3.1 Sources of Chemicals

The polyethyleneimine 2 and 25 kDa (PEI2 and PEI25, respectively) and 15 kDa polyallylamine (PAA15) were purchased from Sigma-Aldrich (St Louis, USA). Cholic acid (ChA), deuterated water (D_2O), Hank's balanced salt solution (HBSS), trypsin/EDTA methylthiazolyldiphenyl tetrazolium bromide (MTT), DNAse I, spectrophometric - grade dimethyl sulfoxide (DMSO), heparin sulfate, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N,N'-dicyclohexylcarbodiimide (DCC) were also purchased from Sigma–Aldrich (St. Louis, USA). Anhydrous chloroform ($CHCl_3$), tetrahydrofuran (THF), dimethyl formamide (DMF) and diethyl ether were purchased from Fisher Scientific (Fairlawn, USA). Sodium chloride was purchased from Fisher Scientific (Alberta, Canada). The Phenol: Chloroform: Isoamyl alcohol mixture (25:24:1 v/v) was purchased from HiMedia Labs (Mumbai, India). 30% Acrylamide/Bis Solution 37.5:1 was obtained from Sigma-Aldrich (St Louis, USA).

SYBR Green I was purchased from Cambrex Bio Science (Rockland, USA). Dulbecco's Modified Eagle Medium (DMEM) cell culture media, Minimum Essential Medium Eagle (MEM), ultrapure nuclease-free water, penicillin/streptomycin (10,000 U/mL/10,000 mg/mL) and Eco RI and Bam HI were purchased from Invitrogen (Grand Island, USA) and HiMedia Labs (Mumbai, India). Fetal bovine serum (FBS) was obtained from PAA Laboratories Inc. (Etobicoke, Canada) and HiMedia Labs (Mumbai, India). The sterile tissue culture plates (24, 48 and 96 well) and sterile tissue culture flasks (25 and 75 cm^2) were obtained from Thermo Scientific and BD Falcon, respectively. The Millex GS syringe filter and Stericup-GP (150 ml, 0.22 μ) was purchased from Millipore, USA.

The Luria broth, Luria agar and agarose were purchased from HiMedia Labs (Mumbai, India). The plasmid gWIZ-GFP (GFP-expressing reporter plasmid) was obtained from Aldevron (Fargo, Canada). The plasmid gWIZ-BMP-2 was constructed in-house in Dr. Uludag Lab, Department of Chemical and Materials Engineering, University of Alberta, Canada. The plasmid p53 (p53-expressing plasmid) was a generous gift from Dr. Bert Vogelstein Lab, Johns Hopkins University (Maryland, USA). The primary antibody p53 (F-8) (# sc-374087) is a mouse monoclonal IgG1 provided at 200 μg/ml (in 1 ml PBS with <0.1% sodium azide and 0.1% gelatin) and secondary antibody goat anti-mouse IgG-HRP (#

sc-2005) were obtained from Santa Cruz Biotech, India. The 2,2,2-Tribromoethhanol (Avertin®), xylazine and ketamine were obtained from Sigma-Aldrich, India.

All the reagents and solvents required for syntheses were purified by general laboratory techniques before use. Melting points were determined using silicon oil bath type melting point apparatus. The completion of the reaction was monitored by thin layer chromatography (TLC) on silica gel pre-coated plates (60F254, Merck, 0.25 mm thickness), visualizing in ultraviolet light (254nm) or iodine vapors.

3.2 Instruments Used

Sonicator:	Modern industrial corporation (Mumbai, India)
Ultracentrifuge:	Sigma 3k 30 (Sigma Centrifuge, USA)
Fluorescence Microscope:	FSX100 (Olympus, USA)
Particle Size Analzyer:	NanoBrook 90Plus (Brookhaven Instruments, USA)
Zeta Potential Measurement:	NanoBrook 90Plus (Brookhaven Instruments, USA)
Flow Cytometer:	QUANTA SC (Beckman Coulter, USA)
Animal Imager:	IVIS Lumina II (Caliper Life Sciences, UK)
Nanodrop:	Nanodrop 2000 (Thermo Scientific, USA)
Transmission Electron Microscope:	TEM Philips/FEI-Morgagni (Philips, Japan)
UV-Visible Spectrophotometer:	UV 1800 (Shimadzu, Japan)
Fluorescence Microplate Reader:	Fluoroskan Ascent CF (Labsystems, USA)
Lyophilizer:	ModulyoD 5 ltr (Thermo Scientific, USA)

