Polyphenolic Nanoparticle Platforms (PARCELs) for In Vitro and In Vivo mRNA Delivery

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1. EXPERIMENTAL SECTION

1.1 Materials

Phenazine methosulfate (PMS), tannic acid (TA), Dulbecco's phosphate-buffered saline (DPBS), calcium chloride, sodium phosphate dibasic, phosphate-buffered saline (PBS, pH 7.4), cholesterol, sodium citrate, citrate acid, penicillin-streptomycin, Triton X-100, NaN_3, filipin from *S. filipinensis,* cytochalasin D, (N-ethyl-N-isopropyl) amiloride (EIPA), ATTO-488 DOPE, DMSO-d6, sodium chloride (NaCl), and 6-(*p*-toluidino)-2-naphthalenesulfonic acid were purchased from Sigma-Aldrich. Hydrochloric acid (HCl) was bought from VWR. 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy-(polyethyleneglycol)-2000] (ammonium salt) (C14-PEG 2000) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti. Moderna SM-102 Lipids was bought from Broadpharm. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, Fetal Bovine Serum (FBS), calcein, LysoTracker Deep Red, 2,3-Bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT), Alexa Fluor 594-wheat germ agglutinin conjugate (AF594-WGA), and trihydrochloride (Hoechst 33342) were purchased from Life Technologies. Firefly luciferase mRNA and Human EPO mRNA were obtained from TriLink Biotechnologies. (-)-Epigallocatechin gallate (EGCG), gallic acid monohydrate (GA), (+)-catechin hydrate (CAT), Quant-iT RiboGreen RNA assay kit, bafilomycin A_1, and Human EPO ELISA kit were purchased from Thermo fisher Scientific. Bright-Glo Luciferase assay system was obtained from Promega. Pitstop 2 was purchased from Abcam. Ultrapure water (Milli-Q) with a resistivity greater than 18.2 MΩ·cm was used in all experiments and obtained from a three stage Millipore Milli-Q Plus 185 purification system. All chemicals were used without further purification.

1.2 PCA analysis

Chemicalize was used for the prediction of 25 physicochemical properties of 73 diverse polyphenols, https://chemicalize.com/, developed by ChemAxon, following the instructions from the website. Then, ClustVis was used to perform PCA analysis on the predicted physicochemical properties to generate heatmap and PCA plots.

1.3 Synthesis of PARCELs

Ethanol phase was prepared by mixing Moderna SM-102 ionizable lipid, dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), cholesterol, 1,2-dimyristoyl-*sn*-glycero-3 phosphoethanolamine-N-[methoxy-(polyethyleneglycol)-2000] (ammonium salt) (C14-PEG 2000) with or without various polyphenols including GA, CAT, EGCG and TA. The aqueous phase was made by dissolving the 0.1 mg mL^{-1} of mRNA in 10 mM citrate buffer (pH 3). Then, the aqueous and ethanol phases were mixed at a ratio of 3:1 into a microfluidic chip device. Finally, the synthesized **PARCEL**s were dialyzed against 1x PBS in a 20,000 MWCO cassette at 4 ºC cold room for 2 h, followed by storing at 4 ºC prior for further use.

1.4 Characterization

Size distribution and zeta potentials of **PARCEL**s were measured using a dynamic light scattering (DLS) measurement (NanoBrook 90 Plus Zeta, Brookhaven, USA). The samples were diluted in

1x PBS (pH 7.4) for the size measurement and 0.1x PBS (pH 7.4) for zeta potential measurement. The FLuc/EPO mRNA encapsulation efficiency was quantified by Quant-iT RiboGreen RNA assay kit using a SpectraMax microplate reader (Molecular Device, USA) with 480 nm excitation wavelength and 520 nm emission wavelength. To characterize the overall mRNA concentration, the **PARCEL**s were fully dissolved with 1x Triton. The ^{1}H NMR results of SM-102, DOPE, cholesterol, C14-PEG-2000, GA, CAT, EGCG and TA were characterized via ^{1}H NMR (Bruker, USA) where the individual components were dissolved in DMSO-d6.

To calculate apparent pK_a of formulations, we used the protocol previously described protocol.[1] Briefly, a series of buffers with pH values varying by 0.5 between 3.0 and 12.0 were prepared by titrating a solution containing 10 mM citrate, 10 mM phosphate, 10 mM borate, and 150 mM NaCl with 1.0 M HCl and aliquoted into a black 96-well plate. Formulations and 6-(*p*-toluidino)-2-naphthalenesulfonic acid were diluted into these buffers at final concentrations of 25 and 5.45 μM, respectively. The plate was covered and equilibrated at room temperature for 20 min. Finally, the Fluorescence intensity was measured using a SpectraMax microplate reader with 325 nm excitation wavelength and 435 nm emission wavelength. The raw fluorescence values were normalized between 0 and 1 and fit using GraphPad Prism to calculate pK_a, which corresponds to the pH value at 50% max fluorescence.

1.5 *In Vitro* Dose Response of FLuc Expression

B16-F10 and DC 2.4 cells were seeded at a density of 1×10^4 cells per well in 96 well plates at 37 ºC. Next day, the cell media was aspirated and 100 μL of 500 ng mL^{-1}, 1000 ng mL^{-1} or 2000 ng mL^{-1} of FLuc mRNA encapsulated **PARCEL**s, and naked FLuc mRNA were added into the seeded B16-F10 and DC 2.4 cells followed by incubation for desired time (2, 4, 24 and 48 h). Subsequently, 100 μL of Bright-Glo Luciferase assay buffer was added into each treated well, and the transfection value was measured using a SpectraMax microplate reader with luminescence function.

1.6 *In Vitro* Dose Response of EPO Expression

B16-F10 and DC 2.4 cells were seeded at a density of 1×10^4 cells per well in 96 well plates at 37 ºC. Next day, the cell media was aspirated and 100 μL of 500 ng mL^{-1}, 1000 ng mL^{-1} or 2000 ng mL^{-1} of FLuc mRNA encapsulated **PARCEL**s, and naked EPO mRNA were added into the seeded B16-F10 and DC 2.4 cells followed by incubation for 24 h. Subsequently, the cell media was aspirated, and the concentration of human EPO protein in the collected cell media was characterized by Human EPO ELISA kits (Thermo Fisher Scientific) followed by manufacturer's protocol.

1.7 Cell Viability by XTT Assay

B16-F10 and DC 2.4 cells were grown in DMEM or RPMI supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 incubator. The cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 100 μL media and incubated overnight. Next day, the cell media was removed and 100 μL of fresh cell media (for cell control), 100 μL of 10x Triton dissolved in DMEM or RPMI (as a negative "kill" control to ensure the assay was properly functioning) and 100 μL of **PARCEL**s with 500 ng mL^{-1} 1000 ng mL^{-1} or 2000 ng mL^{-1}

of mRNA were introduced into cells for desired time (2, 4, 24 and 48 h). Cells treated with 100 µL of 500 ng mL^{-1} 1000 ng mL^{-1} or 2000 ng mL^{-1} of naked mRNA were used as control. Next day, the activated XTT reagent was prepared by mixing 9 mL of 0.2 mg mL^{-1} XTT in cell media and 22.5 µL of 0.6 mg mL^{-1} PMS in DPBS). The old media was aspirated and replaced with 100 µL of activated XTT media for 3 h. Finally, the absorbance of the solution was measured using a SpectraMax microplate reader with 475 nm wavelength with a reference wavelength of 675 nm. Untreated cells containing only fresh DMEM/RPMI media were used as 100% cell viability controls. The cell viability results were then calculated by normalizing absorbance to the untreated cells.

