Supporting information

Chiral 8-AminoBODIPY-based fluorescent probes with site selectivity for the quantitative detection of HSA in biological samples

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Table S1 Photophysical data of **R-PEB**, **S-PEB** and **BB.**

Compound	Solvent	λ_{abs} (nm)	ε_{max} ($10^4 M^{-1}cm^{-1}$)	λ_{em} (nm)	$\Delta\nu$ (cm^{-1})	Φ [a]	τ [b] (ns)	k_{fl} [c] ($10^8 s^{-1}$)	k_{nr} [d] ($10^9 s^{-1}$)
R-PEB	Chloroform	415	2.80	459	2309.89	0.96	-	-	-
	Toluene	411	2.92	462	2685.87	0.71	2.52	2.82	0.12
	Ethanol	405	2.86	449	2419.64	0.11	-	-	-
	Methanol	404	2.69	447	2381.11	0.06	1.44	0.42	0.65
	DMSO	403	2.60	446	2392.37	0.07		-	-
	PBS Buffer	399	2.30	465	3557.28	0.03	0.79	0.38	1.23
	HSA (5 eq) solution	403	2.38	429	1752.63	0.26	3.80	0.68	0.19
S-PEB	Chloroform	415	2.80	459	2309.89	1	-	-	-
	Toluene	411	2.92	462	2685.87	0.64	2.90	2.20	0.124
	Ethanol	405	2.86	449	2419.64	0.11	-	-	-
	Methanol	404	2.69	447	2381.11	0.06	1.44	0.42	0.65
	DMSO	403	2.60	446	2392.37	0.07	-	-	-
	PBS Buffer	399	2.30	465	3557.28	0.03	0.78	0.38	1.24
	HSA (5 eq) solution	404	2.38	433	1752.63	0.18	4.12	0.44	0.19
BB	Chloroform	412	2.89	455	2293.82	1	-	-	-
	Toluene	409	3.05	453	2374.82	0.84	3.45	2.43	0.05
	Ethanol	403	2.98	435	1825.39	0.19	-	-	-
	Methanol	402	2.95	436	1939.84	0.09	0.82	1.09	1.11
	DMSO	401	2.63	435	1949.15	0.10	-	-	-
	PBS Buffer	396	2.44	460	3513.39	0.04	1.29	0.31	0.74
	HSA (5 eq) solution	402	2.56	430	1681.84	0.25	4.21	0.59	0.18

(a) Relative fluorescence quantum yield was obtained using ethanol solution of coumarin 1 ($\Phi = 0.75$) as reference. (b) $\boldsymbol{\tau}$ (average lifetime) = $\sum\boldsymbol{\alpha_i\tau_i^2} / \sum\boldsymbol{\alpha_i\tau_i}$, where $\boldsymbol{\alpha_i}$ and $\boldsymbol{\tau_i}$ are the relative amplitude and lifetime value of i^{th} lifetime component. (c) $\mathbf{k_{fl}}$ (radiative rate constant) = $\boldsymbol{\Phi/\tau}$. (d) $\mathbf{k_{nr}}$ (nonradiative rate constant) = $\boldsymbol{[1-\Phi]/\tau}$.

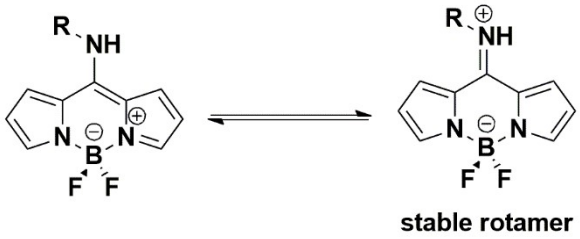

Fig. S1 Cyanine and hemicyanine resonance forms of 8-aminoBODIPY.