3.3 Purification of Solvents

(i) **Toluene**: Analytical grade toluene was dried by continuous refluxing over sodium metal for 4-5h and the distilled solvent was collected after discarding the first fraction of ~50mL. The dried solvent was then stored over 4A type molecular sieves.

(ii) **Ethylene Dichloride (EDC):** AR grade solvent (5L) was refluxed over calcium hydride (2g/L) for 16h, distilled and stored under argon over molecular sieves type 4A (10g/L).

(iii) **Triethylamine (TEA):** AR grade reagent was refluxed with ninhydrin for 4h prior to distillation. The distilled TEA was again refluxed over sodium hydroxide for 4-5h

followed by distillation. The dried reagent was stored over potassium hydroxide in an amber colored reagent bottle.

(iv) **N, N Dimethylformamide (DMF):** Analytical grade solvent was purified by azeotropic distillation with benzene to remove water, followed by distillation under reduced pressure. Subsequently, the distilled solvent was shaken with activated alumina (Grade I), the alumina was removed by filtration and the filtered solvent was stored in an amber colored bottle.

3.4 Protocols for Plasmid Isolation

The plasmid gWIZ-GFP encoding for green fluorescent protein and plasmid CMV-p53 containing p53 gene were amplified and isolated using the methodology and protocols described in Molecular Cloning: A Laboratory Manual, Third Edition, Ed: Sambrook J., Cold Spring Harbor Laboratory Press, 2001.

3.4.1 Bacterial Growth Conditions

E.coli strain (DH5-α) was grown in sterile Luria Bertani (LB) medium supplemented with an antibiotic, ampicillin (100 µg/mL). The culture was grown with constant shaking at 200 rpm at 37˚C for overnight in an orbital shaker incubator (Innova 4330, New Brunswick Scientific Co. Inc., USA).

3.4.2 Preparation of Competent Cells

Competent cells of *E.coli* DH5-α were prepared following the reported method (Cohen *et al.,* 1972). A single colony was inoculated in 5 mL of LB medium and grown overnight at 37˚C, as primary inoculum. LB medium (100 mL) was inoculated as secondary inoculum i.e. 1% from the primary and grown at 37˚C with constant shaking at 200 rpm. Once the absorbance (A_{600}) of the medium reached -0.4-0.6, the culture was centrifuged at 6000 rpm for 10 min at 4 ˚C and the supernatant was discarded. The pellet was resuspended gently in 5mL of 0.1M CaCl2 solution (cold) and incubated for 20 min on ice, then centrifuged at 6000rpm for 10 min with 0.1M CaCl2 containing 10% glycerol solution. Aliquots of 50µL were made and stored at -70˚C.

3.4.3 Transformation

Transformation was carried out by the method described previously (Mandal and Higa 1970). Briefly, to a suspension of competent cells (50 µL), DNA (~200 ng) was added and incubated on ice for 30 min. Cells were then subjected to heat shock at 45˚C for 90 seconds, followed by incubation in ice for 5 min. After incubation, 400-500µL of LB medium was added to the cells and the sample was again kept on ice for 5 min, followed by incubation at 37˚C for 1hr with constant shaking at 200rpm. The cells were centrifuged at 6000rpm for 5min at room temperature and the supernatant was discarded. The cells were resuspended in the remaining medium and plated on LB agar plates supplemented with ampicillin. Ampicillin serves as the marker, because only the cells containing plasmid DNA will grow on this medium.

