SUPPORTING INFORMATION

Polydopamine and dopamine interfere with tetrazolium-based cytotoxicity assays and produce exaggerated cytocompatibility inferences

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Experimental section

1. Dopamine (DA) and polydopamine (PDA) preparation

A 2 mM stock solution of DA was prepared by dissolving dopamine hydrochloride (DA·HCl) in double distilled water. This same solution was used for further reactions with dilution. Further, stock solution of PDA was prepared by polymerizing the 2 mM solution of DA solution at pH 8 for 12 hr using sodium hydroxide solution. Polymerization of DA under basic conditions brought about the color change from transparent to brown black. After that, the pH of the PDA solution was neutralized to ~7.0 using 1N HCl solution. The PDA solution was further purified by dialysis against the distilled water using a 3 kDa cellulose acetate dialysis membrane for 24 hr with four changes of water to remove any unreacted DA·HCl and other salt impurities. Concentration of PDA was estimated by the initial concentration of DA taken for polymerization.

2. Preparation of MTT solution

Solid MTT (5 mg/mL) powder was added in double distilled water in a 15 mL falcon tube and left for 5 min. Then MTT was dissolved by sonication for 10 min followed by vortex mixing for 10 min. This solution was filtered through a 0.22 μm syringe filter and stored at 4 °C for further use.

3.1 Reduction of MTT to formazan in cell free conditions

MTT solution was incubated with either DA or PDA solution at 1:1 molar ratio under cell-free conditions and left reacting under ambient conditions for 3.5 hr, the typical duration of incubating cells with MTT during cell viability assays. Subsequently, the supernatant was discarded and a petri dish with reaction mixture was visualized under an optical microscope for checking the formation of formazan crystals and images were recorded.

3.2 Method validation for limit of detection and limit of quantitation of PDA by MTT

A 10 mM solution of PDA was prepared by base-assisted polymerization of DA in presence of 20 mM NaOH solution in 10 mL deionised water. Separately, 5 mg of solid MTT was dissolved in 10 ml of deionised water to get a 10 mM stock solution of MTT. From the stock solution of 10 mM PDA, different aliquots were taken so as to get final concentrations of PDA as 0.5, 1.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200, 500 μM. 50 μL of MTT stock solution was added to each of these aliquots and the volume of the reaction mixture containing PDA and MTT was made up to 1 mL by using 20 mM phosphate buffer (pH 7.4). For TEM shown in Figure S1 below, the samples were aliquoted from the reaction mixture after about 30 min of incubation of PDA and MTT, and the aliquot was drop cast onto the TEM grid. The reaction mixture was incubated for another 3 hours at room temperature and centrifuged at 5000 RPM for 10 minutes to settle down the formazan crystals. The supernatant was discarded, and the formazan crystals were dissolved in 200 μL of DMSO. The solution of formazan in DMSO was transferred to polystyrene 96 well plates. Further, the absorbance was recorded at 570 nm using 96-well microplate reader. The results are shown in Figure S1 below.