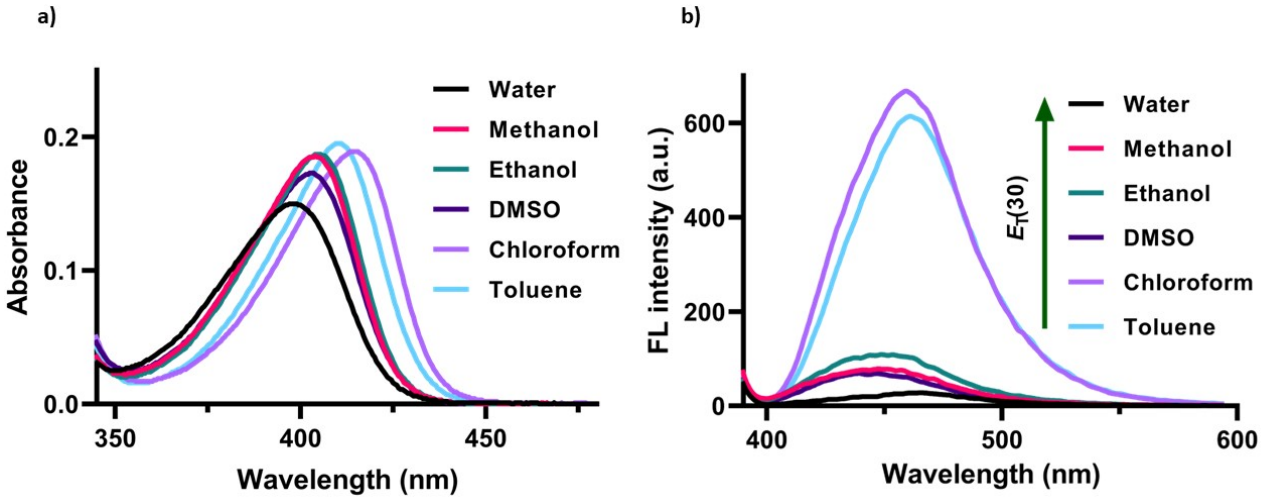

Fig. S2 Absorption spectra of (a) **S-PEB** [6.7μM] and fluorescence spectra of (b) **S-PEB** [1 μM] in solvents with different empirical polarity parameter, E_T (30); λ_{ex} = 385 nm.

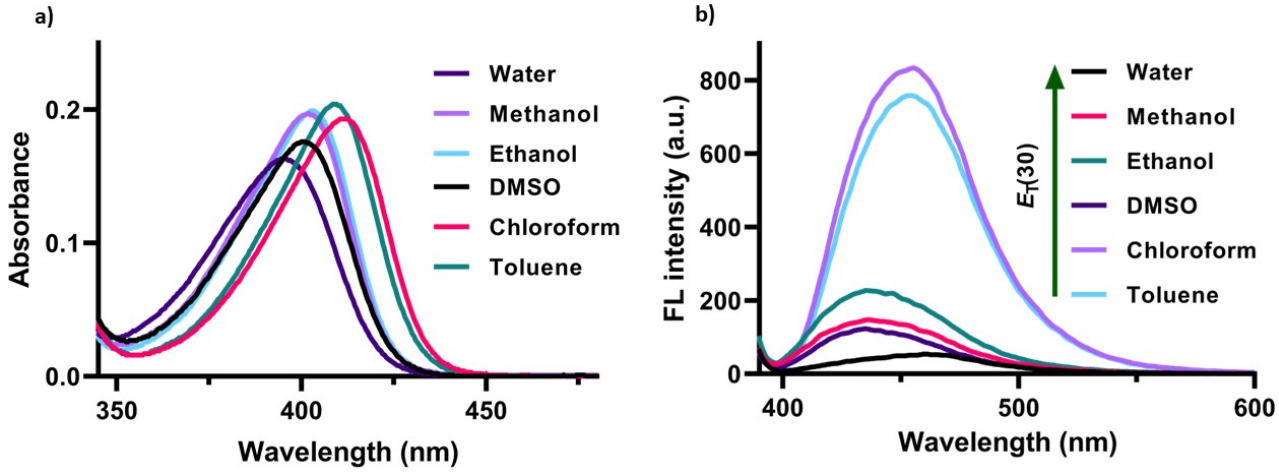

Fig. S3 Absorbance spectra of (a) **BB** [6.7 μM] and fluorescence spectra of (b) **BB** [1 μM] in solvents with different empirical polarity parameter E_T (30); λ_{ex} = 385 nm.