3.2.4 Colony purification

To identify and isolate the high yielding transformant colony, the initial isolation of pDNA was carried out by performing midi preps. In brief, a single colony of recombinant E.coli was inoculated in 10 ml LB medium containing the antibiotic (ampicillin, 100µg/mL) and was grown for 12-16h at 37˚C with shaking at 200rpm. The cells were harvested by centrifugation at 900 rpm at 4˚C for 20min in a centrifuge (Remi, Mumbai). The cell pellets were re-suspended in 0.4mL of a solution 1 or P1 (Resuspension buffer: 50mM Tris-Cl of pH 8.0, 10mM EDTA, 100 µg/mL RNase A) supplemented with 50µg/mL RNase and kept at room temperature for 20min. After this,0.4mL of freshly prepared solution 1 or buffer P2 (Lysis buffer: 200 mM NaOH , 1% SDS w/v) was added, mixed gently by inverting and incubated at room temperature for not more than 5 min. At the end of the incubation period, ice cold 0.4mL of solution 1 or buffer P3(neutralization buffer: 3.0M potassium acetate of pH 5.5) was added and gently mixed by inversion and kept on ice for 20min. The sample was centrifuged at 14000 rpm for 30min. at 4˚C in high speed centrifuge (Sigma 3K30, Sorvall, Germany) to remove the bacterial debris. This step was repeated to remove all the traces of cell debris. The pDNA in the supernatant was precipitated by isopropanol and sample was incubated at room temperature for 5min and centrifuged at 14000 rpm for 20 min. The supernatant was discarded and the pellet was washed with mL of 70% ethanol, allowed to air dry and resuspended in 200 µl of autoclaved distilled water. The concentration and quality of

pDNA was determined by measuring the absorbance at 260 nm and taking the ratio of absorbance at 260/280 nm. This UV ratio is used as a marker of quality and purity of plasmid DNA. The absorbance ratio of 1.8 to 2 for a plasmid DNA solution at 260/280 nm was considered to be good for further experiments.

3.2.5 Mega Preparation of pDNA

After purification of high yielding colonies of transformed E. coli, the pDNA was isolated on a large scale by the alkaline lysis methods. A single colony of recombinant E.coli was inoculated in 1000 ml LB medium containing the antibiotic (ampicillin, 100µg/mL) and was grown for 12-16h at 37˚C with shaking at 200rpm. The cells were harvested by centrifugation at 900rpm at 4˚C for 20min in a high speed centrifuge (Sigma 3K30, Sorvall, Germany). The cell pellets were resuspended in 20 mL of a solution 1 or P1 (Resuspension buffer: 50mM Tris-Cl of pH 8.0, 10mM EDTA, 100µg/mL RNase A) supplemented with 50µg/mL RNase and kept at room temperature for 20min. After this 20 mL of freshly prepared solution 1 or buffer P2 (Lysis buffer: 200mM NaOH , 1% SDS w/v) was added, mixed gently by inverting and, incubated at room temperature for not more than 5 min. At the end of the incubation period, ice cold 20 mL of solution 1 or buffer P3(neutralization buffer: 3.0M potassium acetate of pH 5.5) was added and gently mixed by inversion and kept on ice for 20min. The sample was centrifuged at 14000rpm for 30min. at 4˚C in high speed centrifuge to remove the bacterial debris. This step was repeated to remove all the traces of cell debris. The pDNA in the supernatant was precipitated by isopropanoland sample was incubated at room temperature for 5min and centrifuged at 14000 rpm for 20 min. The supernatant was discarded and the pellet was washed with mL of 70% ethanol and allowed to air dry and resuspended in 10 mL of autoclaved distilled water. The concentration and quality of pDNA was determined by measuring the absorbance at 260 nm and taking the ratio of absorbance at 260/280 nm.

2.3.6 pDNA Digestion Studies

Restriction endonucleases, or restriction enzymes, are used as molecular scissors to cut DNA in a precise and predictable manner. They are members of the class of nucleases that display the general property of breaking the phosphodiester bonds that link adjacent

nucleotides in DNA and RNA molecules. Each restriction endonuclease scans along a DNA molecule, stopping only when it recognizes a specific sequence of nucleotides. To avoid confusion, restriction endonucleases are named according to the following nomenclature (Micklos D.A. and Freyer G.A.-1989):

1) The first letter is the initial letter of the genus name of the organism from which the enzyme is isolated.

