Supplementary Information

Ultra-sensitive Antimicrobial Resistance Gene Detection Using Hybridization Chain Reaction employing Carbon dots.

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Supplementary Information

Section S1: Experimental Section

S1.1 Materials and Methods

Pistachios was collected from the local market and were used after consumption of kernels. Glacial acetic acid (CH_3COOH) was procured form SRL Chemicals Pvt. Ltd., India. Ethylene diamine (EDA) was purchased from Sigma Aldrich. All the chemicals were used as received. Deionized (DI) water was used for hydrothermal treatment and preparation of buffer solutions. Dialysis tubing (benzoylated) with MWCO of 1 KDa and a diameter 32 mm was procured from Sigma Aldrich, USA. All the pictures were captured in bright daylight conditions using iPhone 13. A 50 mL 304 stainless steel autoclave reactor was used for the hydrothermal treatment, with the outer shell having a diameter of 60 mm and 127 mm height and the inner lining of polytetrafluoroethylene (Teflon) with a diameter of 48 mm. Sonication of PCDs before the sensing experiments was performed using Labman Ultrasonic Bath (Model number LMUC-6), operating at a fixed frequency of 40 kHz and power of 150 W. The capacity of the ultrasonic bath is of 5 litres.

S1.2 Probe Designing

HCR probes, H1 and H2 were designed using NUPACK software. Three different HCR probes were designed after careful optimization using the software for sensitive and specific detection of the target genes. First and second set of HCR probes were synthesized as reported. The 3rd set was designed by us. All the 3 sets were purified and purchased from Sigma Aldrich Company. Reconstituted Master DNA oligoes stock was further diluted in SPSC buffer ((50 mM CH3COONa, 1 M NaCl, pH 7.5) to make a working stock of Total Volume 50 µL. Gel electrophoresis (operating conditions of 150 V and 90 minutes) was used for characterising DNA nanostructures. 1.5 % agarose gel, 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA; pH 8.3) 2), SYBR safe (Invitrogen TM DNA Staining Dye), Gel Casting tray and Syngene G: BOX system was used for Gel Electrophoresis.

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Table S1 DNA Sequences Used for the Validation of Fluorescence Assay

Sr. no.	Hairpin probe	Stem length (bp)	Toehold region length(bp)	Loop region (bp)	Oligonucleotide sequences
1.	H1	18	6	6	TTAACCCACGCCGAATCCTAGACTCAAAG TAGTCTAGGATTCGGCGTG
	H2	18	6	6	AGTCTAGGATTCGGCGTGGGTTAACACGC CGAATCCTAGAC TACTTTG
	Target DNA	-			AGTCTAGGATTCGGCGTGGGTTAA
2.	H1	16	15	10	AAACAAAGCCACACCACTGTTCAGGTTGG TGTGGCTTTGTTTGCTTCA TTCATTGCT
	H2	16	10	15	ACCTGAACAGTGGTGTGGCTTTGTTTAGC AATGAATGAAGCAAACAAAGCCACACCA
	Target DNA	-			AGCAATGAATGAAGCAAACAAAGCCACA CCA
3.	H1	16	10	10	GGATCTGGGCGGTCTGGTCATCGGTCAAA AGCCAAAGACCGATGACCAGACC
	H2	16	10	10	GACCGATGACCAGACCGCCCAGATCCGG TCTGGTCATCGGTCTTTGGCTTTT
	Target DNA	-			GACCGATGACCAGACCGCCCAGATCC

Table S2 Oligo synthesized Report of DNA Sequences

Oligo name	purification	Molecular weight (g)	Melting temperature T_m^o	GC %	µg	length	µl for 100µM
H1 (6X6)	HPLC	14737	83.9	50	219.2	48	148
H2 (6x6)	HPLC	14799	83.3	50	153.9	48	104
Target	HPLC	7464	69.2	50	121.4	24	162
H1 (10X15)	HPLC	17491	88.5	43.5	576.9	57	329
H2 (15x10)	HPLC	17603	88.2	43.8	186.5	57	105
Target	HPLC	9517	77.5	41.9	273.2	31	287
H1 (10X10)	HPLC	16073	92.0	57.6	247.5	52	154
H2 (10x10)	HPLC	15938	92.3	57.6	502.0	52	314
Target	HPLC	7895	79.8	65.3	459.7	26	582