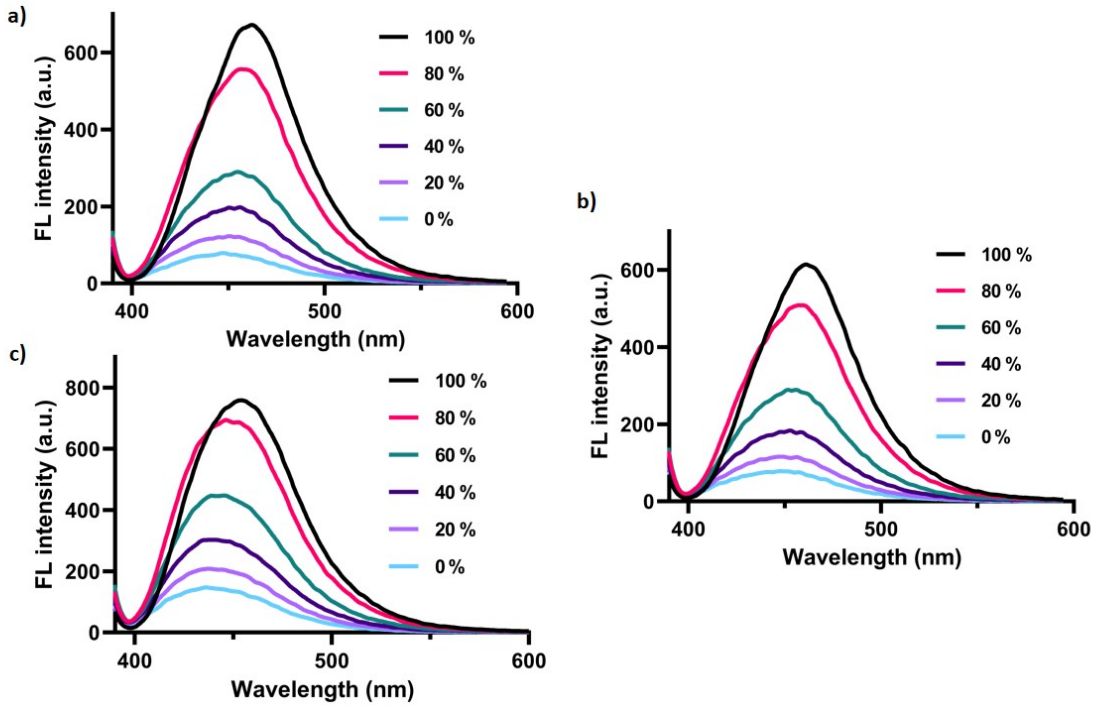

Fig. S4 Fluorescence spectra of 1 µM solutions of (a) **R-PEB**, (b) **S-PEB** and (c) **BB** on increasing the percentages of toluene in methanol. Percentage of toluene is shown in the graph; λ_{ex}= 385 nm.