2) The second and third letters are the initial lettersf therganism's species name. (Sincethey are derived from scientific names, the first three letters of the endonuclease name are italicized.

3) A fourth letter, if any, indicates a particular strain of organism.

4) According to most recent nomenclature, a Roman numeral indicates the order in which enzymes, isolated from the same organism and strain, are eluted froma chromatography column. However, an earlier nomenclature, in which Roman numerals indicate the order of discovery, is still used.

For digestion study of plasmid DNA containing p53, two different restriction enzymes were used which are *Eco*RI and *Bam*HI. *Eco*RI linearises circular pDNA and *Bam*HI selectively cuts the ligated portion encoding for p53.

*Eco*RI E = genus Escherichia
co = species coli
R = strain RY13
I = first endonuclease isolated

*Bam*HI B = genus Bacillus
am = species amyloliquifaciens
H = strain H
I = first endonuclease isolated

20 µl of pDNA (0.1µg/µl) (stored on ice) was incubated for 30 min with 25 µl 2x restriction buffer and 2 µl each of BamHI, EcoRI (stored on ice). The reaction mixture was then incubated at 37˚C for 30 min.The samples were then added with gel loading dye, transferred in separate wells of 0.8% w/v agarose gel and run on gel electrophoresis for 35

min. The pDNA fragments were sStained with ethidium bromide and visualised under UV light (AlphaImager, Innovatech Corp.)

3.5 Protocols for Cell Cultures

The cells HEK 293T (Human embryonic kidney), N2Aa (Neuroblastoma), SKOV3 were obtained from the cell respiratory facility of National Center of Cell Sciences, Pune, India. The cord blood mesenchymal stem cells (CB-MSC) and human bone marrow stromal cells (hBMSC) were obtained from Dr. Uludag lab, University of Alberta, Canada. The NT8e cells were used as available (isolated and developed in the Dr. Rita Mulherkar's Genetic Engineering Lab at Advanced Cancer Treatment, Research and Education Centre (ACTREC), Kharghar, Mumbai). For in vitro transfection , cell lines were maintained at 16h prior to the experiments as monolayer cultures in Dulbecco's modified Eagle culture medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS) and 1% antibiotic (streptomycin + penicillin). Cultures were maintained at 37˚C in a humidified 5% CO_2 atmosphere. The protocols for freezing the cells from existing stock, starting the cell culture from frozen stock, expanding the cell culture and counting the cells are based on the protocols and methodology described in Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, Sixth Edition, Ed: Freshney R. I., Wiley- Blackwell Publication, 2010:

3.5.1 Freezing Cells from Existing Stock

Solutions:

Dulbecco's Modified Eagle Medium with 10% FBS

Penicillin/Streptomycin

Cell Culture Freezing Medium DMSO

HBSS: Hanks' Balanced Salt Solution

1) The medium and HBSS were warmed up in the water bath at 37°C.
2) The cells were taken from incubator and observed for cell growth under the microscope (made sure cells have grown ~80% confluence).

3) Medium was removed with the help of vacuum from the T-flask and 5 mL HBSS was added to the flask and allowed to sit for 30 s-1 min.
4) Again HBSS was removed with the help of vacuum, 1 mL Trypsin solution was added and allowed to stand for 5 min occasionally swirling to dissociate cells.
5) Then 5 mL medium was added to stop the trypsin activity and the cell suspension was transferred into 50 mL tube.
6) The cells were centrifuged at 600 rpm for 6 min.
7) Supernatant was removed with the help of vacuum leaving only the undisturbed pellet.
8) 50 mL Cell Culture Freezing Medium & DMSO was added into the tube.
9) The cell suspension was splitted into small vials (1 mL into each vial).
10) The vials were put into a box and frozen for 1 hour.
11) The box was stored under liquid Nitrogen until further use.