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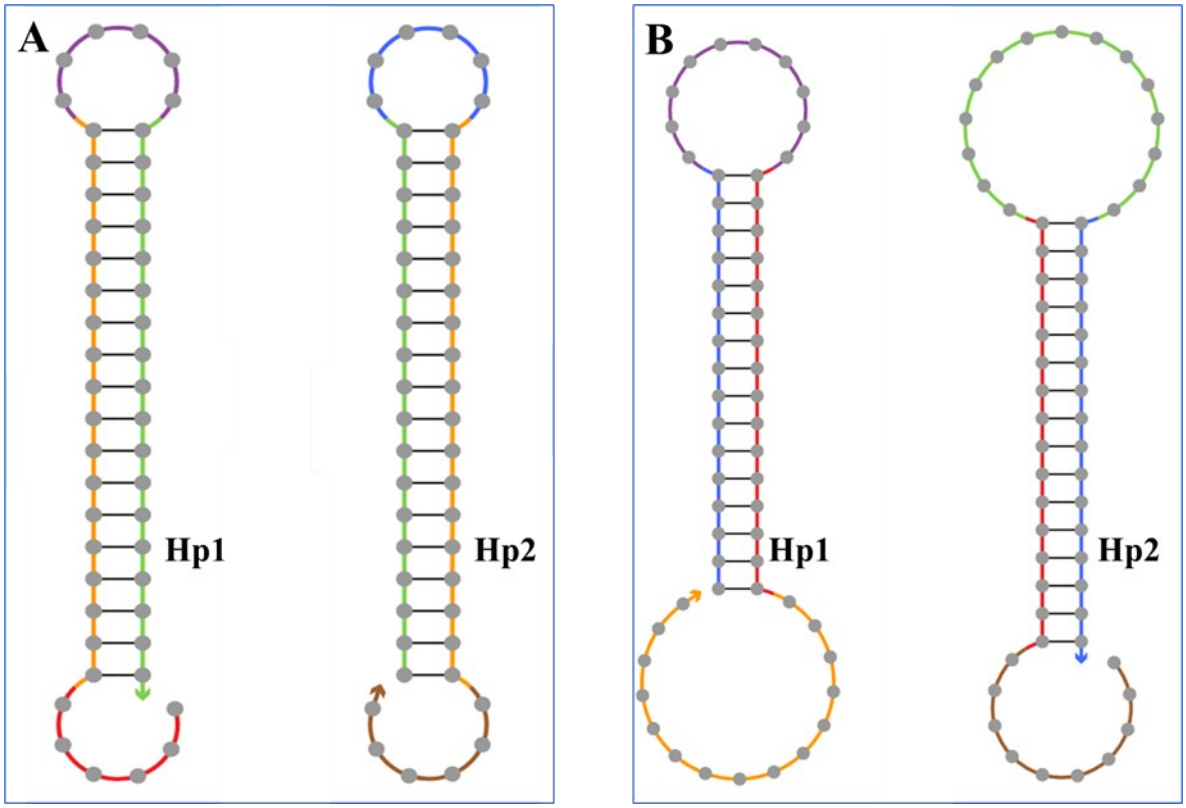

Figure S1 Hairpin structure (A) H1 and H2 :6 bp loop, 6 bp toehold and 18 bp stem length, (B) H1: 15 bp toehold length, 10 bp loop length and 16 bp stem length; H2: 10 bp toehold length, 15 bp loop length and 16 bp stem length.

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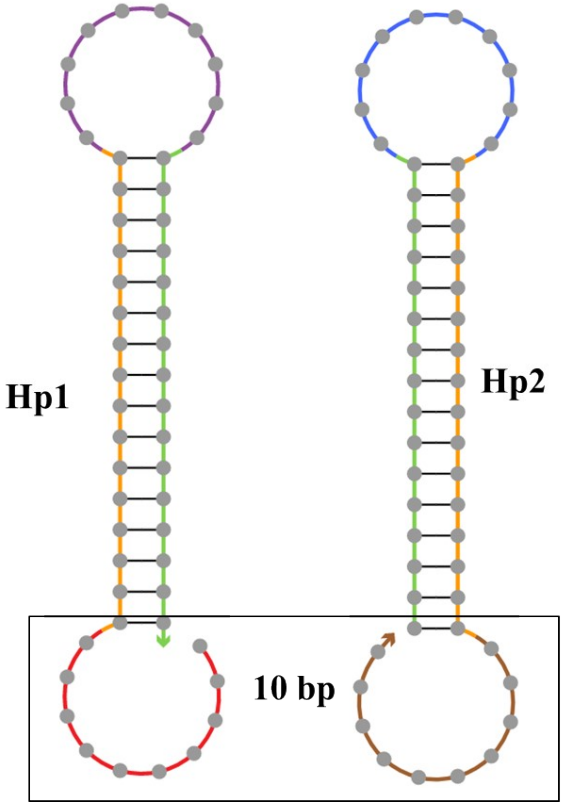

