Supporting Information

Non-Viral Vaccination Through Cationic Guanidium Polymer-pDNA Polyplex Mediated Gene Transfer

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1) Synthesis of polymers

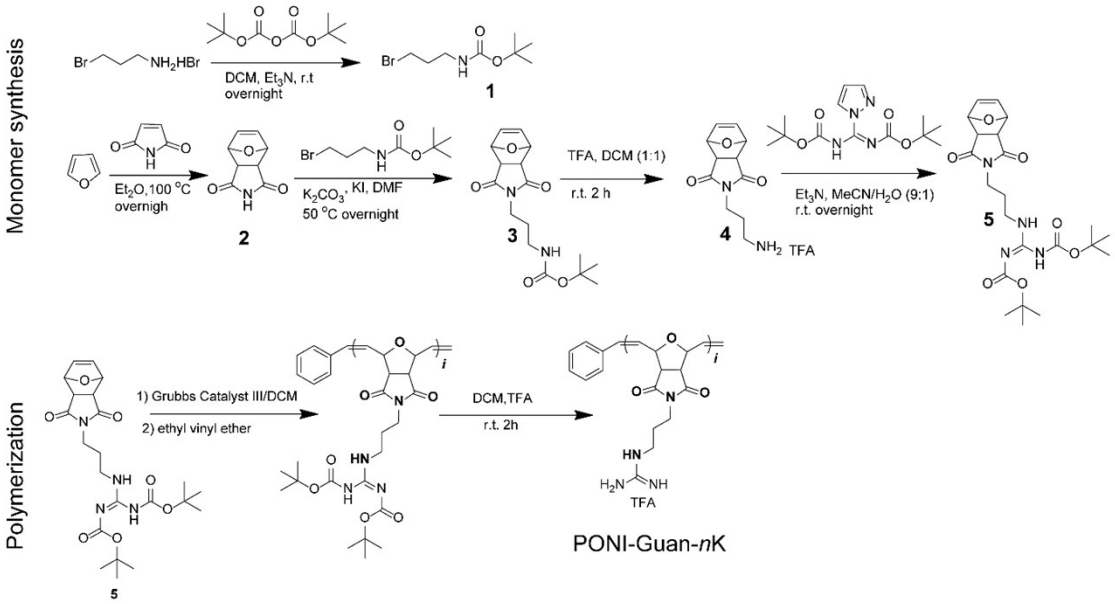


Figure S1. General synthetic scheme for PONI-Guan polymers.