1.8 Cellular Association

B16-F10 and DC 2.4 cells were seeded in a 24-well transparent well plate at a density 8×10^4 cells per well and incubated in complete DMEM/RPMI media at 37 ºC for 24 h. Subsequently, 400 µL of 500 ng mL^{-1} FLuc mRNA encapsulated ATTO-488 labeled **PARCEL**s were added to the cells followed by incubation for desired time (2, 4, 24 and 48 h) at 37 ºC. After desired times, the cells were washed with DPBS three times and dissociated using trypsin-EDTA. Finally, the samples are analyzed on Attune Flow Cytometer (Thermofisher Scientific, USA). The cellular association and geometric mean fluorescence intensity (GMFI) were assessed by using the percentage of cell with stronger fluorescence intensity than the control untreated cells.

1.9 Intracellular Trafficking Evaluated by Confocal Microscopy

B16-F10 and DC 2.4 cells were seeded at a density of 4×10^4 cells per well in µ-slide 8-well coverslip slides at 37 ºC overnight to allow the cellular adhesion to the substrate. After 24 h, the cell media was aspirated, and 300 µL of ATTO-488 labeled **PARCEL**s were added into the cells at a mRNA concentration of 500 ng mL^{-1}, followed by incubation at 37 ºC. After desired times (2, 4, 24 and 48 h), the supernatant was removed, and cells were washed with DPBS three times. Then 300 µL AF594-WGA (5 µg mL^{-1}) was introduced and incubated for 15 min to stain the cell membrane at 37 ºC. Subsequently, cells were washed with DPBS for three times followed by introducing 1 µg mL^{-1} of Hoechst 33342 to cells for 10 min to stain the nucleus at 37 ºC. Finally, the live cell imaging was performed by confocal laser scanning microscope (CLSM) (Leica SP8X, USA) with 63 × oil immersion objective. The images were further processed by WCIF Image J software.

1.10 Endocytosis Mechanism

DC 2.4 cells were seeded in a 24-well transparent well plate at a density 8×10^4 cells per well and incubated in complete RPMI media at 37 ºC for 24 h. Subsequently, Endocytosis inhibitors (pitstop 2, EIPA, filipin from *S. filipinensis*, NaN3, and cytochalasin D) were added to the cells to achieve final concentrations of 5, 15, 5 µg mL^{-1}, 16.7 mM, and 20 µM, respectively. After 15 min incubation with the endocytosis inhibitors, 300 µL of 500 ng mL^{-1} FLuc mRNA encapsulated ATTO-488 labeled **PARCEL**s were added to the cells followed by incubation for 2 h at 37 ºC. Then the cell media was removed, and the cells were washed with DPBS three times and dissociated using trypsin-EDTA. Finally, the cells were analyzed using an Attune Flow Cytometer (Thermofisher Scientific, USA). The percentage of inhibition was quantified by normalizing the cellular association of the cells treated with inhibitors with the control cells treated without inhibitors at 2 h.

1.11 Endosomal Escape

DC 2.4 cells were seeded at a density of 4×10^4 cells per well in µ-slide 8-well coverslip slides at 37 ºC overnight to allow the cellular adhesion to the substrate. Next day, the cell media was aspirated and 300 µL of ATTO-488 labeled **PARCEL**s were added into the cells at a FLuc mRNA concentration of 500 ng mL^{-1}, followed by incubation at 37 ºC. After 24 h, the cells were washed with DPBS three times, and 300 µL 100 nM of LysoTracker Deep Red, dissolved in culture media (prewarmed at 37 ºC), was introduced and incubated for 1 h. Subsequently, cells were washed with DPBS three times, and 1 µg mL^{-1} of Hoechst 33342 was added to cells for 10 min to stain the nucleus. Finally, the live cell imaging was performed by confocal laser scanning microscope (CLSM) (Leica SP8X, USA) with 63 × oil immersion objective. The images were further processed by WCIF Image J software. Experiments were performed in triplicates, five representative cell images (> 20 cells) were used to calculate the PCC value.

1.12 Endosomal Escape Mechanism Study with Bafilomycin A_1

DC 2.4 cells were seeded at a density of 4×10^4 cells per well in µ-slide 8-well coverslip slides at 37 ºC overnight to allow the cellular adhesion to the substrate. Then, the culture media was replaced with 180 µL of fresh media or media containing bafilomycin A_1. Calcein (20 µL, 1.5 mg/mL) was added to each well to obtain a final concentration of 150 µg mL^{-1} for calcein and 100 nM for bafilomycin A_1. Non-fluorescently labeled **PARCEL**s were incubated with the treated cells for 4 h at a FLuc mRNA concentration of 500 ng mL^{-1}. After incubation, the samples were gently washed four times with DPBS to remove excess materials. Finally, cells were imaged and captured by CLSM with 40 × oil immersion objective.

1.13 Buffering Capacity

For the titration of **PARCEL** suspensions, 100 µL of FLuc mRNA encapsulated **PARCEL**s were prepared and dispersed in 4.9 mL of Milli-Q water. The pH was subsequently adjusted to ~7.4 to mimic physiological pH. Then, each **PARCEL** was gradually added 5 µL of 0.1 M hydrochloric acid (HCl) dropwise to the nanoparticle suspensions followed by stirring, and the corresponding pH was measured using an Orion Star A214 pH meter (Themo Scientific).

1.14 Animal Studies of FLuc mRNA encapsulated PARCELs

All animal studies were approved by the UNC Institutional Animal Care and Use Committee, were consistent with local, state, and federal regulations as applicable, and were supported within the UNC Lineberger ASC at the University of North Carolina at Chapel Hill.

FLuc mRNA encapsulated **PARCEL**s were prepared by microfluidic mixing. Naked FLuc mRNA and PBS injection were used as control groups. Each group was then injected via the tail vein of female C57 black 6 mice (Jackson Laboratory, 18-21 g) at a dose of 0.6 mg kg^{-1}. After 24 hours, mice were injected intraperitoneally with 130 µL of D-luciferin (30 mg mL^{-1} in PBS). After 15 min, mice were euthanized and organs (pancreas, spleen, liver, kidneys, ovaries, lung and heart) were removed and imaged with an in-vivo imaging system (IVIS) (Perkin Elmer, Waltham, MA). Luminescence was quantified using AuRA software (Spectral instruments imaging). The liver, spleen, and lung were then fixed in 10% neutral buffered formalin after imaging and were then routinely processed to paraffin. Histological sections were evaluated using hematoxylin and eosin stain. Briefly, Paraffin embedded tissue blocks were sectioned at 5 µm onto positively charged

slides. In order to proceed with histological staining, samples were first baked at 60 °C for 60 min minimum and were then deparaffinized in xylene and hydrated with graded ethanol before continuing with the H&E stain. H&E stains were performed using the autostainer XL from Leica Biosystems. The sections were stained with Hematoxylin (Richard-Allen Scientific, 7211) for 2 min and Eosin -Y (Richard-Allen Scientific, 7111) for 1 min. Clarifier 2 (7402) and Bluing (7111) solutions from Richard-Allen Scientific were used to differentiate the reaction. After staining, slides were then dehydrated and coverslipped with Cytoseal 60 (8310-4, Thermo Fisher Scientific). Histological analysis was then performed on each sample.