Figure S2 (A) H1 and H2 :10 bp loop, 10 bp toehold and 16 bp stem length

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NDM-1 gene sequence was retrieved and the target region was selected. The NDM-1 gene was retrieved from NCBI database (*Achromobacter sp*. NF518 betalactamase NDM-1 (blaNDM-1) gene, complete cds) in Fasta format as: >KJ018857.1 *Achromobacter sp*. NF518 beta-lactamase NDM-1 (blaNDM-1) gene, complete cds

ATGGAATTGCCCAATATTATGCACCCGGTCGCGAAGCTGAGCACCGCATTAGCCC
TGCATTGATGCTGAGCGGGTGCATGCCCGGTGAAATCCGCCCGACGATTGGCCA
GCAAATGGAAACTGGCGACCAACGGTTTGGCGATCTGGTTTTCCGCCAGCTCGCA
CCGAATGTCTGGCAGCACACTTCCTATCTCGACATGCCGGGTTTCGGGGCAGTCG
CTTCCAACGGTTTGATCGTCAGGGATGGCGGCCGCGTGCTGGTGGTCGATACCGC
CTGGACCGATGACCAGACCGCCCAGATCCTCAACTGGATCAAGCAGGAGATCAA
CCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAGATGGGCGG
TATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAAC
CAGCTTGCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCC
GCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTA
TTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCA
CCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCG
GCAATCTCGGTGATGCCGACACTGAGCACTACGCCGCGTCAGCGCGCGCGTTTG
GTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGCCATTCCGCCCCCGATAG
CCGCGCCGCAATCACTCATACGGCCCGCATGGCCGACAAGCTGCGCTGA

The coding script were pasted in the NUPACK software as represent in **Figure S3** to generate possible target sequences for HCR as represented in **Table S3**, considering parameters like pairing probability, Gibbs free energy, equilibrium concentration and Ensemble paring matches.

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Figure S3 NUPACK software window with generated Candidate hairpin probes.

Table S3 ndm DNA Sequences for fluorescence assay

Sr. no.	Hairpin probe	Stem length (bp)	Toehold region length(bp)	Loop region (bp)	Oligonucleotide sequences
1.	H1	16	10	10	GAGGATCTGGGCGGTCTGGTCATC GGTCACACCTACACGACCGATGAC CAGACCGC
2.	H2	16	10	10	GACCGATGACCAGACCGCCCAGA TCCTCGCGGTCTGGTCATCGGTCG TGTAGGTGT
3.	Target DNA *ndm*_T	-			GACCGATGACCAGACCGCCCAGA TCCTC
4.	Random DNA *ndm*_R	-			GATTGATGACCAGACGCACgCAGA CTGA
5.	Single Mismatch *ndm*_SM	-			GACCGATGACCGGACCGCCCAGA TCCTC

S1.3 Characterization

Ilshin Bio Base TFD 8503 Lyophilizer was used for lyophilization of PCDs. A Bruker Alpha IR spectrophotometer was used for FTIR determination in the range of 4000−400 cm^{-1}. DLS and Zeta potential was analysed using Malvern Zetasizer (Nano-ZS 90). A Jasco FP-6300 fluorescence spectrophotometer was used to record the fluorescence spectra of PCDs. An Agilent Technologies make Cary 60 UV-Vis Spectrophotometer was used to determine the absorbance of PCDs. A Jeol (Jem-2100F) electron microscope operated (200 kV) was employed for high resolution transmission electron microscopy (HRTEM) imaging. For HRTEM imaging, samples were dispersed on a copper grid coated with carbon. These grids were air-dried overnight at ambient temperature. XPS analysis was done on ESCA, Omicron Nanotechnology under ultra-high vacuum (UHV) conditions using X-rays AlKα with 1486 eV.