2) Characterization of polymers

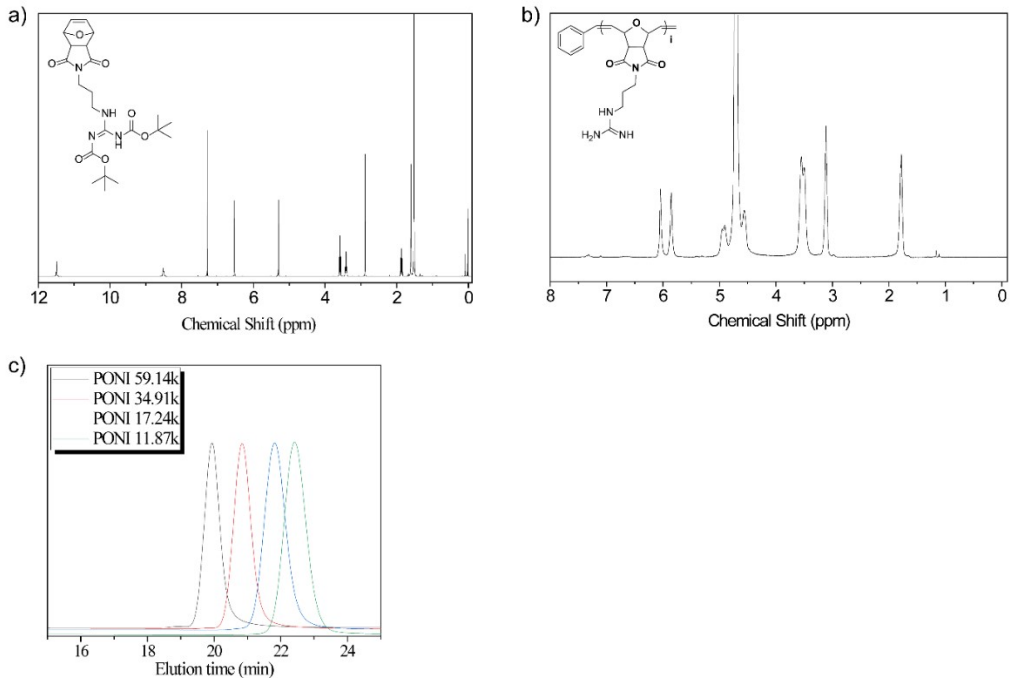


Figure S2. (a) Nuclear magnetic resonance (NMR) spectra of compound **5 in** $CDCl_3$ and PONI-Guan polymer in D_2O. (c) Gel permeation chromatography (GPC) traces of different MW PONI-Guan polymers against PMMA standards.

3) Polyplex characterization

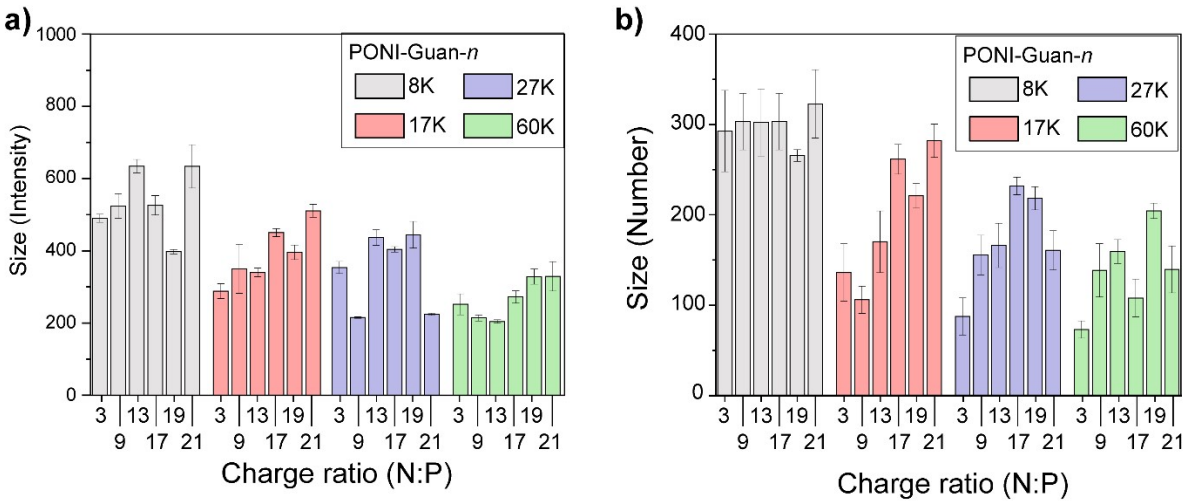


Figure S3. Average hydrodynamic radius (nm) of polyplexes measured by DLS, presented by (a) Intensity average and (b) Number average.