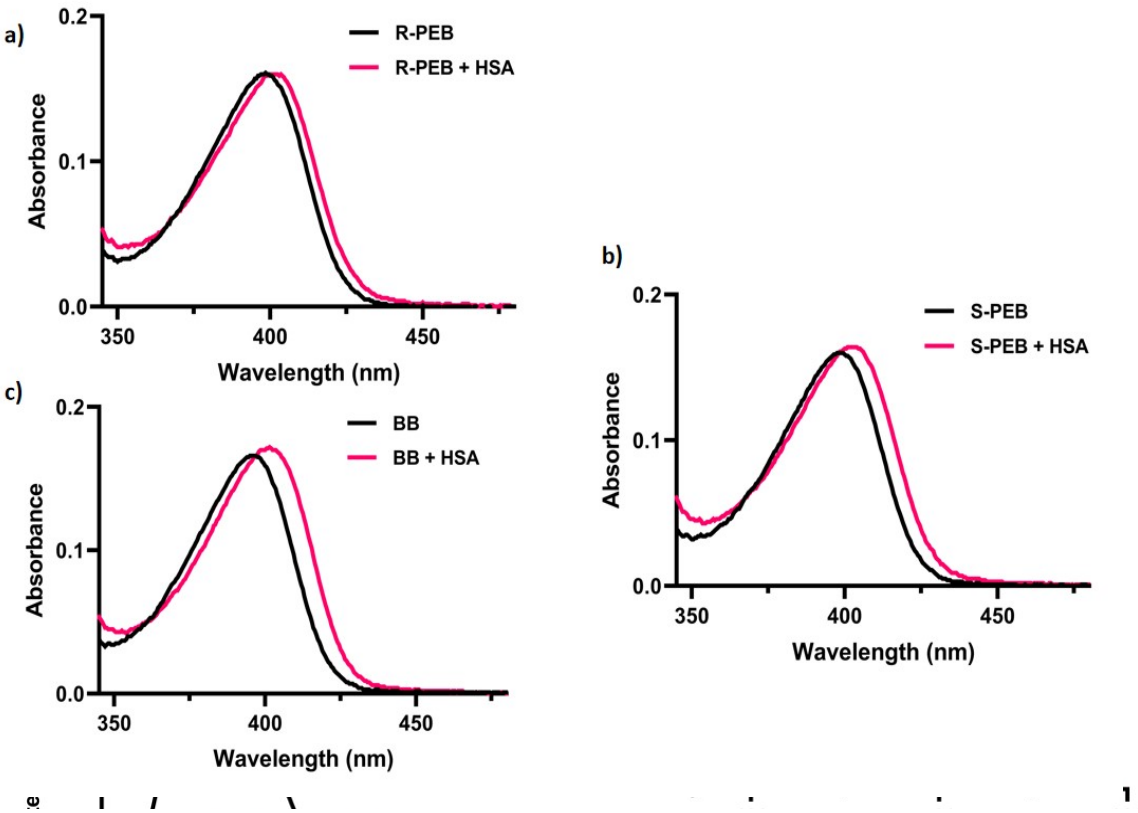

Fig. S7 Absorption spectra of 6.7 µM solutions of (a) **R-PEB**, (b) **S-PEB** and (c) **BB** in the presence of HSA (5 eq) in PBS buffer (1 mM, pH 7.4).

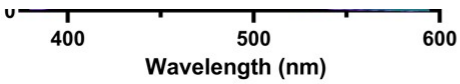

Fig. S5 Fluorescence spectra of 1 µM solutions of (a) **R-PEB**, (b) **S-PEB** and (c) **BB** on increasing the percentage of glycerol in water. Percentage of glycerol is shown in the graph; λ_{ex}= 360 nm.

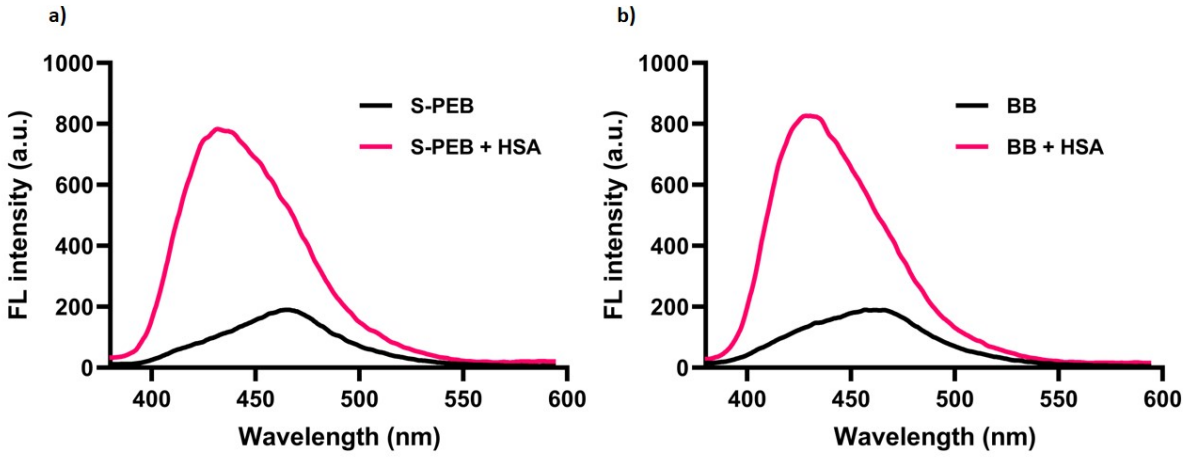

Fig. S6 Fluorescence response of 1 μM solutions of (a) **S-PEB** and (b) **BB** on addition of HSA (5 eq) in PBS buffer (1 mM, pH 7.4); λ_{ex}= 360 nm (**S-PEB**), 355 nm (**BB**).