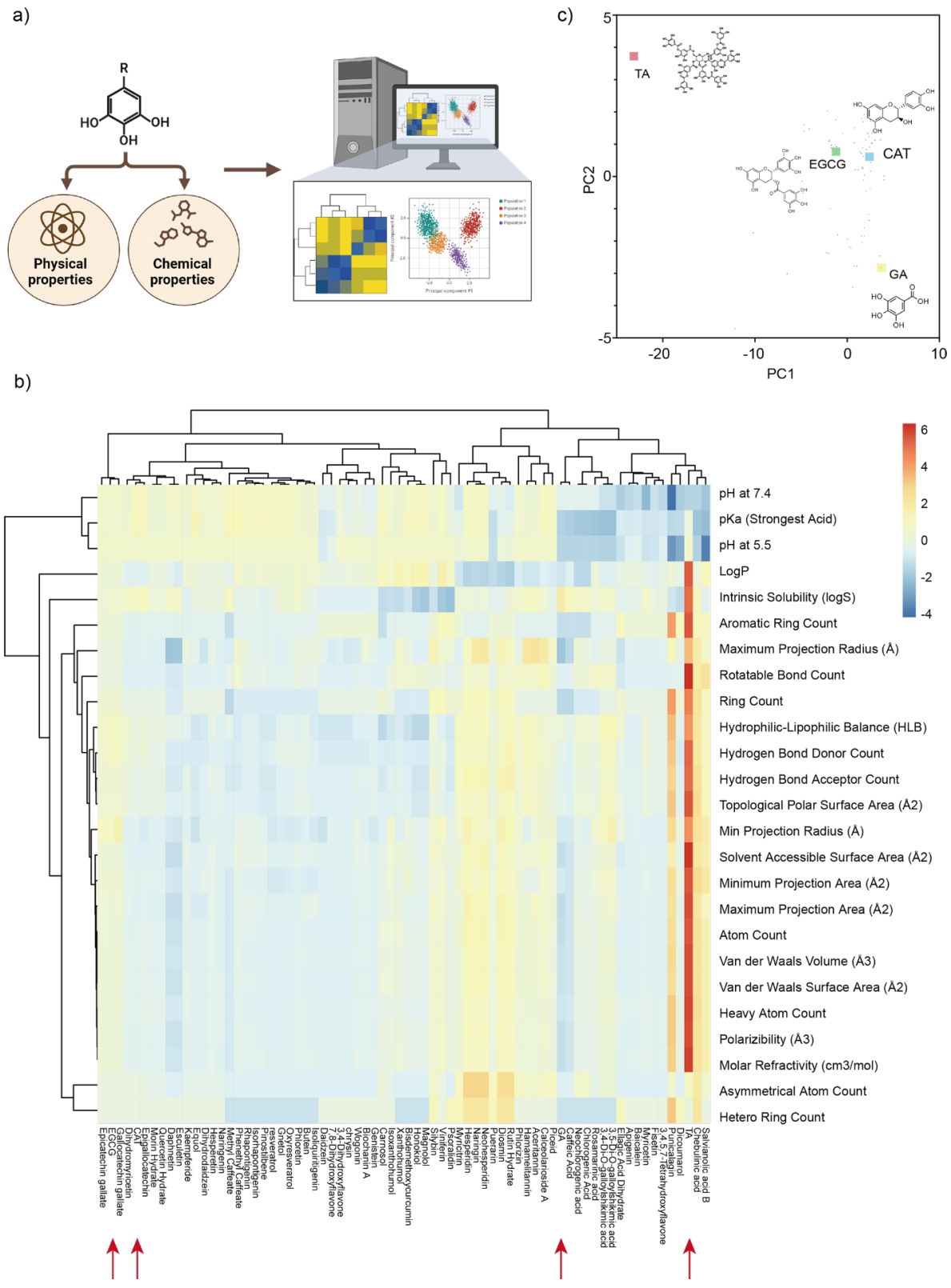
1.15 Animal Blooding Studies

FLuc mRNA encapsulated **PARCEL**s were prepared by microfluidic mixing. Naked FLuc mRNA and PBS injection were used as controls. Each group was then injected via the tail vein of female C57 black 6 mice (Jackson Laboratory, 18-21 g) at a dose of 0.6 mg kg^{-1}. After 24 hours, mice were injected intraperitoneally with 130 µL of D-luciferin (30 mg mL^{-1} in PBS). 250 µL blood to be collected from each animal by cardiac puncture, in which 60 µL into tubes with K_2EDTA (BD microcontainer) and 190 µL into Minicollect tube (Greiner bio-one) at room temperature. Then the serum was collected by centrifuging the blood in Minicollect tubes at 1300 rcf for 10 min. Then the complete blood count and liver and kidney functions from the blood in each mouse was evaluated.

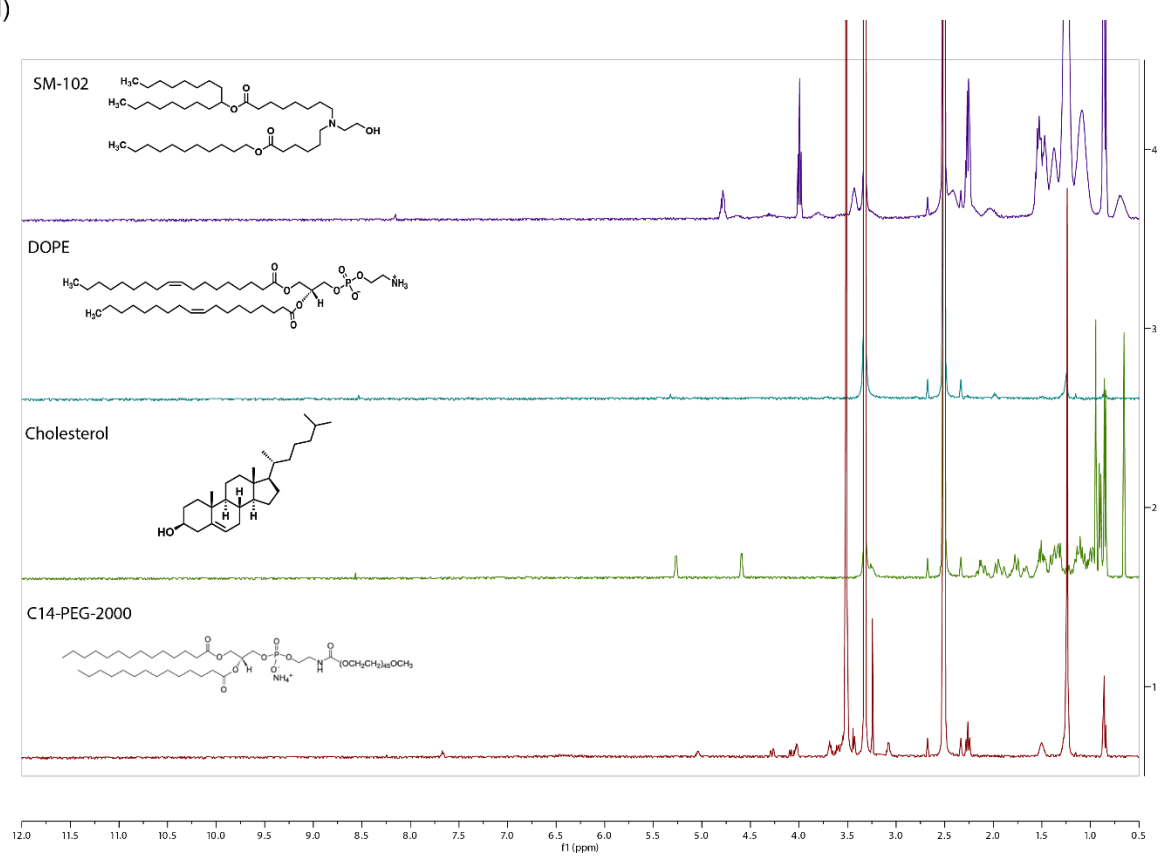
1.16 Animal Studies of EPO mRNA encapsulated PARCELs

EPO mRNA encapsulated **PARCEL**s were prepared by microfluidic mixing. Naked EPO mRNA and PBS injection were used as controls. Each group was then injected via the tail vein of female C57 black 6 mice (Jackson Laboratory, 18-21 g) at a dose of 0.3 mg kg^{-1}. After 24 hours, cardiac puncture was performed to collect the maximal blood sample for each mouse into Minicollect tubes (Greiner bio-one) at room temperature. Then the serum was collected by centrifuging the blood at 1300 rcf for 10 min. Then the dilution of each sample was performed, and the concentration of human EPO protein was characterized by Human EPO ELISA kits (Thermo Fisher Scientific) followed by manufacturer's protocol.