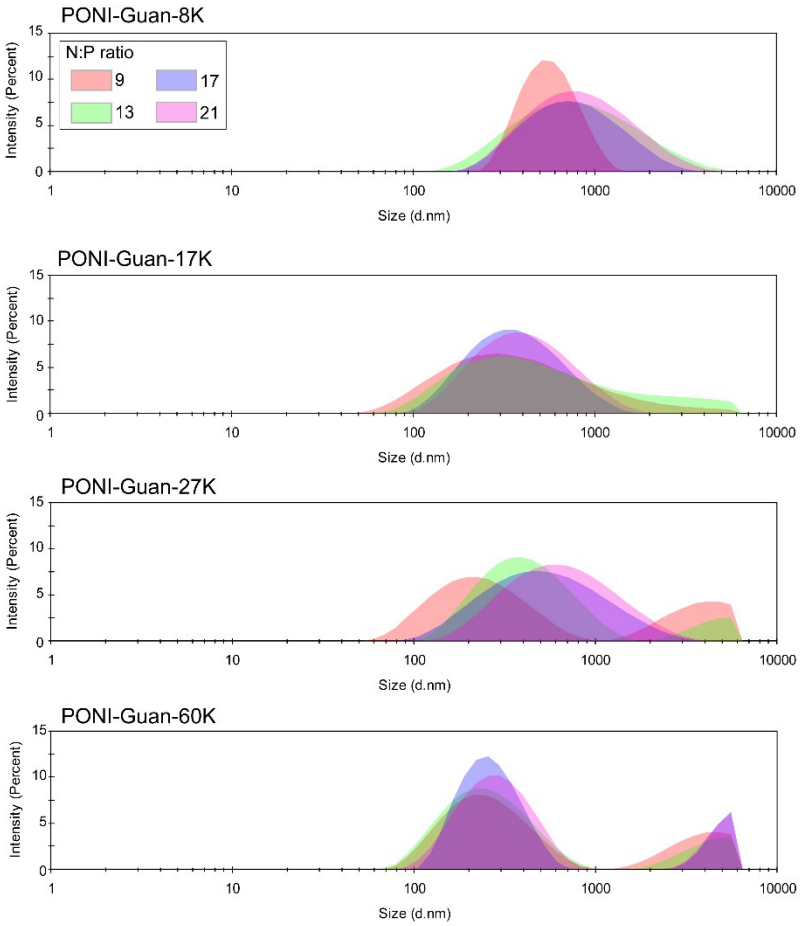


Figure S4. Hydrodynamic radius determined by DLS (Intensity). Representative histograms shown for 4 N:P ratio formulations. Key applies to all four spectra.

Table S1. Polyplex average size by hydrodynamic radius (Intensity, nm). Values are average of 2 experimental replicates and 3 technical replicates, per. Polydispersity index (PDI) represents average across all values. Deviation is shown as standard deviation by population.

N:P	PONI-8K	PDI	PONI-17K	PDI	PONI-27K	PDI	PONI-60K	PDI
3	490.1 ± 11.4	0.66	288.3 ± 20.5	0.31	353.9 ± 16.7	0.65	252.0 ± 29.2	1.00
9	524.1 ± 34.3	0.43	350.2 ± 68.0	0.68	215.2 ± 2.1	0.51	214.2 ± 8.4	1.00
13	634.3 ± 18.7	0.36	340.0 ± 12.0	0.37	436.8 ± 21.7	0.32	204.2 ± 4.7	0.62
17	526.1 ± 26.7	0.27	450.6 ± 10.9	0.30	404.2 ± 7.6	0.45	272.2 ± 17.2	0.46
19	222.5 ± 6.3	0.25	396.0 ± 20.3	0.37	444.5 ± 37.0	0.34	328.7 ± 20.9	0.38
21	808.7 ± 60.1	0.28	510.7 ± 17.7	0.30	224.4 ± 3.1	0.38	329.1 ± 40.7	0.45

Table S2. Polyplex average size by hydrodynamic radius (Number average, nm). Values are average of 2 experimental replicates and 3 technical replicates, per. Deviation is shown as standard deviation by population.

N:P	PONI-8K	PONI-17K	PONI-27K	PONI-60K
3	292.8 ± 44.9	136.2 ± 31.7	86.3 ± 20.5	73.1 ± 9.6
9	303.1 ± 31.2	106.1 ± 15.1	155.4 ± 22.0	138.6 ± 29.5
13	302.3 ± 37.0	170.2 ± 34.1	166.1 ± 24.5	159.3 ± 13.2

17	303.1 ± 31.2	261.5 ± 16.5	231.9 ± 9.7	108.0 ± 20.9
19	355.6 ± 6.6	221.1 ± 13.7	218.2 ± 12.8	204.5 ± 8.5
21	384.7 ± 37.9	282.0 ± 18.5	160.8 ± 21.7	139.6 ± 26.0

4) Polyplex size distribution in 10% FBS

PONI-Guan/pDNA polyplexes were generated as described and diluted either directly in DPBS for immediate measurement (0h) or in DPBS supplemented with 10% FBS for stability measurement. Samples were incubated at 37˚C with shaking (100 RPM). Results suggest polyplexes were stable in serum for at least 4h. An increase of ~50 nm in polyplex size was observed due to protein corona formation.