3.5.2 Starting the Cell Culture from Frozen Cells

1) The cell culture vial was taken from liquid N2 storage and transferred on dry ice (-60°C) to the lab.
2) The cells were thawed in the water bath at 37°C.
3) 5 mL medium was taken and added to 50 mL centrifuge tube.
4) The cells were thawed, added to 50 mL tube and washed the vial with medium.
5) The cell suspension was centrifuged at 600 rpm for 6 min.
6) The supernatant was removed and added 20 mL medium to the pellet and re-suspended the cells.
7) The cell suspension was equally splitted between two T-25 cm^2 flasks (~10 mL into each).
8) The flask was placed on its side in the incubator. It was made sure that the cap was loose, so as to avoid limiting oxygen supply.

3.5.3 Expanding the Cell Culture

1) Medium and HBSS were warmed up in the water bath at 37°C.
2) The cells were taken from incubator and observed for cell growth under the microscope (made sure cells have grown ~80% confluence).

3) Medium was removed with the help of vacuum from the T-flask and 5 mL HBSS was added to the flask and allowed to sit for 30 s-1 min (added to non-cell side and allowed to stand on cell side).
4) HBSS was removed with the help of vacuum from the T-flask (away from cells) and added 1mL of trypsin to the flask (on non-cell side) for ~30 s for 293T, ~1 min for CB-MSC and ~3 min for hBMSC, occasionally swirling to dissociate cells. The 5 mL medium was added to the flask.
5) The cell detachment was monitored under the microscope.
6) The Trypsin/medium/cell suspension was collected in a 15 mL test tube and centrifuged for 6 min (at 600 rpm) so that the cells settle at the bottom of the test tube as a pellet.
7) Supernatant was removed with the help of vacuum (trypsin/medium) getting as close as possible to the pellet (without touching the cells).
8) 10 mL of medium was added to the test tube and mixed.
9) The cell suspension was splitted equally between 10 T-25 flasks (1 mL in each flask).
10) 9 mL medium was added to each flask.
11) The flask were put (on its side) in the incubator (made sure that the cap is loose).

3.5.4 Cell Counting in Multiwell Plates

1) Medium was removed and cells were washed with 0.5 mL (48 wells) or 1 mL (24 wells) HBSS.
2) HBSS was removed and 50 µL of Trypsin solution was added (48 wells) or 100 µL (24 wells) to each well and incubated for 5 minutes. Observed cell detachment under the microscope.
3) Cells were pooled from the first well to the other wells of the same kind.
4) Each well was washed with 100 µL tissue culture medium and combined all solutions and measured the volume of cells.
5) The cells were mixed by gently flushing the cell/medium mixture up and down with a pipet.
6) Two independent 1:10 dilutions were made (if necessary) of the cell suspension.
7) Cells were counted with hemocytometer (did two counts from each dilution).

*It should be noted that, only those cells were counted which were within two inner lines of the triple line boundary of the four 1 mm^3 squares (each of these squares is made of 16 small squares).

The number of cells per mL = Number of cells divided by 4 X 10,000 X dilution factor. For example, if average count is 96 cells in all four squares and cells were undiluted:

Number of cells/mL = 96/4 X 10^4 X 1 = 24,000 cells/mL

3.5.5 Seeding 48-well plates with cells

1) The medium was removed from the T-flask using vacuum and rinsed the flask with 6 mL sterile HBSS.
2) 1 mL trypsin was added and the flask was gently shaken observing cell detachment under the microscope.
3) After the cells were detached, 9 mL of basic medium (with 10% FBS) was added.
4) The cell suspension was pipetted out of the T-flask and transferred it to the 50 mL centrifuge tube.
5) The cell suspension was centrifuged at 1000 rpm for 7 minutes.
6) The supernatant was removed and small amount of medium was added to re-suspend the cells.
7) Sufficient medium was added till enough volume of cell suspension was achieved to seed the plate.
8) 500 µL of cell suspension was added into each well for 48-well plate (1000 µL for 24-well plate) and incubated at 37^oC, 5% CO_2 for 24-48 h (depending on the cell concentration).

3.6 *In vitro* transfection

For monitoring the in vitro gene expression in the mammalian cell lines, reporter gene encoding for Green fluorescent Protein (GFP) was used.