2. ADDITIONAL DATA



d)


SM-102
DOPE
Cholesterol
C14-PEG-2000
f1 (ppm)

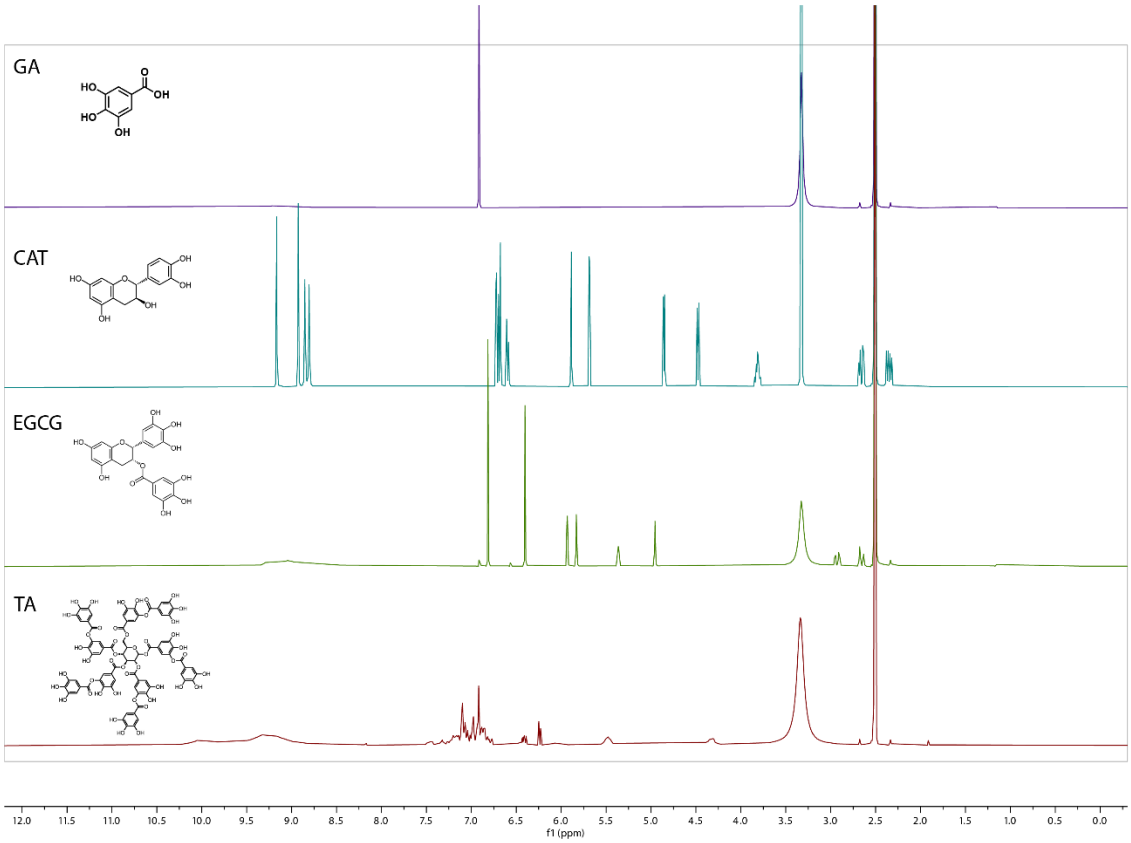

GA
CAT
EGCG
TA
f1 (ppm)

Figure S1. (a) Schematic illustration of computational analysis of polyphenols based on physical and chemical properties. **(b)** Heatmap of 25 physicochemical properties of 73 different polyphenols. In this study, we select **GA, CAT, EGCG** and **TA**, highlighted with red arrows. The meaning of each physicochemical property has been included in **Table S1**. **(c)** PCA score plot of the 73 polyphenols. Data points are separated into the principal components based on their differences in physicochemical properties. **(d)** ^{1}H NMR spectra of respective components used to formulate **PARCEL** formulations.

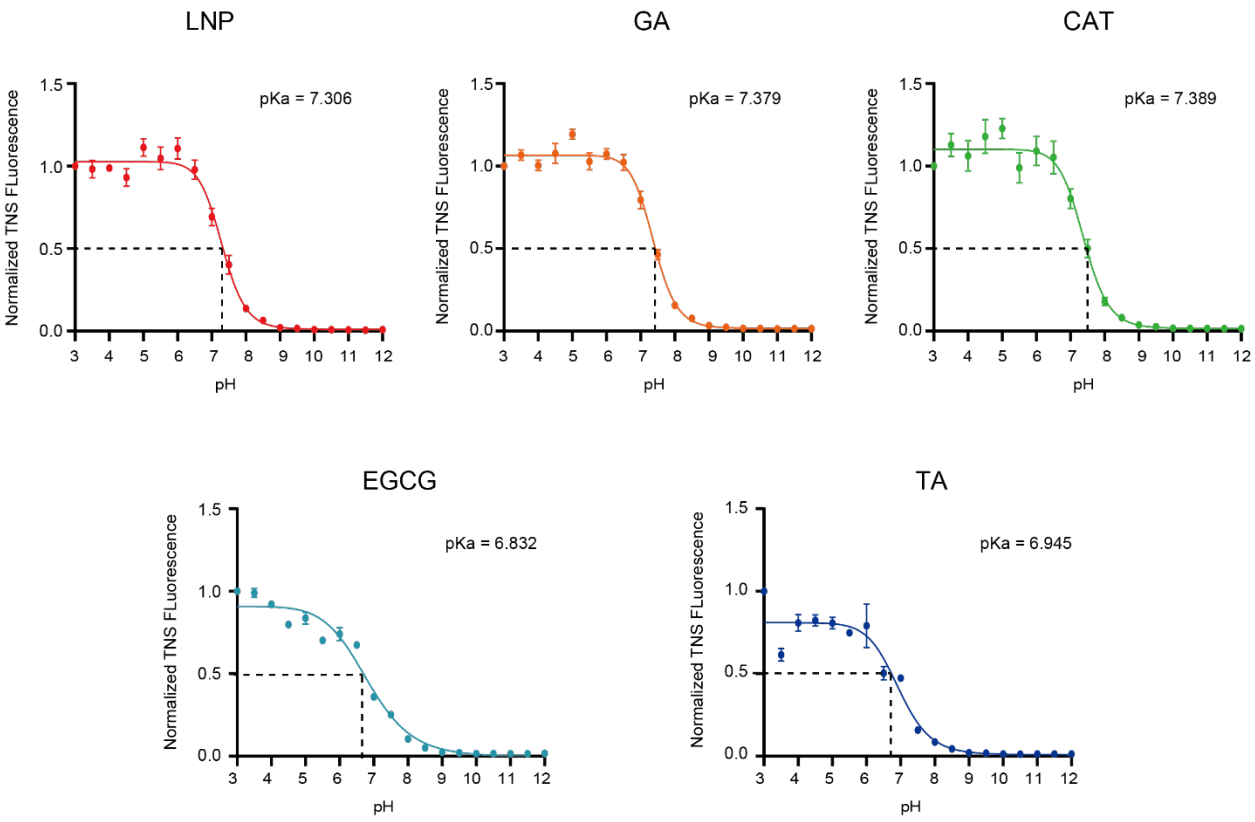


Figure S2. TNS curves of LNP and **PARCEL** formulations.