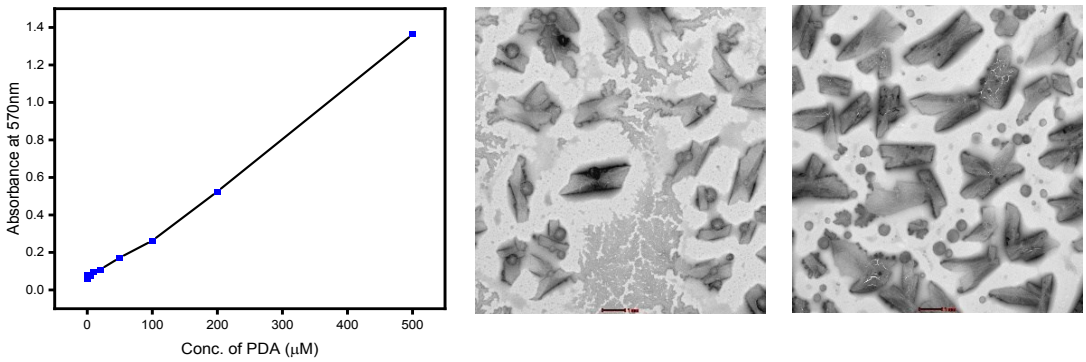

Figure S1. (**Left**) Changes in absorbance at 570 nm due to formation of formazan upon varying the concentration of PDA (at constant MTT concentration of 500 μM). The TEM images of aliquot taken 30 minutes after mixing MTT and DA (**Middle**), and MTT and PDA (**Right**), respectively, are also provided.

4. Electron Spin Resonance (ESR)

Electron Spin Resonance (ESR) spectra of aqueous solution of DA and PDA was recorded after freezing it at 180 K. First a 2 mM DA solution in pH 7.4 phosphate buffer (20 mM) was taken in ESR tube after that it was kept for 20 min for freezing at 180 K in ESR spectrometer. An equimolar mixture of DA/PDA and MTT was also prepared in the same medium and the time dependent ESR signal of a frozen samples was recorded (Figure S1A-B).

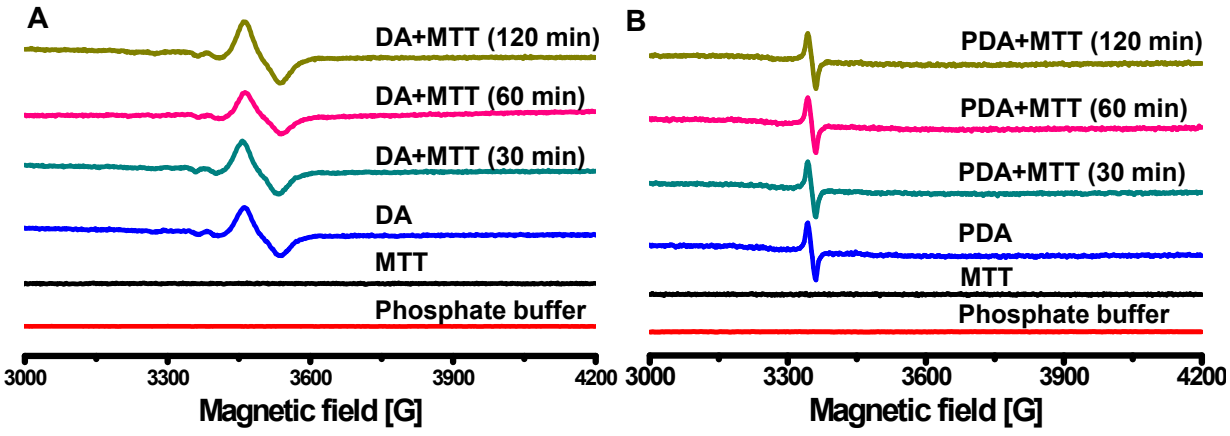

Figure S2. ESR signals of samples prepared in pH 7.4 20 mM phosphate buffer containing **(A)** dopamine (DA), MTT and their mixture at different time intervals; and **(B)** polydopamine (PDA) and MTT and their mixtures at different time intervals.

5. Cyclic voltammetry (CV)

Cyclic voltammetry (CV) profiles of DA, PDA, MTT and the reaction mixture of DA/PDA and MTT in 0.1 M KCl as electrolyte using the platinum electrode as counter electrode and Ag/AgCl reference electrode with glassy carbon as working electrode.[49] Further the time dependent voltammogram following the reaction between the DA and MTT reaction mixture was also recorded at different time points. pH of 7.4 was maintained using a 20 mM phosphate buffer during the CV measurements. CV profile was recorded in the range of -0.9 V to +0.9 V and at fixed scan rate of 0.05 V/s. All the readings were recorded after calibration of the instrument and after saturation of the electrodes.