Green fluorescent Protein (GFP): It is a fluorescent protein isolated from coelenterates, such as pacific jellyfish, Aequoria victoria (Morin J.G. and Hastings J.W.-1971). By energy transfer, it transduces the chemiluminiscence of another protein aequorin into green fluorescent light (Ward W.W.- 1979). GFP has expressed in bacteria, yeast, slime molds,

plants, drosophila, and zebra fish and in mammalian cells. It can act as a protein tag, as it tolerates N-and C-terminal fusion to broad variety of protein many of which have been shown to retain native function. In mammalian cells, wild type GFP expression is distributed throughout the cytoplasm and nucleus, but excluded from the nucleolus and vesicular organelles. The enormous flexibility of GFP as a non –invasive marker in living cells paves way to numerous another applications such as a cell lineage tracer, reporter of gene expression and as potential measure of protein- protein interactions (Mitra R.D. et al.- 1996). GFP is composed of 238 amino acids. Wild type GFP from jellyfish has two excitation peaks, a major one at 395nm and a minor one at 475nm with extinction coefficient of 30,000 and 7,000 Lmol-1cm-1, respectively. Its emission peak is at 509nm in the lower green portion of the visible spectrum. In GFP, the fluorophore originates from an internal Ser-Tyr-Gly sequence which is post translationally modified to a 4(p-hydroxybenzylidene) –imidazolidin -5-one structure.

Cells were seeded into 96-well plates at a density of 10,000-15,000 cells per well and incubated at 16 h for adherence. Subsequently, the cells were treated with complexes prepared by taking a known amount of pDNA, mixed with various concentrations of polymer or polymer conjugates to form nanoplexes (or nanoparticles) in 150 mM saline and incubated for 4 h in an incubator. The transfection media was replaced with 200 μL of normal growth medium and cells were further incubated for 36 h.

3.7 GFP Expression Studies

3.7.1 Protocol for GFP Expression by Fluorimetry

1) Cells were seeded in 48 well tissue culture plates in 10% FBS containing DMEM media and incubated overnight in incubator at 37 ˚C and 5% CO_2.
2) Post 24 h, the cells were treated with the nanoplexes with polymer or polymer conjugates (experiment also contained positive and negative controls; untreated cells or cells treated with naked gWIZ-GFP were used as negative controls and nanoplexes with PEI25 were used as positive control).
3) The cells were allowed to incubate at 37 °C in a CO2 incubator for further 24 h,
4) After 24 h the media was removed and cells were washed with sterile HBSS.
5) The plates were read in a fluorescence plate reader (λEX, 485 nm, λEM, 527 nm).

6) The results were summarized as GFP fluorescence (in arbitrary units) per well.

3.7.2 Protocol for GFP Expression by Flow Cytometry

1) Cells were seeded in 48 well tissue culture plates in 10% FBS containing DMEM media and incubated overnight in incubator at 37 ˚C and 5% CO_2.
2) Post 24 h, the cells were treated with the nanoplexes with polymer or polymer conjugates (experiment also contained positive and negative controls; untreated cells or cells treated with naked gWIZ-GFP were used as negative controls and nanoplexes with PEI25 were used as positive control).
3) The cells were allowed to incubate at 37 °C in a CO2 incubator for further 48 h.
4) After 48 h, media was removed and cells were washed with sterile HBSS.
5) The cells were trypsinized and fixed in 3.7% formaldehyde in HBSS (250 µl).
6) The GFP expression was quantified by Beckman Coulter QUANTA SC flow cytometer using FL1 channel (3000-5000 events/sample). Instrument settings were calibrated for each run so as to obtain a background level of the GFP expression of 1% for the control samples (i.e., untreated cells).
7) The results were presented as the mean GFP fluorescence for the total population and mean GFP fluorescence for GFP positive cells.

3.8 BMP-2 Production in hBMSC cells

The human Bone Morphogenetic Protein 2 (BMP-2) production assay was performed in hBMSC cells using DuoSet ELISA Development Kit (#DY355).

Capture Antibody: 180 µg/mL of mouse anti-human BMP-2 when reconstituted with 1.0 mL of PBS. After reconstitution, stored at 2-8 ˚C for up to 60 days. Diluted to a working concentration of 1.0 µg/mL in PBS, without carrier protein.