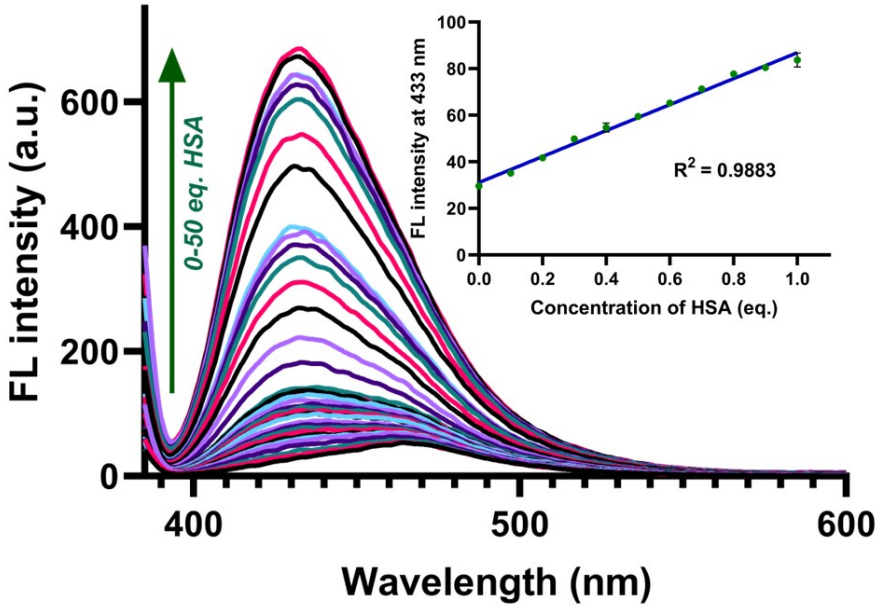

Fig. S8 Fluorescence spectra of **S-PEB** (2 μM) on addition of HSA (0-50 eq) in PBS buffer (1 mM, pH 7.4); inset- linear relationship between concentration of HSA and the corresponding fluorescence intensity at 433 nm (values are presented as mean ± SD, n = 3); λ_{ex}= 380 nm.