S1.4 Synthesis of PCDs

PCDs were synthesized through hydrothermal treatment of Pistachio shells. Briefly, the shells were washed thoroughly using DI water and dried in oven at 60 ºC. The dried shells were crush using grinder to yield Pistachio shell powder. 2 g of powder was added to 25 mL of the DI water, sonicated for 10 minutes and then the suspension was transferred to Teflon lined stainless steel autoclave. 500 µL of ethylene diamine was added to the suspension as a nitrogen dopant. The reaction mixture was subjected to hydrothermal treatment which was optimized as represented in figure S4. After hydrothermal carbonization, the autoclave was cooled to room temperature. The resulting suspension was filtered using 0.22 µ filter paper and centrifuged at 10000 rpm to separate the filtrate containing PCDs. PCDs were then purified using dialysis against water for 48 hours. The PCDs were stored at 4 ºC for further studies.

S1.5 Quantum Yield (QY) measurement QY measurement.

The quantum yield of PCDs was calculated using relative method. The standard used for QY determination was quinine sulfate (QS) with QY = 54% and η = 1.33. PCDs were diluted with

DI water (η = 1.33), and QS solution was diluted using 0.1 M H_2SO_4. The excitation spectra of PCDs and QS solution were recorded at their respective wavelengths. The absorbance was maintained at 0.05 in a 1 cm quartz cuvette. With the same concentration of these solutions used to record the absorbance spectra, the PL spectra were plotted. The integrated fluorescence intensities obtained for PCDs and films were compared to the QS according to the corresponding equation:

$$QY_x = QY_{std} I_x A_{std} n_x^2 / I_{std} A_x n_{std}^2$$

Here, "I" represents the measured integrated emission intensity, "n" stands for the solvent's refractive index, and "A" represents the absorbance. The "std" subscript makes a reference to the QS standard with known QY and "x" for the PCD. The quantum yield of the PCDs was obtained to be 24, which is higher than that of the previously reported biogenic carbon dots.

S1.6 Gel Electrophoresis.

The stock solutions of H1, H2 and target (T-DNA) were diluted, thawed and stored at 4 ºC before HCR studies. The diluted oligos were subjected to heat treatment at 95 ºC for 2 mins and allowed to cool at room temperature (25 ºC). The products of Hybridization Chain Reaction (HCR), obtained after injecting equimolar ratio of H1, H2 and T-DNA and incubating for 1 hour, was evaluated using Gel electrophoresis performed in 1.5 % agarose gel. The gel was run using 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA; pH 8.3) operating at 150 V and 90 minutes. Gel imaging was performed on Syngene G: BOX system under UV radiation.

S1.7 Fluorescent DNA Assay.

For the HCR based fluorescent assay, 1.5 µL of H1 (10 µM) and 1.5 µL of H2 (10 µM), was incubated with 1.5 µL of T-DNA (10 µM) in the SPSC buffer for 3 h. To this mixture, optimized concentration of WCD sensor solution was added and diluted to 15 µL using SPSC

buffer. Fluorescent measurements were performed after incubation of 10 mins, to monitor the HCR process and the final T-DNA concentration. The excitation and emission wavelengths were 300 nm and 420 nm, respectively. Control experiments were performed using pristine oligos (H1, H2 and T-DNA) and mixture of oligos (H1-H2 mixture and a mixture of H1, H2 and T-DNA). These studies were performed using the same optimised conditions as mentioned in the fluorescent assay studies.

Qualitative studies/selectivity studies were performed for the WCD sensor towards the T-DNA. For this, 1.5 µL of 10 µM solution of each H1 and H2 probes were incubated separately with 1.5 µL of T-DNA (10 µM), 1.5 µL of single-base mismatch DNA (SM, 010 µM) and 1.5 µL of random DNA (R, 10 µM), respectively, in SPSC buffer for 3 h. To all the 3 solutions, 500 µL of PCDs was added and incubated for 15 min, and fluorescence measurements were recorded. All the studies were performed at excitation and emission wavelengths were 350 and 460-470 nm, respectively. Determination of detection limits of the sensor towards T-DNA was done using various concentration of T-DNA (0-10 µM). In these studies, 1.5 µL of H1 (10 µM), 1.5 µL of H2 (10 µM) and 1.5 µL of T-DNA (0-10 µM), in SPSC buffer. After incubation of these solutions for 10 mins, fluorescence intensities were recorded. Based on these studies, Stern-Volmer plot, based on the following equation was obtained:

$$I_0/I = K_{SV}C$$

Here, "I_0" and "I" represent PL intensity of PCDs in absence and presence of T-DNA, "K_{SV}" represents the Stern−Volmer constant, and "C" is the concentration of T-DNA. From this plot, LOD and LOQ was determined to demonstrate the sensitivity of the sensor.