6. Determination of the kinetic parameters

For obtaining kinetic parameters the two sets of reaction between DA and MTT were kept for time intervals ranging from 2 to 25 minutes. The first set of reaction concentrations of MTT (600 µM) was fixed and concentration of DA (200 µM, 400 µM, 600 µM) was varied. In the second set of reactions, fixed concentrations of DA (600 µM) were incubated with varied concentrations of MTT (200 µM, 400 µM, 600 µM). All the reactions were carried out in aqueous medium. The volume and pH was maintained at 7.4 using 20 mM phosphate buffer. The formazan crystals formed at different time points were dissolved in DMSO and absorbance was measured at 570 nm in UV-vis spectroscopy. The concentration of formazans was calculated from obtained absorbance values. Further, the initial rate of the reaction, order and rate constant of the reaction was determined by following the rate law equation shown below.

$r = k[A]^{x}\cdot[B]^{y}$

where r= rate of the reaction; [A]= [MTT]; [B]= [DA]; k= Rate constant; "x" and "y" are the order of the reaction with respect to MTT and DA respectively.

7. Comparison of kinetics of MTT reduction in presence of DA and PDA

Rate of reaction was determined by the incubating 500 µM of MTT with 500 µM DA and 500 µM PDA. Readings were taken in triplicates. Further, formazans formed during the reactions were quantified by recording the absorbance at 570 nm of formazans by dissolving them in DMSO at different time points. Further, rate of the reaction was determined by estimating the rate of formation of formazans. Rate of reaction = $+d[\text{formazans}]/dt$, where: t=time and [formazans] denotes concentration of formazans (Figure S3A-B). Rate of reaction was found to be 2.81×10^{-7} $M\cdot min^{-1}$. Molar extinction of formazans was taken as 13000 M^{-1} cm^{-1}.

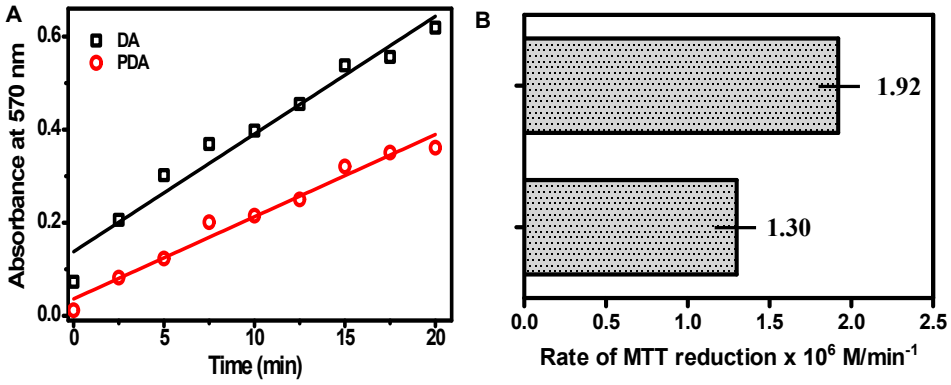

Figure S3. (A) MTT (500 µM) reduction in presence of DA and PDA concentration (500 µM) at pH = 7.4 (20 mM phosphate buffer); **(B)** Comparison of rate of MTT reduction in presence of DA and PDA.

8. pH-dependent kinetics of MTT reduction with DA and PDA

DA and PDA were incubated with MTT at different pH in aqueous medium. Concentration of reactants was fixed at 500 µM for all. The pH of the medium was adjusted to 5.6, 7, 7.4 and 8 using 20 mM phosphate buffer solution. Initial rate of reduction of MTT to formazans was calculated in accordance with the procedure mentioned above (Figure S3A-B).