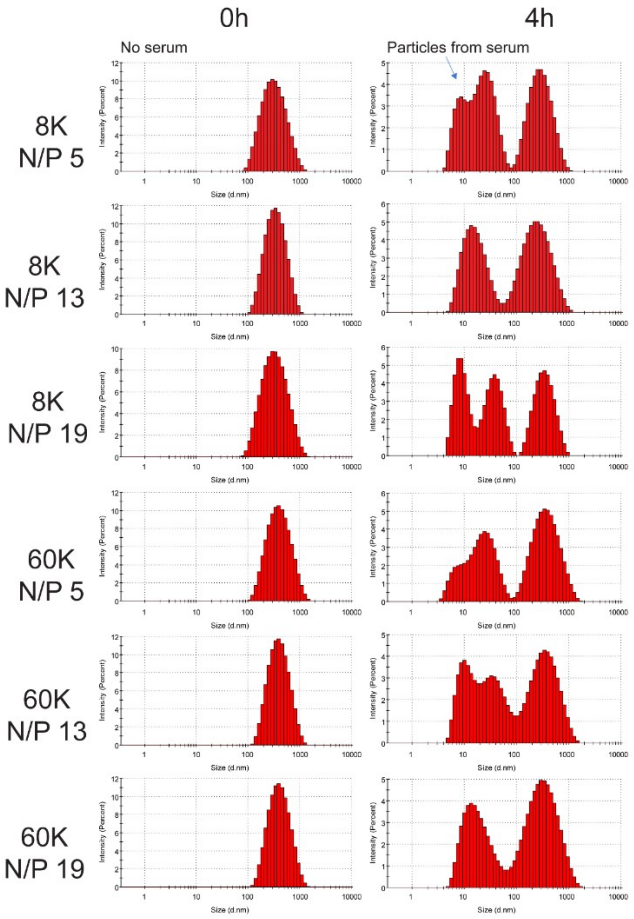
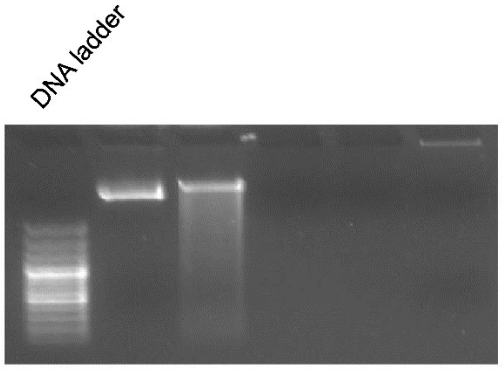


Figure S5. Polyplex stability in 10% FBS, measured immediately after formation (0 h) and after 4 h of incubation in serum conditions. The distinct polyplex peak indicates lack of observable aggregation. Similar results were obtained for PONI-Guan-17K and -28K (data not shown).

5) Polyplex protection against nucleases

To assess protection against nucleases, PONI-Guan/pDNA polyplexes were prepared as described at N:P 9 and incubated at 37˚C for 1h with or without DNase I (10 U). After incubation, 10% SDS (1% v/v) and heparin (50 µg) were added to inactivate DNase I and displace pDNA, respectively. Samples were loaded on 0.8% agarose gel (0.5 µg/mL EtBr) and run at 150V for ~25 min. Imaging was performed using a Bio-Rad Gel Imaging system.

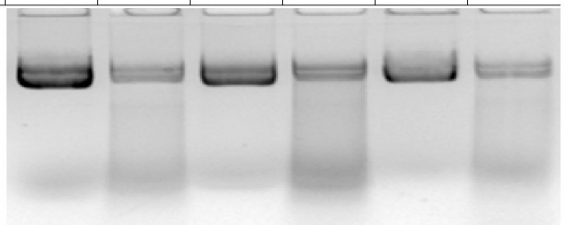


PONI-Guan-17K (0.56 nmol)	-	-	+	+	+
pDNA (500 ng)	+	+	+	+	+
DNase I	-	+	-	+	+
Heparin (50 µg)	-	-	-	-	+
SDS (10% w/v)	-	+	-	+	+

Figure S6. PONI-Guan grants condensed pDNA protection against degradation by DNase I. Gel image shows control lanes with and without assay components. pDNA was partially dissociated from the PONI-Guan polymer in the presence of heparin.

a)

PONI-Guan-nK	17	17	8	8	28	28
pDNA (500 ng)	+	+	+	+	+	+
Polymer (nmoles)	0.56	0.56	1.13	1.13	0.45	0.45
DNase I (units)	-	+	-	+	-	+



b)

PONI-Guan-nK	60	60
pDNA (500 ng)	+	+
Polymer (nmoles)	0.23	0.23
DNase I (units)	-	+

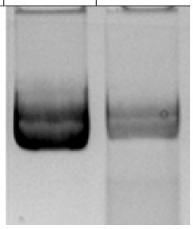


Figure S7. PONI-Guan grants condensed pDNA protection against degradation by DNase I. Lanes depict conditions with/without DNase I treatment.