Supplementary Information

Section S2: Results and Discussion

S2.1 Synthesis of PCDs

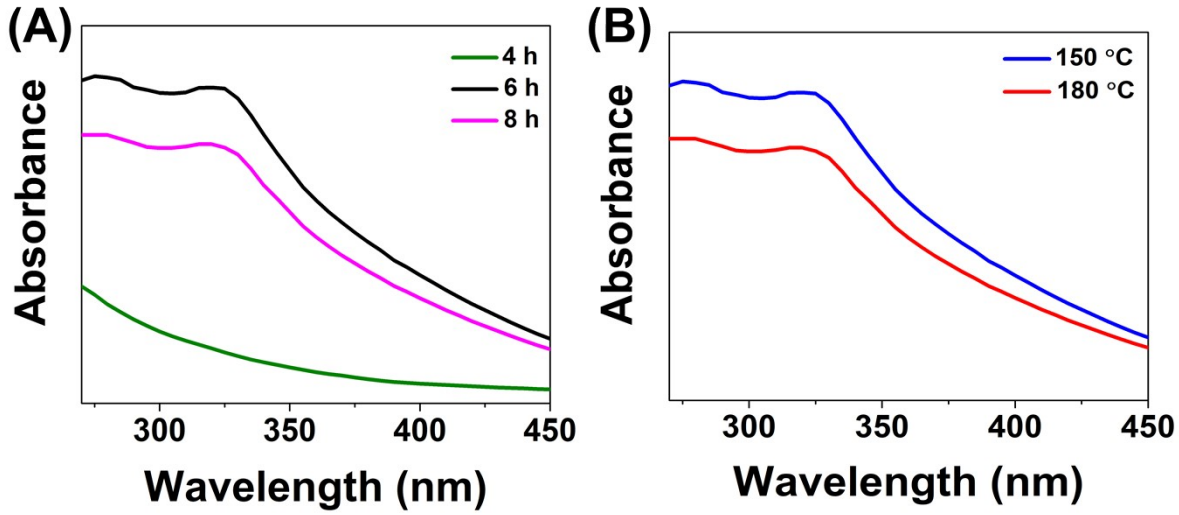

Figure S4 Optimization of (A) Time (at 150 ºC) and (B) Temperature of PCD synthesis.

S2.2 Characterization of PCDs.

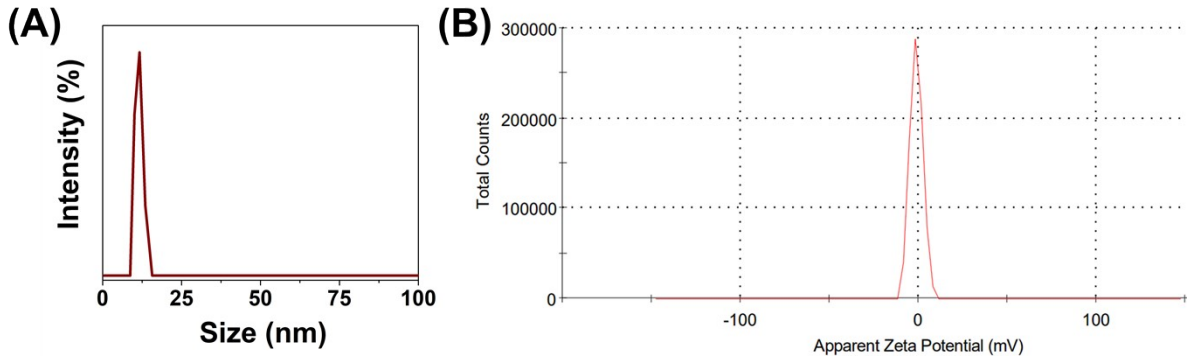

Figure S5 (A) DLS Spectra and (B) Zeta Potential of PCDs.