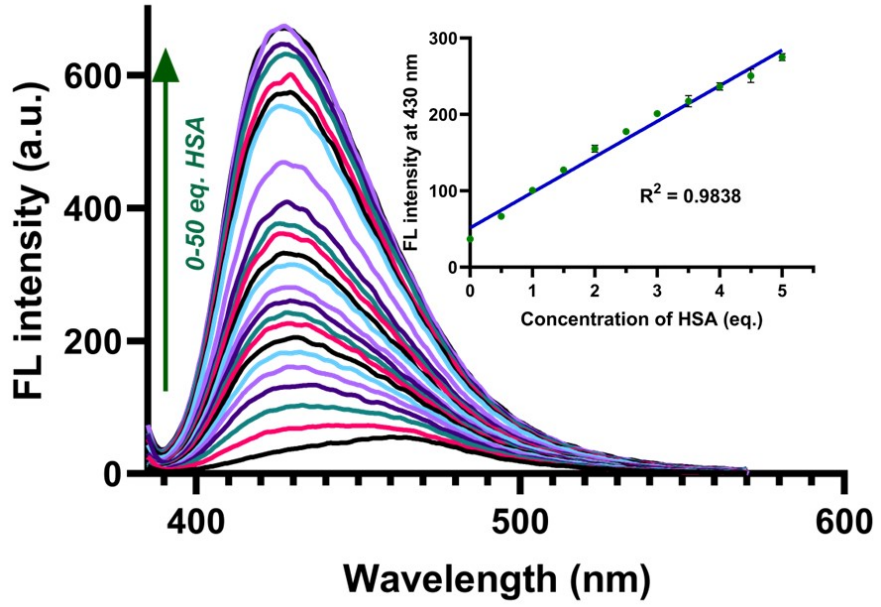

Fig. S9 Fluorescence spectra of **BB** (2 μM) on addition of HSA (0-50 eq) in PBS buffer (1 mM, pH 7.4); inset- linear relationship between concentration of HSA and the corresponding fluorescence intensity at 430 nm (values are presented as mean ± SD, n = 3); λ_{ex}= 375 nm.

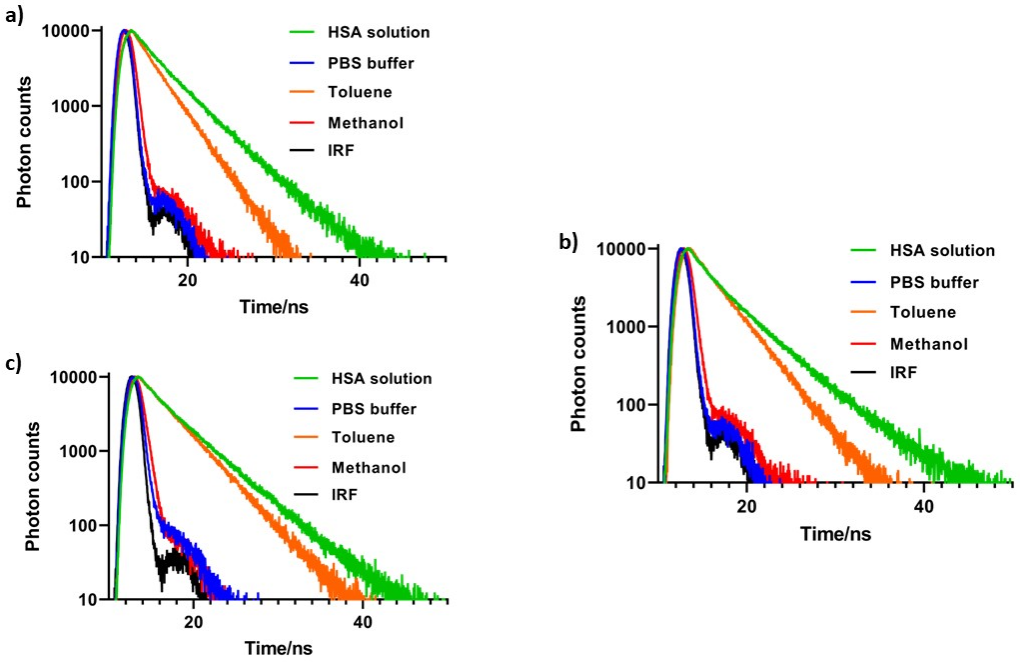

Fig. S10 Fluorescence decay profiles of (a) **R-PEB** [5 μM], (b) **S-PEB** [5 μM] and (c) **BB** [5 μM] in methanol, toluene, water and HSA solution (5 eq). Data were recorded at their respective λ_{em} using 330 nm nanoLED excitation source. IRF -instrument response function.