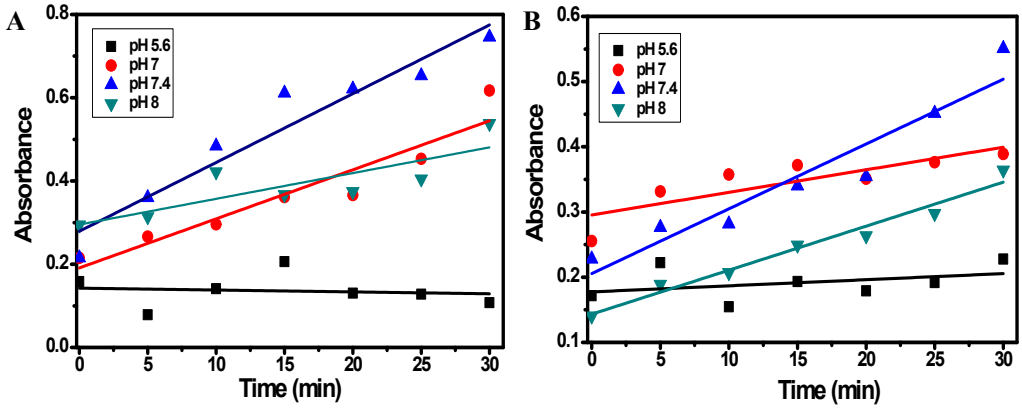

Figure S4. pH-dependent formation of formazan from MTT in the presence of **(A)** DA; **(B)** PDA.

9. MTT reduction under de-aerated conditions

500 µM concentration of each DA and MTT dissolved in 20 mM phosphate buffer, pH 7.4 was taken in separate 5 mL glass vials and purged with N_2 gas for 30 min. Then, the two solutions were mixed together and the reaction vessel was kept under positive N_2 pressure by continuous

flow of N_2 gas during the course of reaction between MTT and DA. The rate of MTT reduction was monitored by the amounts of formazans formed at different time points. Absorbance of formazans formed were plotted against time and subsequently the rate of reaction was calculated. The rate of formazan formation or MTT reduction in presence of air and N_2 was calculated as 2.83 x 10^{-6} M·min^{-1} and 2.53 x 10^{-7} M·min^{-1}, respectively, (Figure S5).

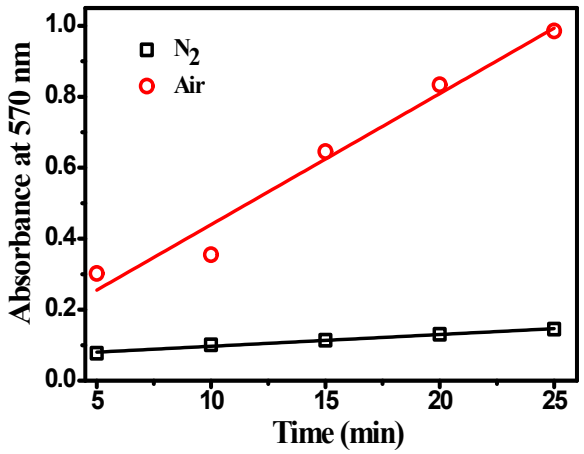

Figure S5. Absorbance at 570 nm due to the formation of formazans by the reduction of MTT with DA in presence of air (red circles) and in deaerated conditions (black squares).

10. Reduction of Nitro Blue Tetrazolium (NBT): Solutions NBT and DA/PDA were mixed together in different sets of reaction vials. The overall concentration of both the reactants were adjusted to 500 µM at pH 7.4 in 20 mM PB buffer. Formazan crystals formed at different time intervals were dissolved in DMSO and the absorbance at 570 nm was plotted against the time at which the sample aliquot was taken (Figure S5). The rate of reaction was calculated using the formula +d[formazans]/dt. The rate of NBT reduction with DA was calculated as 2.01 x 10^{-7} M·min^{-1}, while the rate of reduction in presence of PDA was 2.96 x 10^{-7} M·min^{-1}.

