,43]. Results also showed that antibody titers decreased over time, but were successfully boosted by additional vaccination, which is highly relevant for vaccination in humans.

Anti-ZIKV E IgG titers from the 5- and 10-μg dose groups were similar, though the 5-μg dose group showed incomplete protection against virus challenge at 2 days post-infection. Neutralization titers were lower at 5 μg than for the 10-μg dose, which may affect efficacy. It is also possible that non-neutralizing functional antibodies (such as those that induce ADCC) may be involved in protection, and T cells could contribute a role.

This novel synthetic delivery system allowed rapid prototyping and assessment of vaccination efficiency among different ZIKV antigens designed by bioinformatics from ZIKV genome sequence analysis, and showed that the different antigen designs have disparate effects on immunogenicity. Together, the genome region that begins after the Capsid protein, which is cleaved by viral serine protease, and includes the full-length E protein, constitutes an immunogenic construct that can be expressed on host cell surfaces without dependence on viral factors.

Current understanding of flavivirus structural biology indicates the prM-E complex is necessary for native conformation of E on the mature, smooth virion [13,35,44,45]. We included the precursor with the intention to foster expression of native-like dimeric E pro-

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tein complexes. Published reports using a similar immunogen DNA construct, either naked [16] or delivered by electroporation [19], which appeared after we had begun this pilot study, suggested that including prM with the Envelope facilitates antigen expression and increases immunogenicity over constructs that lack it. Larocca et al. [16] showed protection during peak immunogenicity (week 4) in mice immunized with a single dose of a DNA vaccine expressing the prM-E antigen. Larocca et al. [16] emphasized the importance of the prM-E sequence to induce binding and neutralizing antibodies against envelope protein. They did not evaluate M-E in the absence of the precursor (pr), an approach that parallels our own.

Our results show high immunogenicity and neutralization activity in sera from animals immunized with a plasmid DNA encoding the full, naturally occurring sequence of ZIKV premembrane and envelope proteins delivered by ABC. Use of this natural prM-E sequence for cross-neutralization against at least three different ZIKV isolates could be of interest to avoid undesired cross-reactivity with other proteins, which could lead to adverse safety events, though this remains to be validated.

It is always difficult to compare precisely with other reported studies the neutralizing antibody titer obtained against ZIKV with other nucleic acids-based vaccine approaches. In the present study, we report a PRNT80 titer at day 56 of about 1:6000, which corresponds to a 3.8 $\log_{10}$ reduction. With comparable immunization schemes in mice, mRNA-encoding prM-E complexed through a formulation process with liposomes made with at least four different lipids, led to a PRNT50 of 2.8 logs [22] and about 1:1000 [23]. For plasmid DNA delivered by electric pulses, a PRNT50 of 2.2 logs was described [18]. Thus, the prM-E sequence designed in the present study from fully natural occurring ZIKV genome formulated with a simple, self-assembling mechanism led to more potent responses than were obtained with mRNA or plasmid DNA-based vaccines, because differences would be even greater than shown had results presented in the current study been expressed as PRNT50. Indeed, greater serum dilution and lower titer is needed to obtain 50%, rather than 80%, plaque reduction.

Building on the foundation established by Larocca et al. [16], Abbink et al. [43] recently showed the lack of protection durability, both in mice and monkeys, immunized with DNA vaccine. Interestingly, Abbink et al. [43] also showed that the prM-E DNA vaccine was less protective than a similarly constructed M-E DNA vaccine, which deleted the precursor region, and tested a different M-E construct than we did. We did not study use of complete M region (pr-deleted, Fig. 1), while Abbink et al. [43] used the 75 amino-acid M protein, which starts at a furin cleavage site. It remains unclear why Abbink et al. [43] obtained greater potency and durability outcomes without the precursor protein. Possibly better antigen presentation is attained by independence from host furin cleavage. It is also possible that the untethered M-E polypeptide chain is more accessible to antibodies than one anchored at both ends, i.e. prM-E, at least until it is restricted by a host protease. We hope future structural studies will compare the resulting Envelope assemblies, and evaluate the antibody specificities that result from DNA vaccination both with and without the precursor protein.

Abbink et al. [43] also found the DNA vaccine formulations did not perform as well as purified, inactivated virus or a replicating, adenovirus vector-based vaccine [43]. This suggests more research is still needed before the potential of DNA vaccination will be fully realized.

Our results demonstrate that assembly of ABC with DNA prM-E led to protection against viremia after Asian clade ZIKV challenge in a mouse model, as was observed with the DNA vaccine delivered by electric pulses currently under human clinical trials [46]. Electroporation of ZIKV DNA-based vaccine has already been shown as preclinical proof-of-principle, but remains to be tested in humans and to report positive outcomes in clinical trials. Further development of the DNA vaccine approach presented here may be justified. It does not require an electroporation device for delivery. Subsequent efforts to increase durability, and consider indications for seasonal administration or for use by travelers into endemic zones, could increase the commercial viability of this candidate vaccine. Delivery of antigen plasmid DNA with this synthetic polymer is an important step toward development of a pharmaceutically acceptable, synthetic vaccine formulation, because it can be produced at scale under Good Manufacturing Practices and has shown excellent safety profile, with no safety concerns in a recent regulatory-enabling Good Laboratory Practices safety evaluation of the synthetic polymer, both alone and formulated with the highest DNA dose intended in future human clinical trials (unpublished results).

These findings open new avenues to develop DNA vaccination strategies with polyvalent DNA, which encodes different antigen variants, to promote broader cross-reactive immune stimulation against highly variable pathogens [47]. The strategy of combining multivalent or highly polyvalent immunogens formulated with ABC delivery is hypothesized to induce potent cross-reactive breadth and protective immunity against multiple flaviviruses, without the adverse outcomes expected from incomplete neutralization and antibody-dependent enhancement.

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## Conflict of Interest

B.P. owns stock in In-Cell-Art, which commercializes ABC, and is an inventor on several patents and patent applications related to use of block copolymers for gene delivery. P.H. is a co-inventor on patent applications for polyvalent immunogens against HIV-1.

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