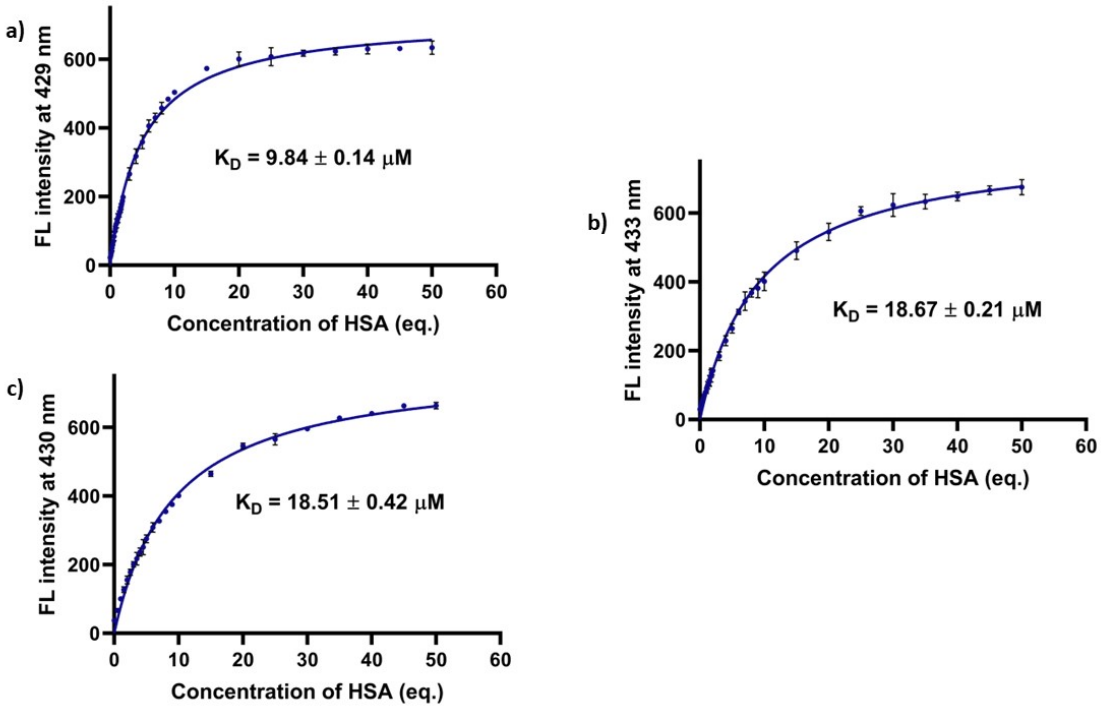

Fig. S11 Binding curves of (a) **R-PEB** [2 μM], (b) **S-PEB** (2 μM) and (c) **BB** (2 μM) with HSA (0-50 eq) in PBS buffer (1 mM , pH 7.4); λ_{ex}= 380 nm (**R-PEB**, **S-PEB**), 375 nm (**BB**). (values are presented as mean ± SD, n = 3)