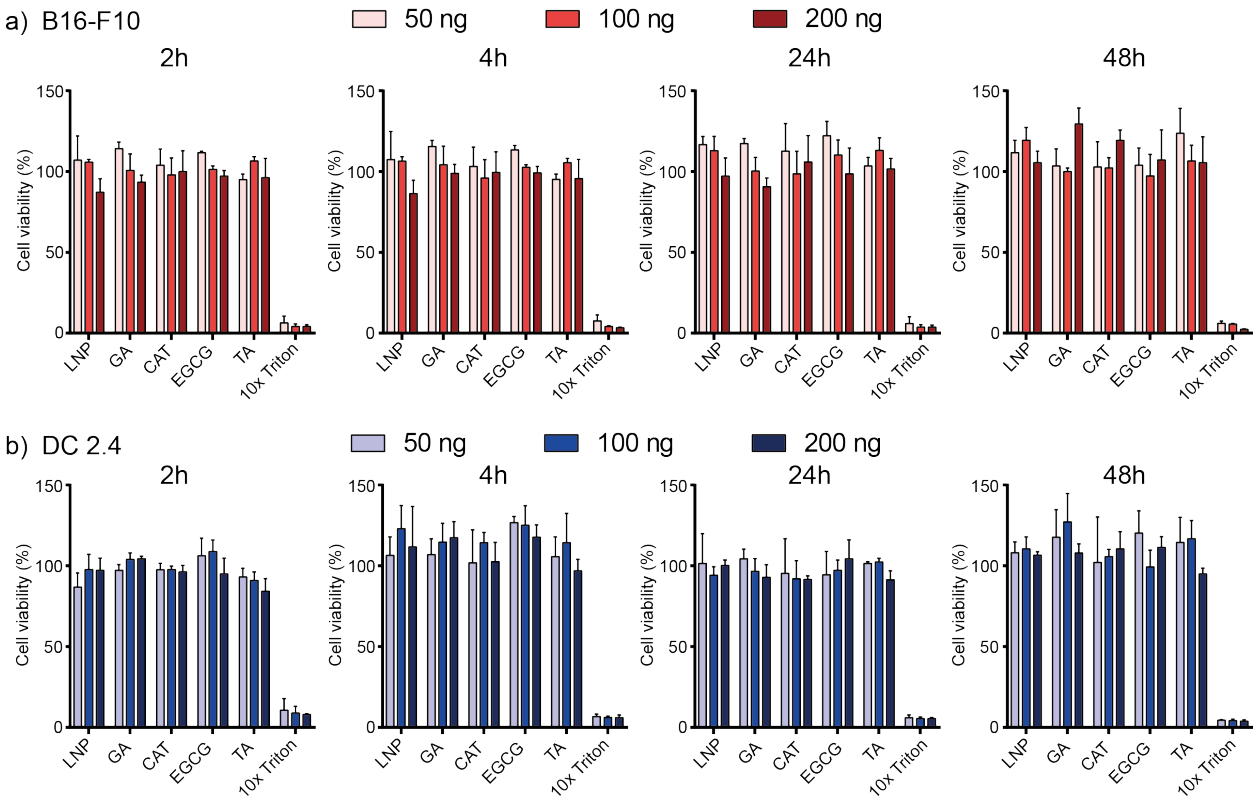


Figure S3. Cell viability of FLuc mRNA encapsulated **PARCEL**s with **(a)** B16-F10 and **(b)** DC 2.4 cells at 50, 100 and 200 ng doses per well for desired times (2, 4, 24 and 48 h) at 37 ºC. 10x Triton dissolved in cell media was used as negative control. (Data are shown as the mean ± SD, n = 3).

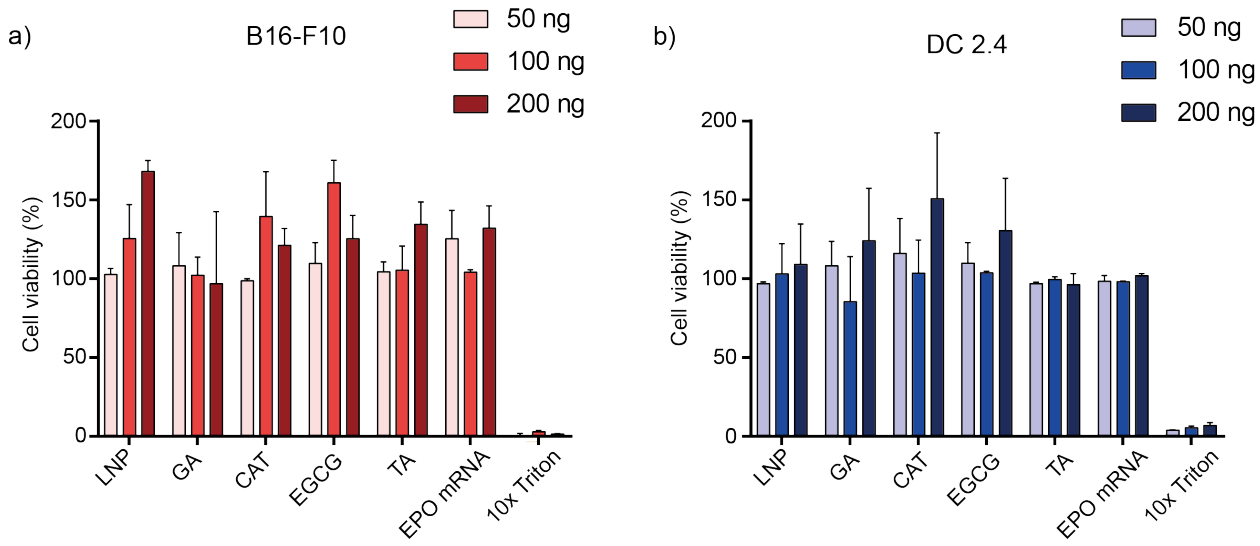
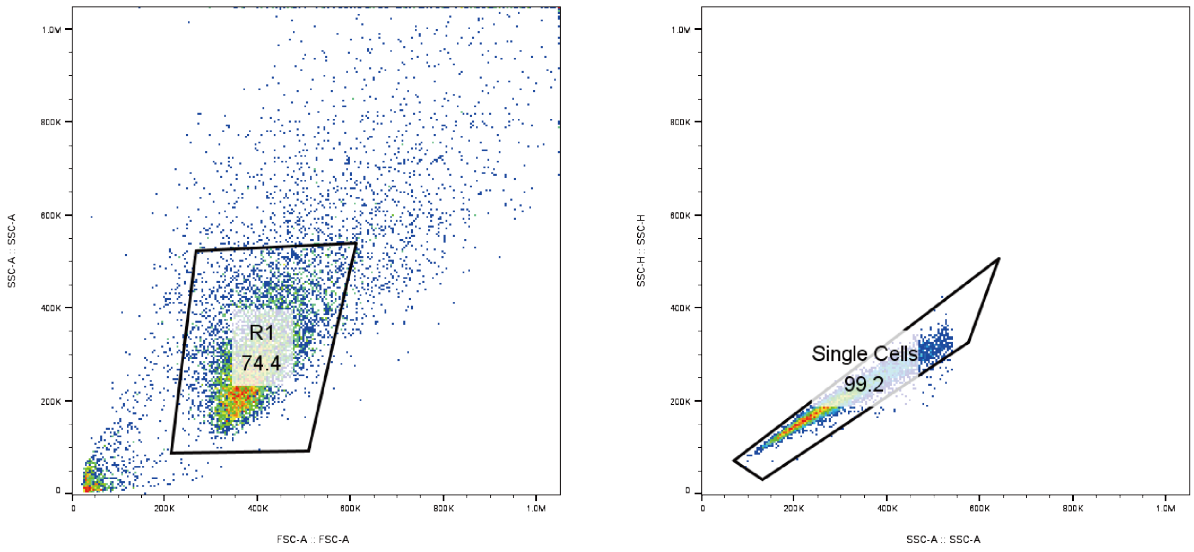


Figure S4. Cell viability of EPO mRNA encapsulated **PARCEL**s with **(a)** B16-F10 and **(b)** DC 2.4 cells at 50, 100 and 200 ng doses per well for 24 h at 37 ºC. Cells treated with naked EPO mRNA were used as control. 10x Triton dissolved in cell media was used as negative control. (Data are shown as the mean ± SD, n = 3).