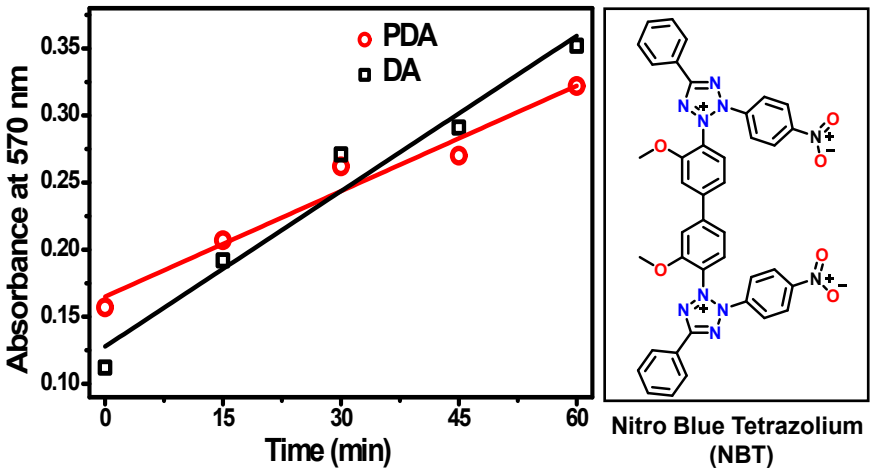

Figure S6. NBT (500 µM) reduction in presence of DA and PDA concentration (500 µM) at pH = 7.4 (20 mM phosphate buffer).

11. Cell Culture

Cancerous cell line HeLa and noncancerous HEK 293T cell line were obtained from ATCC. All cell lines were cultured at 37 ºC and 5 % CO_2 in complete media containing Dulbecco's Modified Eagle medium, 10 % Fetal Bovine Serum and 1 % penicillin-streptomycin antibiotic solution.

12. MTT assay

HeLa and HEK 293T cell lines were seeded at 4000 cells/ well in a 96 well plate. After seeding, cells were allowed to adhere to the surface overnight prior to DA/ PDA treatment. After 24 hr, the cells were treated with different concentrations of DA and PDA followed by incubation for 24 hr at 37 ºC. The treatment was given to cells in triplicates. 5 mg/mL MTT stock solution prepared in PBS in sterile conditions. A volume of 10 µL of MTT (5 mg/mL) was added to each well of the plate and incubated for 3.5 hr at 37 ºC in dark. The media was carefully removed from each well and the formazan crystals formed were dissolved in 200 µL of DMSO. Then the absorbance of formazan crystals was recorded at 570 nm in a microplate reader. The cell viability after treatment with DA and PDA was determined by using untreated cells as a negative control.

13. Effect of preparation protocol of PDA on MTT reduction

MTT (500 μM) with PDA samples (500 μM) prepared using different protocols incubated together at pH 7.4 using a 20 mM phosphate buffer. Reduction of MTT to formazans was measured by recording the absorbance of formazans formed at different time points. A linear increase in absorbance was observed with increased time intervals. PDA prepared by 20 mM sodium periodate solution showed the highest rate of MTT reduction. Rate of reduction in presence of sodium periodate and PDA-L1 were found to be 2.69 x 10^{-6} M·min^{-1} and 2.0 x 10^{-6} M·min^{-1} respectively. While in the case the case of PDA prepared by the 20 mM tris buffer and sodium hydroxide at pH 8, the rate of MTT reduction amounts to 1.84 x 10^{-6} M·min^{-1} and 1.38 x 10^{-6} M·min^{-1} respectively.

14. Total Phenolic Content (TPC):

(a) Preparation of Folin-Ciocalteu's phenol reagent:

4 g Sodium tungstate and 1 g sodium molybdate were dissolved together in 17 ml water. After that 1.5 ml 85% phosphoric acid and 2.5 ml conc. HCl was added to this solution. This mixture was refluxed for 10 hr. Subsequently, heating bath under the vessel was removed and it was allowed to cool down ambiently. Then, 4 g of sodium sulphate, 2 ml water and one drop of bromine were added to it. This mixture was further refluxed for 15 min, cooled to room temperature and diluted with water to bring the volume to 25 ml.