6) Polyplex cytotoxicity assay

Cells were seeded (5×10^5 cells/well) in a 96-well plate 24 h prior to the experiment. On the day of experiment, cells were washed by PBS and treated with varied concentrations of PONI-Guan polymers or PONI-Guan/pDNA polyplexes followed by incubation for additional 48 h with complete media (10% FBS, 1% antibiotic). The cell viability was measured using the AlamarBlue assay (Invitrogen, CA) by following the manufacturer's instruction**.**

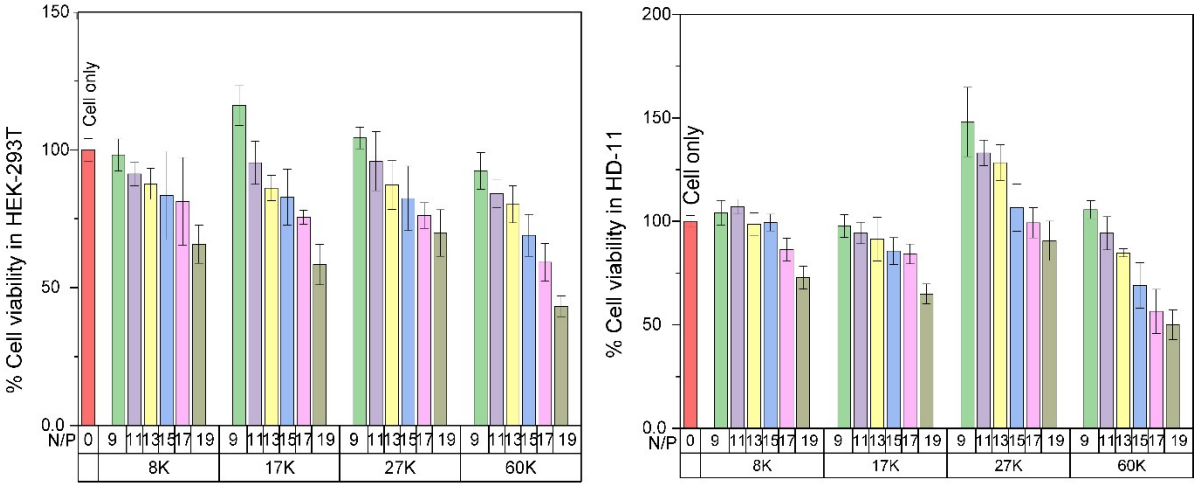


Figure S8. Viability of HEK-293T and HD-11 cells after 48 h exposure to PONI-Guan/pDNA polyplexes at varied N/P ratios

7) Confocal microscopy of transfection in HEK-293T cells at different ratios with all polymers

Polyplexes were prepared as described and incubated with HEK-293T cells plated at 10000 cells per well in a 96-well plate one day prior to delivery. After 24 hours cells were washed and were incubated another 24 hours in complete (10% FBS) culture media to allow sufficient time for protein expression. Additional incubation time did not notably effect efficiency of expression (data not shown). Confocal microscopy imaging was performed using a A1SP-FLIM: Nikon TiE stand with A1 Spectral Detector Confocal and FLIM/FCS Module.