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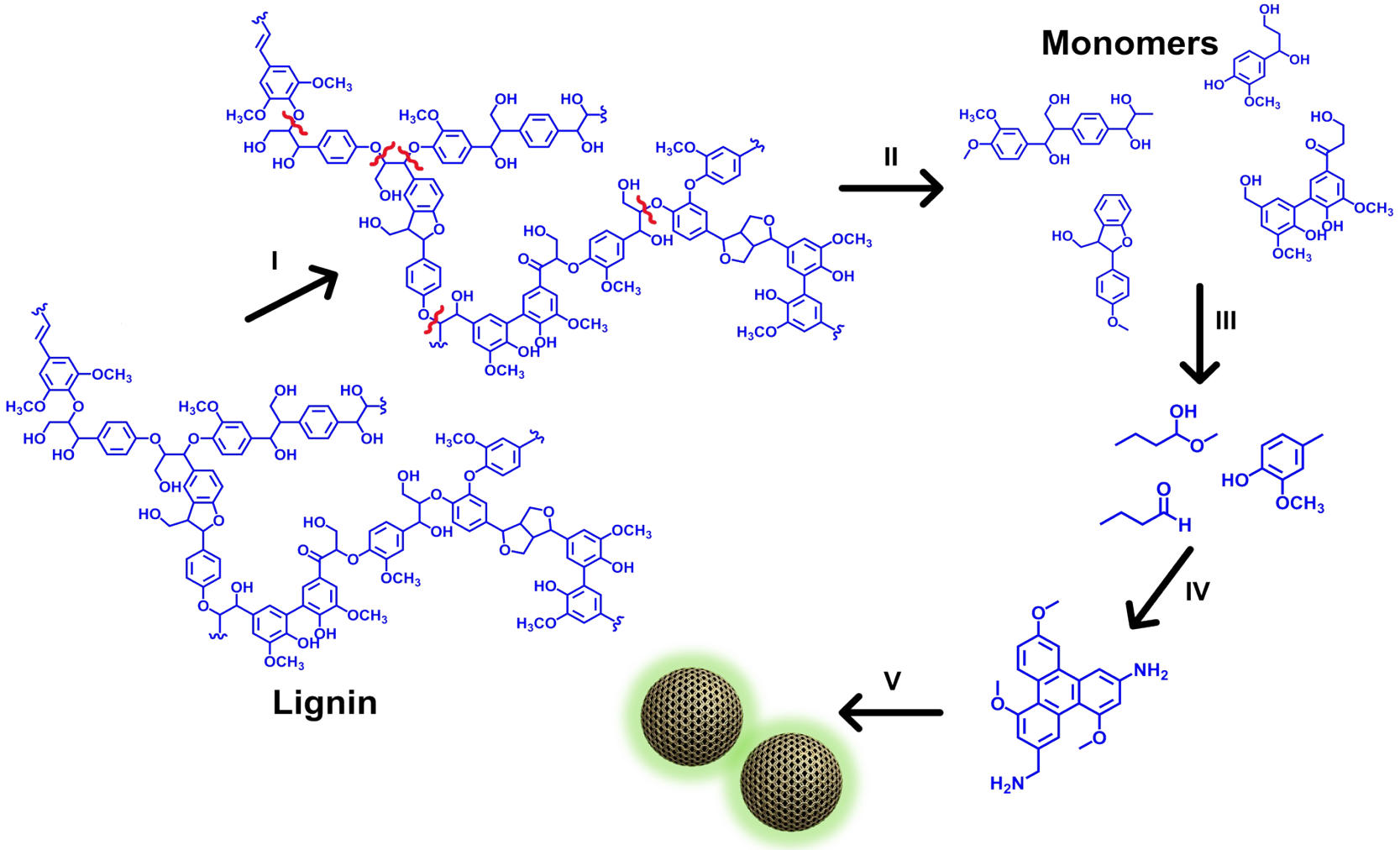

Figure S6 Mechanism of formation of PCDs

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S2.2 Characterization of HCR Product using Agarose Gel Electrophoresis.

HCR is target-DNA initiated Amplification technique, which needs to validated via Gel electrophoresis. Gel electrophoresis experiments revealed no amplification in the absence of target DNA. This is evident by low molecular weight bands in lane 1 (hp1), lane 2 (hp2) and lane 3 (hp1+ hp2) as shown in **Figure S8**. When target DNA was added with hp1 and hp2, large molecular bands was observed (lane 4). This indicates occurrence of HCR producing lengthy nicked dsDNA.