B16-F10



DC2.4

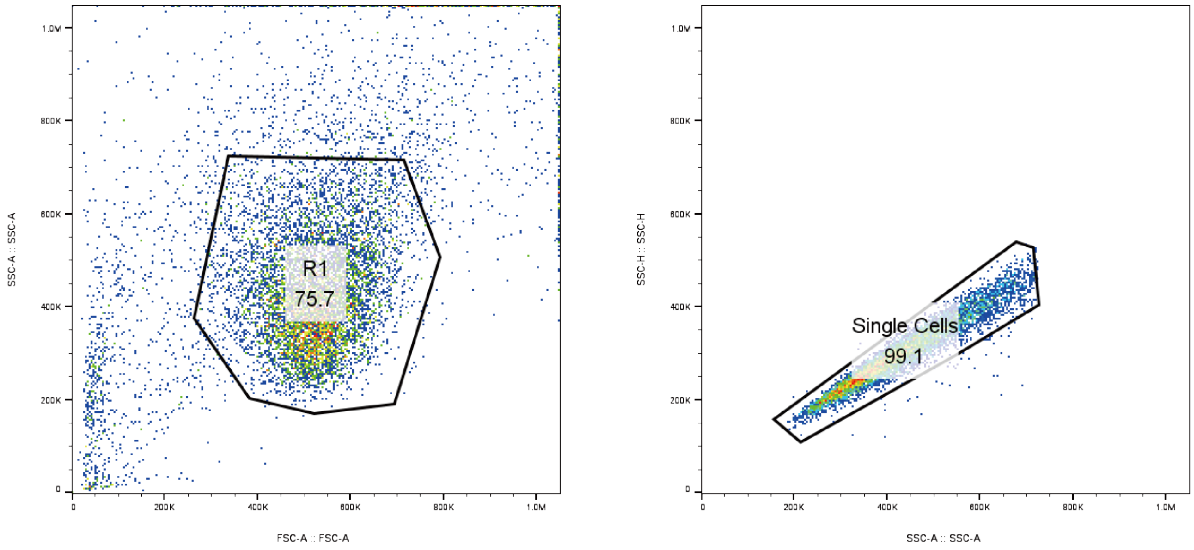


Figure S5. Original gating to quantify cellular association percentages for B16-F10 and DC2.4 cells.

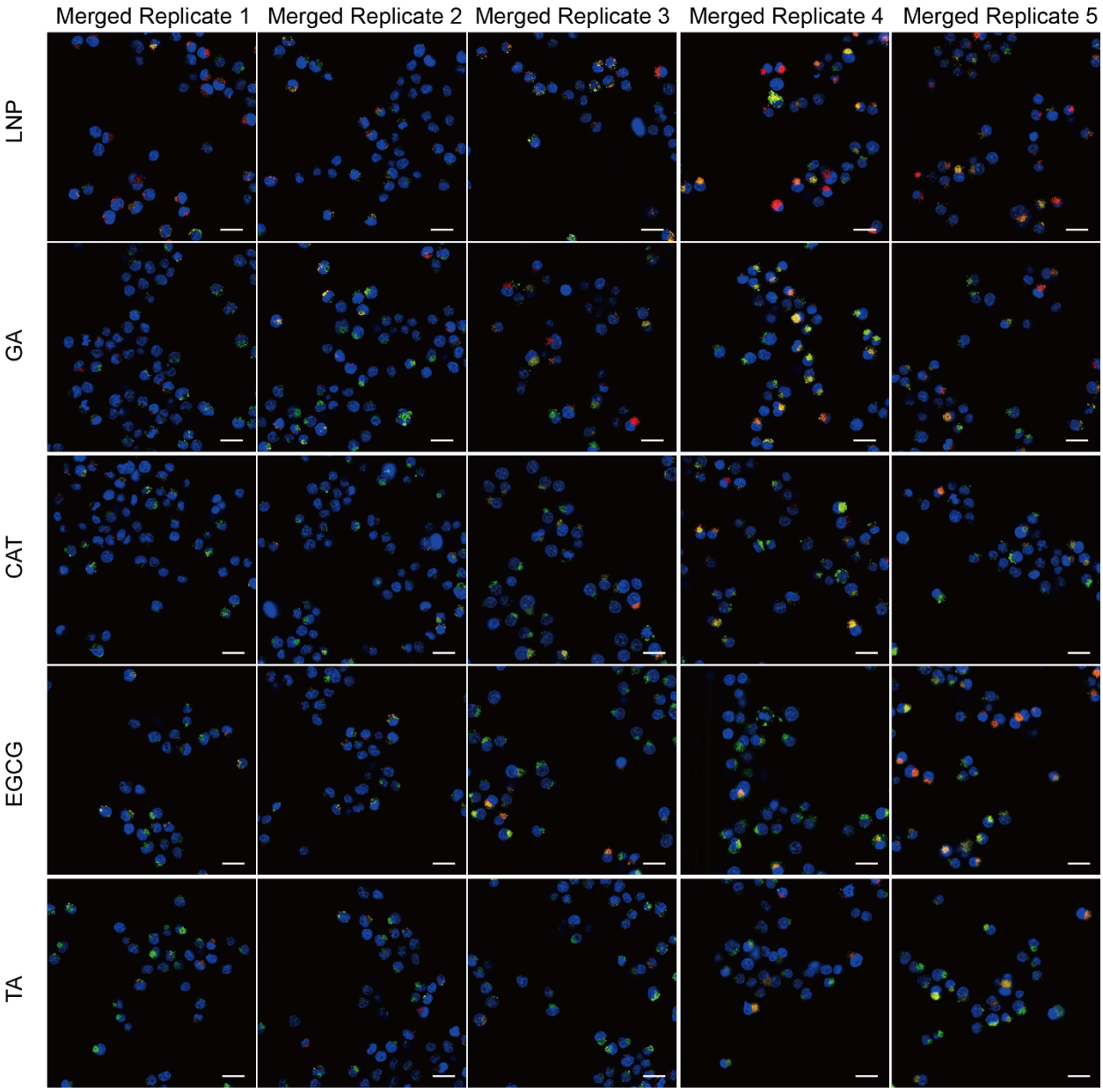


Figure S6. Confocal microscope images to generate the Pearson Correlation Coefficient (PCC) data in **Figure 4c**. Confocal images of DC 2.4 cells treated with ATTO-488 labeled **PARCEL** (green). Endo/lysosomes (red) were stained with LysoTracker Deep Red. Nuclei (blue) were stained with Hoechst 33342. Scale bars are 10 μm.