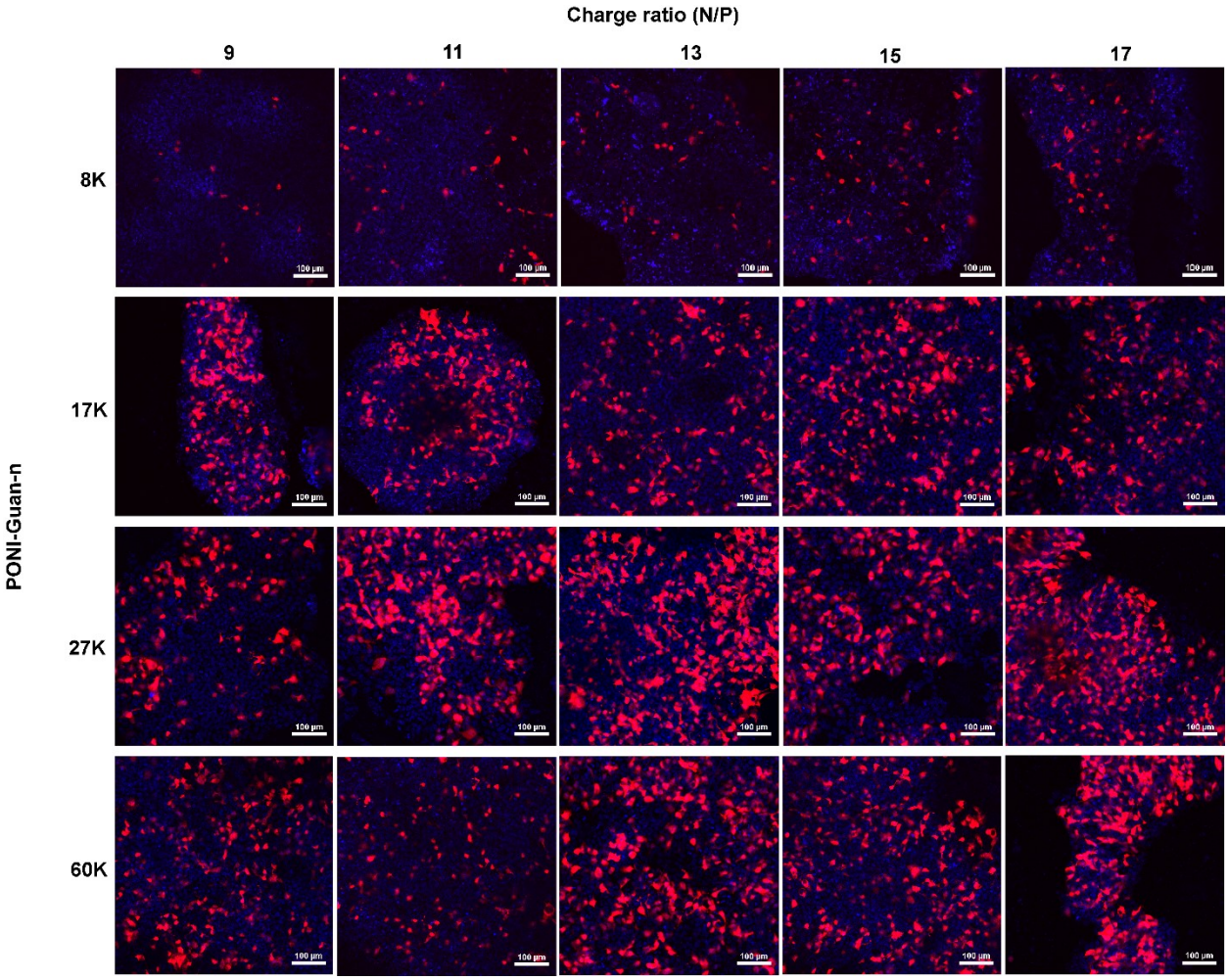


Figure S9. Representative 20x fluorescence micrographs of HEK-293T cells transfected with PONI-Guan/pDNA polyplexes encoding mCardinal after 48h of inclubation. General increase in transfection efficiency is observed with increasing N/P ratio and increasing polymer MW. HEK-293T is a loosely adherent cell line; PONI-Guan-60K at N/P 17 shows cell detachment from the surface. Red channel represents cells with positive mCardinal transfection and blue channel represents nuclei stained with Hoechst 33342 solution. Scale bars: 100 µm

9) Confocal microscopy of transfection with lysotracker satining

Confocal microscopy imaging was performed using a A1SP-FLIM: Nikon TiE stand with A1 Spectral Detector Confocal and FLIM/FCS Module. For all confocal studies, HEK-293T cells were seeded at $1.5x10^5$ cells in #1.5 4-chamber Lab-Tek II chambered coverglass (Thermo) for imaging 24h prior. Cell staining was performed ~20 min. prior to imaging with Hoechst 33342 solution (Thermo) using manufacturer instructions, to visualize nuclei or Lysotracker Deep Red (Thermo) to visualize endo/lysosomal compartments. All mCardinal pseudocoloring presented in orange; Lysotracker pseudocoloring in purple; Hoechst nuclear staining in blue.