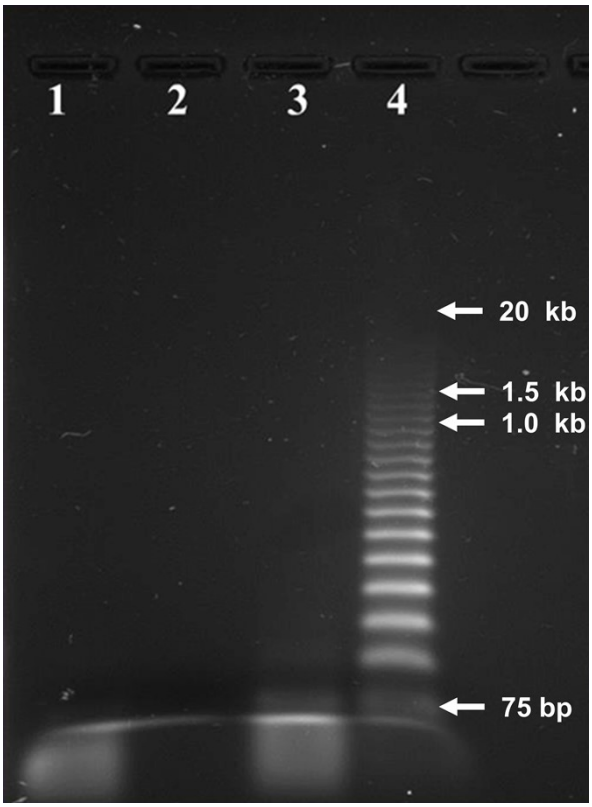

Figure S7 gel electrophoresis of HCR, lane 1: hp1, lane 2: hp2, lane 3: hp1+hp2 and lane 4: hp1+hp2+target DNA.

HCR reaction was also validated at different concentrations of SPSC buffer via Gel Electrophoresis. The studies indicated decreasing amplification efficiency with decrease in buffer concentration. As depicted in **Figure S9**, lane 2 has higher bands than lane 4 and lane 6. Lane 2,4 and 6 systems were prepared with 1x SPSC buffer, 0.1x and 0.01x SPSC buffer. Hence for further studies, 1x buffer was used.

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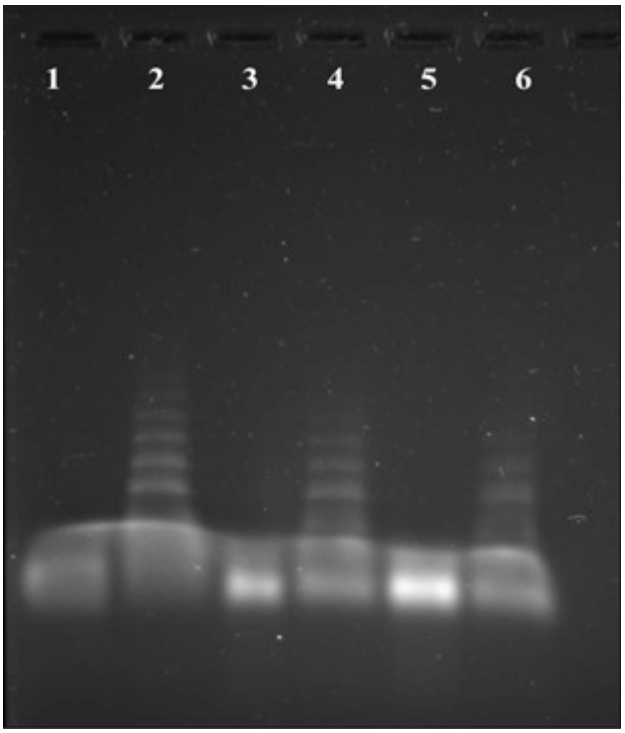

Figure S8 HCR at different buffer concentration. lane 1 and 2: 1X SPSC buffer; lane 3 and 4: 0.1x SPSC buffer; lane 5 and 6: 0.01x SPSC buffer.