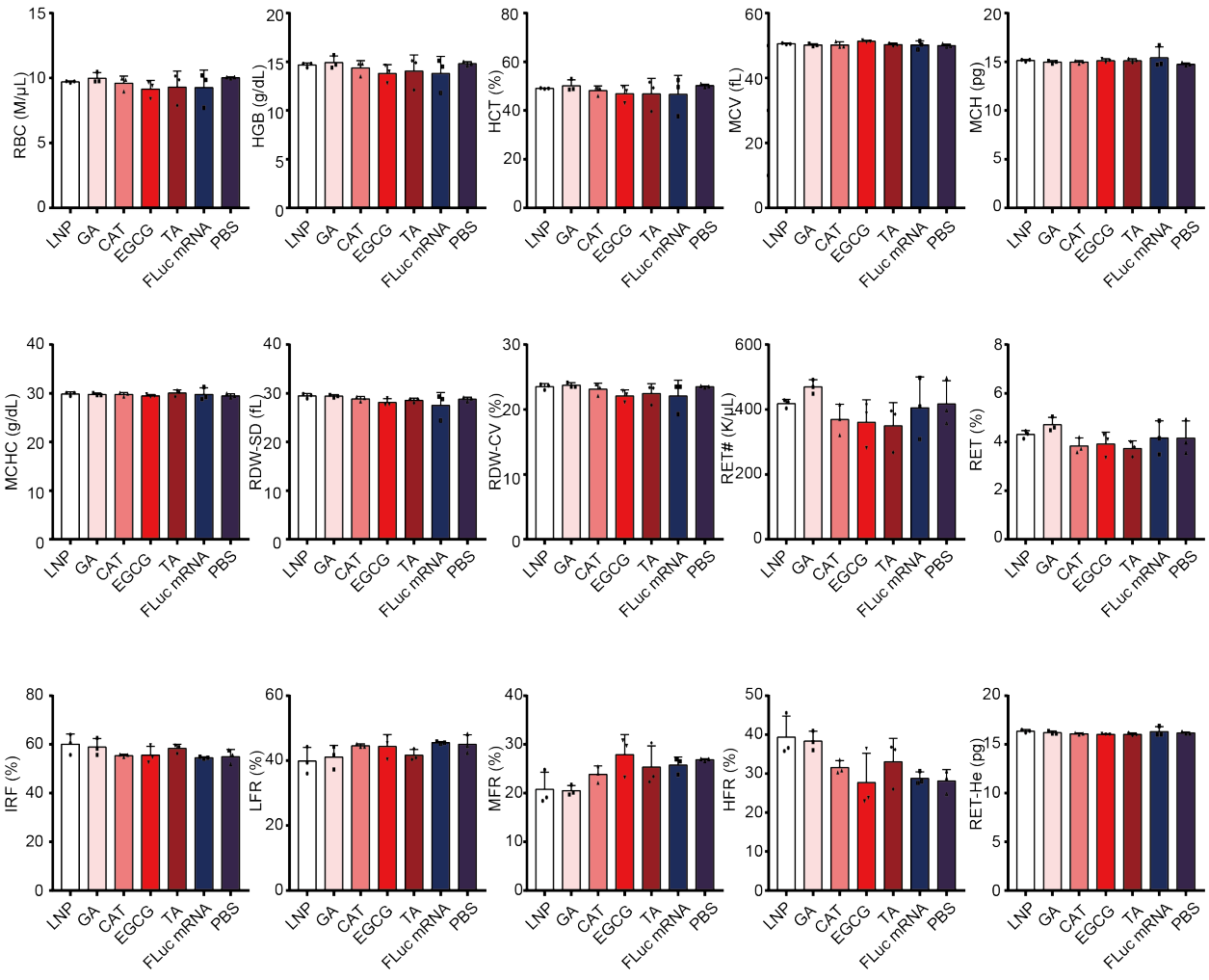


Figure S7. Complete blood test result of **PARCEL**s. Mice only injected with naked FLuc mRNA and PBS were used as controls (Data are shown as the mean ± SD, n = 3).

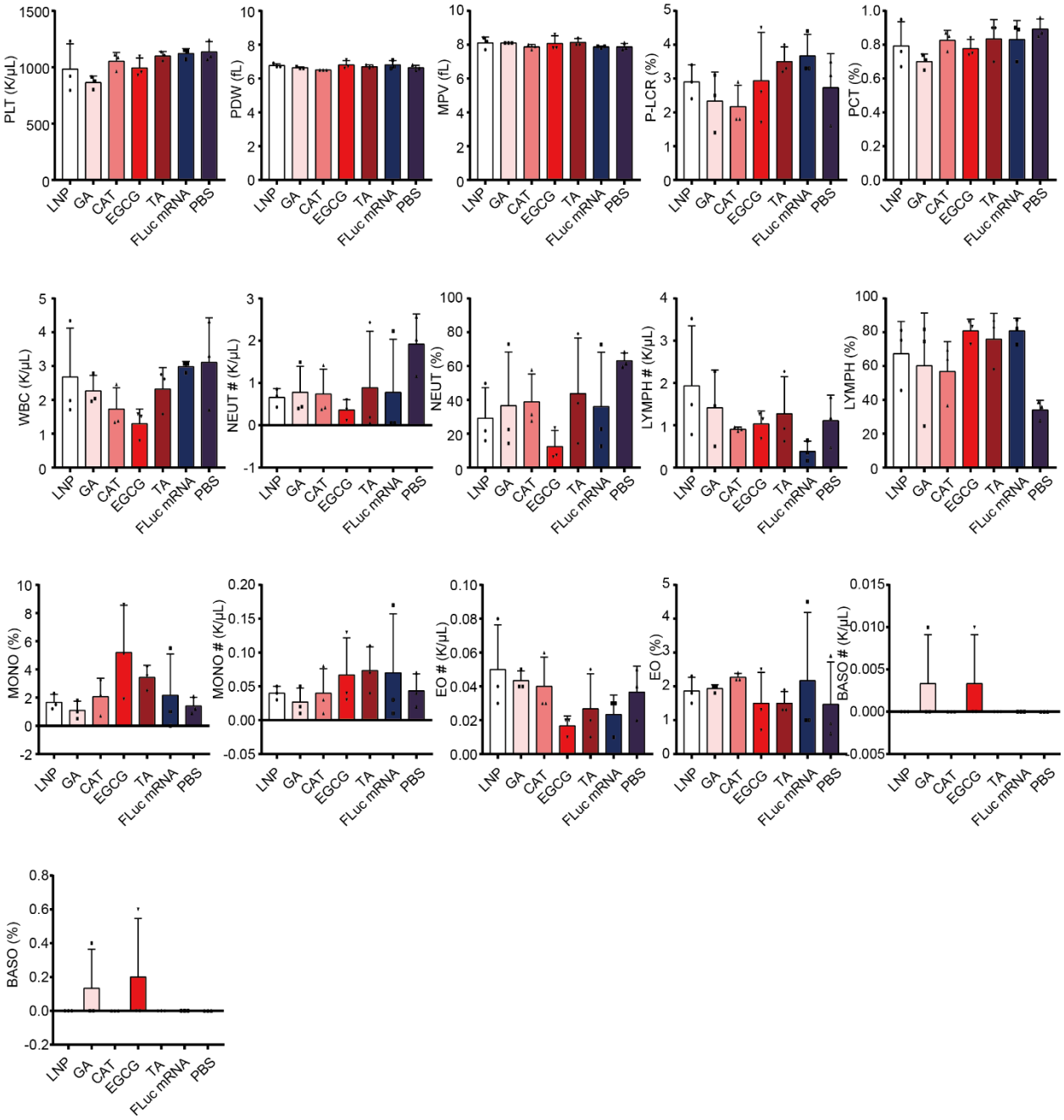


Figure S8. Complete blood test result of **PARCEL**s. Mice only injected with naked FLuc mRNA and PBS were used as controls (Data are shown as the mean ± SD, n = 3).