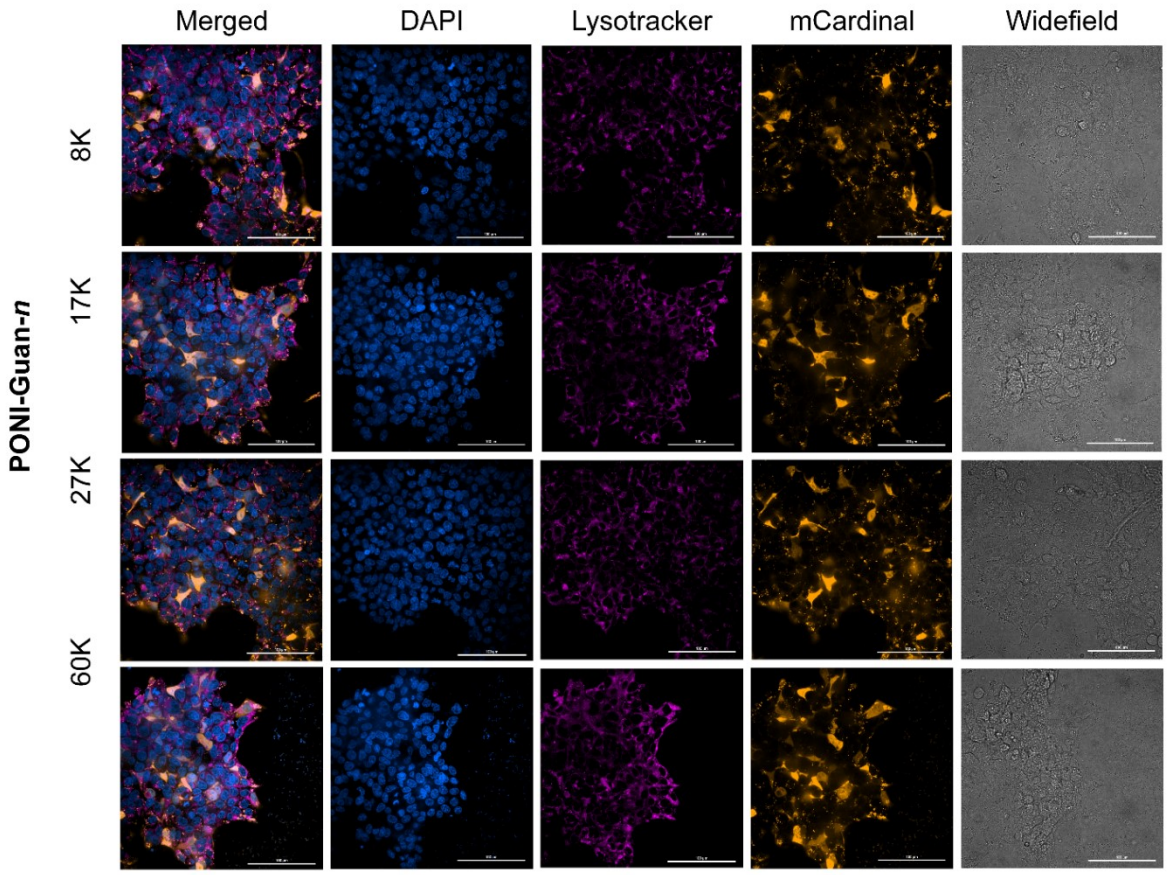


Figure S10. 60x merged confocal micrographs of HEK-293T cells transfected to express mCardinal. Scale bar: 100 µm

10) Animal experimental procedures

One-day-old SPF White Leghorn layer chickens were used for vaccination, as these are relevant targets for NDV livestock vaccination. All chickens were healthy in appearance, from the same source and hatch. NDV-f pDNA vaccine encoding the VEEV Replicon RNA were previously screened *in vitro* for proper antigen expression. IM route of administration was used as this has been extensively demonstrated to provide optimal seroconversion efficiency. Vaccines were applied using Micro-Fine 1.0 ml insulin syringe (Becton-Dickinson).