S2.3 Fluorescence Sensing Studies

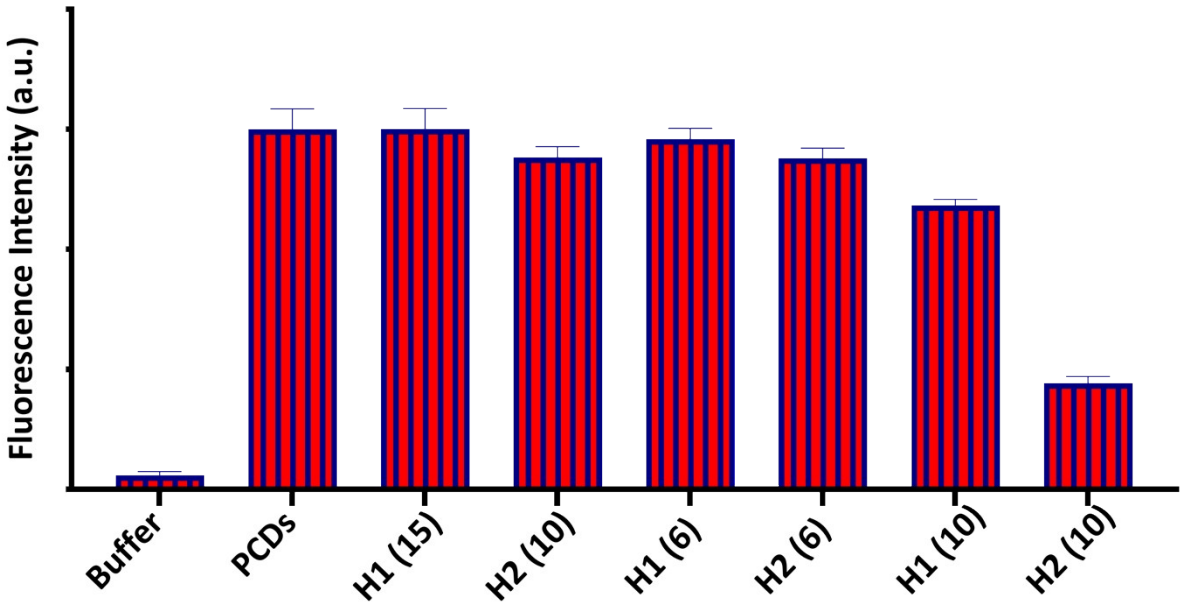

Figure S9 PL quenching of PCDs in presence of different sets of probes

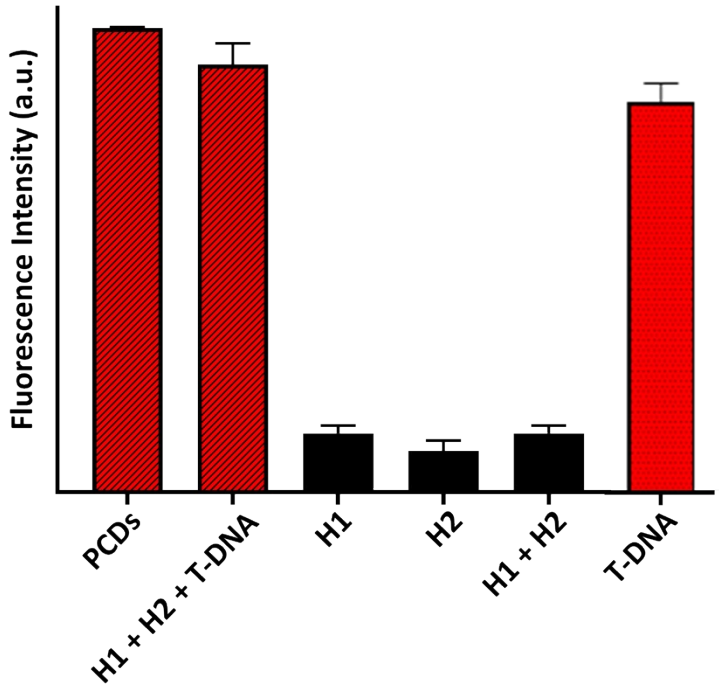

Figure S10 Fluorescence intensities of (I) PCDs, (II) PCDs + H1 + H2 + T-DNA; (III) PCDs + H1; (III) PCDs + H2, (IV) PCDs + H1 + H2 and (V) PCDs + T-DNA.

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Table S4 Comparison of Different materials for DNA detection.

Sr. No.	Material	Fluorescent Probe	Comments	Detection Limit (LOD)	Ref.
1.	Graphene oxide (GO)	FAM	Complementarity	2 nM	[1]
2.	g-C_3N_4	FAM, ROX	exonuclease III amplification	81 pM	[2]
3.	MoS_2	FAM	hybridization chain reaction	15 pM	[3]
4.	Nitrogen and Sulphur doped GO Quantum dots	HBV and HIV probe	DNA hybridization	0.17 nM, 0.19 nM	[4]
5.	terbium−carbon dots nanocomposite (CDsTb)	CDs-Tb	surface charge variations	0.012 pM	[5]
6.	silicon nanodots (SiNDs) and Ru(bpy) 2 $(dppx)^{2+}$	silicon nanodots (SiNDs) and Ru(bpy) 2 $(dppx)^{2+}$	-	4.3 nM	[6]
7.	heteroatom doped graphitic carbon nanodots (CDs)	heteroatom doped graphitic (CDs)	Base pair specific binding	-	[7]
8.	Pistachio Shell derived Carbon dots (PCDs)	No external probe required	hybridization chain reaction	16.17 pM	-

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