(b) Determination of the total phenolic content:

0.250 g Gallic acid was dissolved in 50 ml water as stock solution. For standard calibration curve, 0.1, 0.2, 0.3, 0.5 and 1 ml of this stock solution was taken and diluted with water to 10 ml to obtain final Gallic acid concentrations of 50, 100, 150, 250, and 500 μg/ml, respectively. From each solution, a 20 μl aliquot was taken into separate vials and then 1.58 ml water, and 100 μl of the Folin-Ciocalteu reagent was added and mixed well. After 5 min, 300 μl of 20% Na_2CO_3 solution was added and kept the solutions at 40 °C for 30 min. Thereafter, the absorbance of each solution was measured at 770 nm and a plot of absorbance vs. concentration generated (Figure S7). Subsequently, 20 μl of each PDA sample was mixed separately with same reagents, as performed for construction of standard calibration curve. Determined the absorbance at 770nm and then created the calibration curve and determined total phenol content in each PDA samples and Results reported as Gallic acid equivalent (GAE)

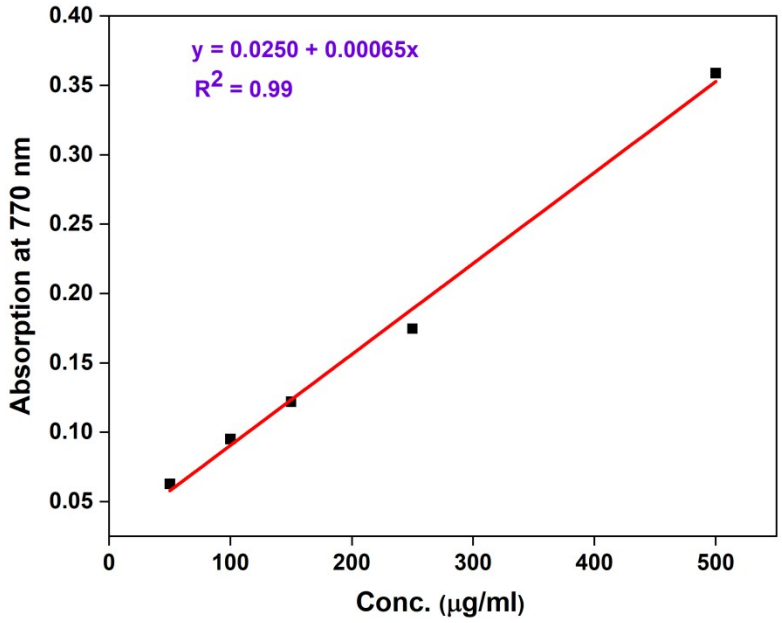

Figure S7. Standard calibration curve of absorbance at 770 nm vs. the concentration of Gallic acid upon treatment with Folin-Ciocalteu reagent.

Table S1. Summary of variation in MTT reduction rate, total phenol content, estimated hydrodynamic diameter by DLS and Zeta potential for the PDA samples prepared by different protocols

Method of PDA preparation	Rate of MTT reduction ($\mu M \cdot min^{-1}$)	TPC (μg GAE$\cdot mL^{-1}$)	Particle Size (nm)	Zeta Potential (mV)
PDA-$NaIO_4$	2.69	280	125	9.2
PDA-Tris	1.84	152	1780	29.9
PDA-NaOH	1.38	255	>6000	-5.6

Trypan Blue assay

Trypan blue assay was carried out following the previously reported protocol.[1]

1. W. Strober, *Curr. Protoc. Immunol.,* **1997**, *21*, A3.B.1–A3.B.3.