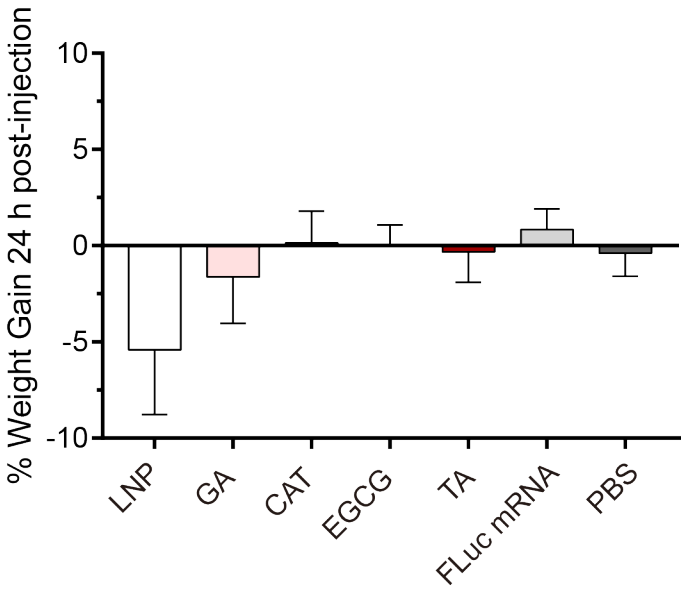


Figure S9. Percent weight gain reported as mean ± SD (n = 3) after 24 hours respective intravenous injection of FLuc mRNA encapsulated **PARCEL**s into mice.

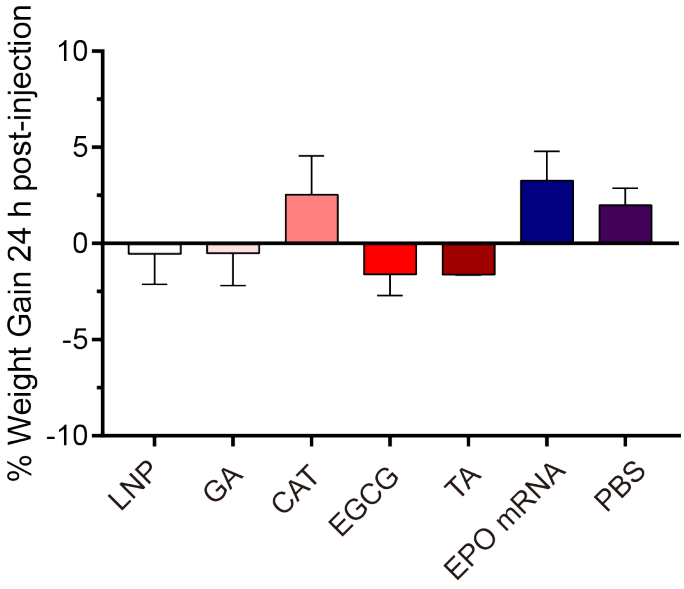


Figure S10. Percent weight gain reported as mean ± SD (n = 3) after 24 hours respective intravenous injection of EPO mRNA encapsulated **PARCEL**s into mice.

Table S1. Physicochemical properties of polyphenols

Physicochemical properties	**Meaning**
LogP	Octanol/water partition coefficient
Intrinsic Solubility (logS)	Solubility of a compound in its free acid or free base form
Aromatic Ring Count	The number of cyclic structures with alternating single and double bonds, meeting aromaticity criteria, present in a chemical compound
Maximal Projection Radius	Calculates the radius for the maximal projection area of the conformer (in Å)
Rotatable Bond Count	The number of flexible, freely rotating bonds in a chemical compound
Ring Count	The number of distinct ring structures in a chemical compound
Hydrophilic-Lipophilic Balance	Measure of the degree of a molecule being hydrophilic or lipophilic
Hydrogen Bond Donor Count	The sum of the atoms in the molecule which have H donor property
Hydrogen Bond Acceptor Count	The sum of the acceptor atoms. An acceptor atom always has a lone electron pair/lone electron pair that can establish a H bond
Topological Polar Surface Area	A molecular property that quantifies the exposed polar surface area in a chemical compound
Min projection radius	Calculates the radius for the minimal projection area of the conformer (in Å)
Solvent Accessible Surface Area	ASA calculated using the radius of the water molecule (1.4 Å)
Minimal projection area	Minimum of projection areas of the conformer, based on the van der Waals radius (in $Å^2$)
Maximal projection area	Maximum of projection areas of the conformer, based on the van der Waals radius (in $Å^2$)

Atom Count	The total number of atoms present in a chemical compound
Van der Waals Volume	Van der Waals volume of the conformer (in $Å^3$)
Van der Waals Surface Area	Van der Waals surface area of the conformer (in $Å^2$)
Heavy Atom Count	The total number of non-hydrogen atoms in a chemical compound
Polarizability	Relative tendency of an electron cloud (a charge distribution) of a molecule to be distorted by an external electric field
Molar Refractivity	A molecular property representing the sum of atomic polarizabilities in a compound, providing information about its interaction with light
Asymmetrical Atom Count	The number of non-equivalent atoms in a chemical compound
Hetero Ring Count	The number of rings in a chemical compound that contain at least one heteroatom

Table S2. Size, PDI, zeta potential, mRNA encapsulation efficiency (%) of **PARCEL**-EPO mRNA for *in vitro* experiments

	Size (nm)	PDI	Zeta potential (mV)	EPO mRNA Encapsulation Efficiency (%)
LNP	172.8±4.0	0.23±0.08	-2.38±5.30	82.2±1.7
GA	195.6±8.8	0.29±0.02	2.00±3.77	73.7±1.4
CAT	162.3±4.4	0.29±0.01	0.69±1.96	88.1±1.0
EGCG	189.5±17.2	0.25±0.07	2.31±5.43	90.4±1.9
TA	203.6±6.0	0.30±0.02	-1.87±4.58	85.8±1.6

Table S3. Size, PDI, zeta potential, mRNA encapsulation efficiency (%) of ATTO-488 labeled **PARCEL**-FLuc mRNA for *in vitro* experiments

	Size (nm)	PDI	Zeta potential (mV)	FLuc mRNA Encapsulation Efficiency (%)
LNP	147.1±4.2	0.25±0.02	-2.30±2.66	77.7±0.1
GA	102.3±2.0	0.14±0.05	0.39±1.44	82.9±0.2
CAT	124.4±1.5	0.17±0.06	0.20±0.41	74.7±0.8
EGCG	128.8±1.3	0.22±0.04	-0.27±1.51	88.0±0.2
TA	153.2±3.1	0.25±0.03	2.59±4.21	81.1±0.4

Table S4. Size, PDI, zeta potential, mRNA encapsulation efficiency (%) of **PARCEL**-FLuc mRNA for *in vivo* experiments

	Size (nm)	PDI	Zeta potential (mV)	FLuc mRNA Encapsulation Efficiency (%)
LNP	147.2±4.1	0.27±0.01	1.91±2.44	85.3±0.1
GA	133.2±4.1	0.26±0.03	1.71±3.26	90.1±3.2
CAT	133.2±5.4	0.24±0.06	-0.78±2.94	89.0±0.4
EGCG	142.7±1.0	0.18±0.01	-1.33±0.75	92.0±0.3
TA	162.8±2.9	0.28±0.01	1.88±1.89	85.3±0.6

Table S5. Size, PDI, zeta potential, mRNA encapsulation efficiency (%) of **PARCEL**-EPO mRNA for *in vivo* experiments

	Size (nm)	PDI	Zeta potential (mV)	EPO mRNA Encapsulation Efficiency (%)
LNP	126.1±0.5	0.26±0.02	0.03±1.53	88.9±0.9
GA	134.3±1.5	0.28±0.02	5.48±5.77	87.3±0.3
CAT	130.5±11.9	0.26±0.03	1.60±3.11	85.2±0.1
EGCG	132.8±1.7	0.25±0.01	1.98±2.24	90.8±0.6
TA	181.1±3.7	0.21±0.04	4.77±6.01	92.2±0.7

REFERENCES

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