Detection Antibody: 180 µg/mL of biotinylated mouse anti-human BMP-2 when reconstituted with 1.0 mL of Reagent Diluent. After reconstitution, stored at 2-8 ˚C for up to 60 days. Diluted to a working concentration of 1.0 µg/mL in Reagent Diluent.

Standard: Each vial contained 190 ng/mL of recombinant human BMP-2 when reconstituted with 0.5 mL of Reagent Diluent. The standard was allowed to stand for a minimum of 15

min, with gentle agitation prior to making dilutions and the reconstituted standard was stored at 2 - 8° C.

Streptavidin-HRP: 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Stored at 2 - 8° C after initial use.

Solutions Required

PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2 - 7.4, 0.2 µm filtered.

Wash Buffer: 0.05% Tween 20 in PBS, pH 7.2 - 7.4

Reagent Diluent: 1% BSA in PBS, pH 7.2 - 7.4, 0.2 µm filtered.

Substrate Solution: 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine).

Stop Solution: 2 N H2SO4.

3.8 General ELISA Protocol

i) Plate Preparation:

1) The Capture Antibody was diluted to the working concentration in PBS without carrier protein and immediately coated a 96-well microplate with 100 µL per well of the diluted Capture Antibody. The plate was sealed and incubated overnight at room temperature.
2) Each well was aspirated and washed with wash buffer; the process was repeated two times for a total of three washes. After the last wash, removed any remaining wash buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3) The plates were blocked by adding 300 µL of Reagent Diluent to each well and incubated at room temperature for a minimum of 1 hour.
4) The aspiration/wash as in step 2 was repeated to make plates ready for sample addition.

ii) Assay Procedure

1) 100µL of sample or standards in Reagent Diluent was added per well and covered with an adhesive strip and incubated for 2 hours at room temperature.
2) The aspiration/wash steps were repeated.
3) 100 µL of the Detection Antibody diluted in Reagent Diluent was added to each well and again covered with a new adhesive strip and incubated 2 hours at room temperature.
4) The aspiration/wash steps were repeated.

5) 100 µL of the working dilution of Streptavidin-HRP was added to each well and again covered the plate andincubated for 20 minutes at room temperature.
6) The aspiration/wash steps were repeated.
7) 100 µL of Substrate Solution was added to each well and incubated for 20 minutes at room temperature.
8) 50 µL of Stop Solution was added to each well and gently tapped the plate to ensure thorough mixing.
9) The optical density of each well was determined immediately, using a microplate reader set to 450 nm. As the wavelength correction was not available, subtracted the readings at 540 nm from the readings at 450 nm. This subtraction did correct for optical imperfections in the plate.

3.9 Cytotoxicity assay in Multiwell Plates

1) Cells were grown in 48-well plates with 0.5 mL medium (1 mL for 24-well plates).
2) At the time of assay, the medium was removed and cells were washed with HBSS (sterile) once.
3) 0.2 mL of DMEM was added for 48-well plates (0.4 mL for 24-well plates).
4) 20 µL of polymer/polymer conjugate solution was added (dissolved in sterile ultrapure water) at desired concentration and incubated the plates for 24 hours
5) Post 24 h of treatment, the cells were washed twice with HBSS.
6) Just before 24 hours were complete, MTT was dissolved in HBSS (5 mg/mL) and filtered the solution using a Millipore sterile 0.22 µm filter and warmed the solution to 37°C.
7) 40 µL of MTT solution was added into each well that contains 0.2 mL medium. This gave an MTT concentration of 1 mg/mL (for 48-well plates). The MTT volume was adjusted if different multiwell plates were used.
8) The plate was incubated at 37°C for 2 hours and then removed the supernatant.
9) The plate was dried by dabbing on tissue paper (do not wash) and added 0.5 mL (48-well) or 1mL (24-well) DMSO.
10) The plate was incubated for 5 min in the dark and mixed to dissolve the crystals.
11) Absorbance was read on ELISA plate reader at 570 nm and calculated mean (-/+ SD) of absorbance as a measure of cell activity/cell number.