Eleven groups of n=5 animals were vaccinated on D1 (prime vaccination) and D20 (boost vaccination) using plasmid DNA formulated with PONI-Guan polymers. Groups vaccinated with positive control VEEV replicon particles (RP) encoding for NDV-f were used for comparison. RPs were single, round infectious particles possessing the same VEEV Replicon RNA as coded by the pDNA vaccines. All dosing was performed according to Supporting Table S3. Chickens were housed in negative-pressure isolators under DM-I conditions, with Feed and water was available *ad libitum*.

At D1 T=0 blood was taken from SPF hatch mates. At D20, D40, D54, and D68 blood samples (~1 ml) was taken from the wing vein from all groups. At D82 (end of study), blood samples (~1-2 ml) were taken after anesthesia (prior to euthanasia) from the wing vein, neck or heart puncture.

For serology protocols, after clotting at ambient temperature, serum was collected from the blood samples by centrifugation. The serum samples were heat-inactivated for 30 min at 56°C and stored at < -15°C. Serum samples were tested for the absence/presence of NDV-f specific antibodies using the ID Screen® Newcastle Disease Indirect ELISA (ID-Vet) according to manufacturer instructions.

Table S3. Overview of groups and vaccine administrations.

Group	Formulation	Admin. route	Volume / Dose
1	PONI-Guan-8K	IM	0.1 mL / 5 µg

2	PONI-Guan-17K	IM	0.1 mL / 5 µg
3	PONI-Guan-27K	IM	0.1 mL / 5 µg
4	PONI-Guan-60K	IM	0.1 mL / 5 µg
5	RPs (Pos. control)	IM	0.1 mL / 1.0E^{8} RP

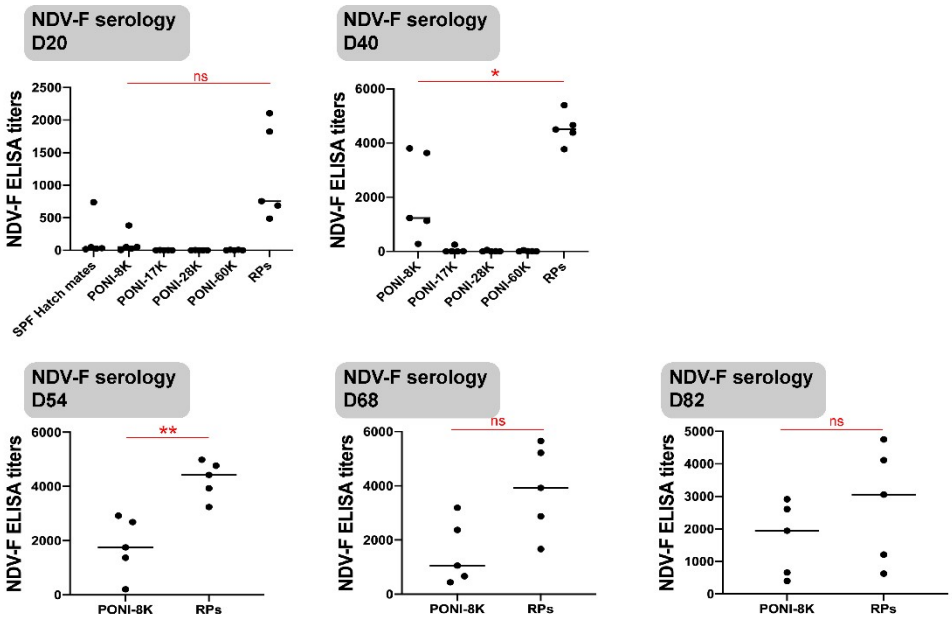


Figure S11. Individual IgY ELISA serology data of NDV-F conversion at specified time points. Error bars represent the mean ± the standard error of the mean (SEM) (n = 5, one-way Anova and Tukey multiple comparisons, **p ≤ 0.01, *p ≤ 0.05, ^{ns} p> 0.05)