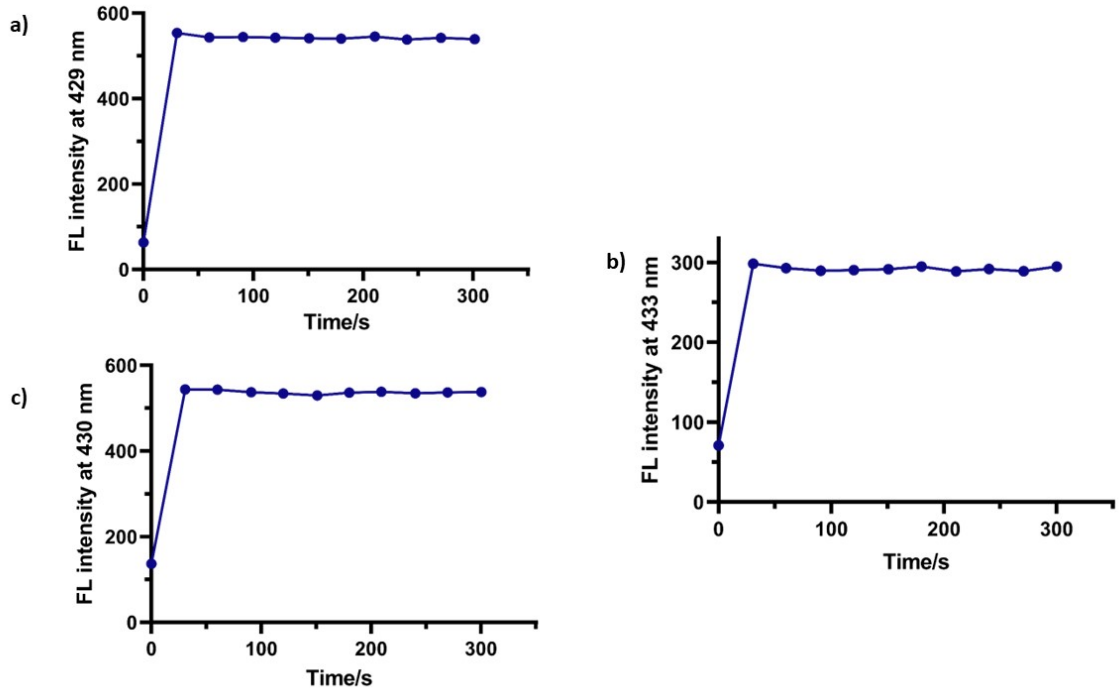

Fig. S12 Time-dependent fluorescence of 1 μM solution of (a) **R-PEB**, (b) **S-PEB** and (c) **BB** on addition of HSA (5 eq) in PBS buffer (1 mM, pH 7.4); λ_{ex}= 360 nm.

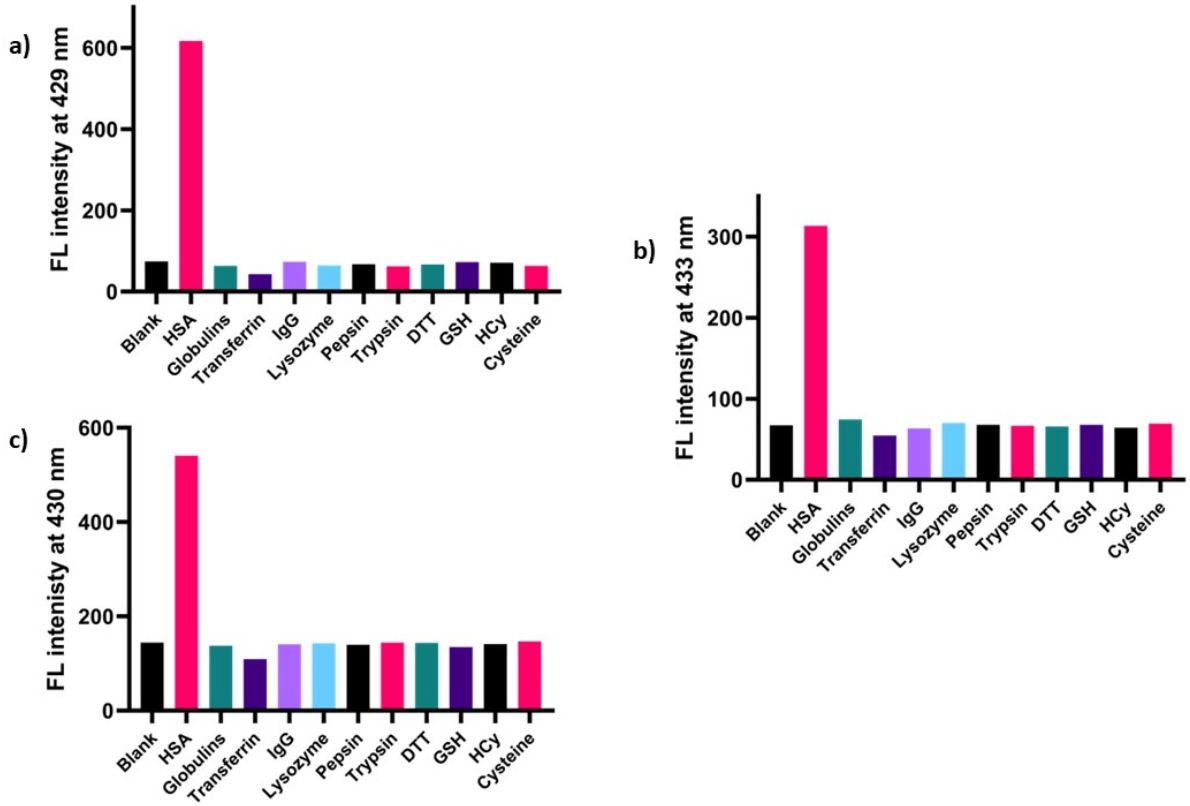

Fig. S13 Fluorescence response of 1 μM PBS solution of (a) **R-PEB**, (b) **S-PEB** and (c) **BB** in the presence of various proteins and thiols (5 eq); λ_{ex}= 360 nm.

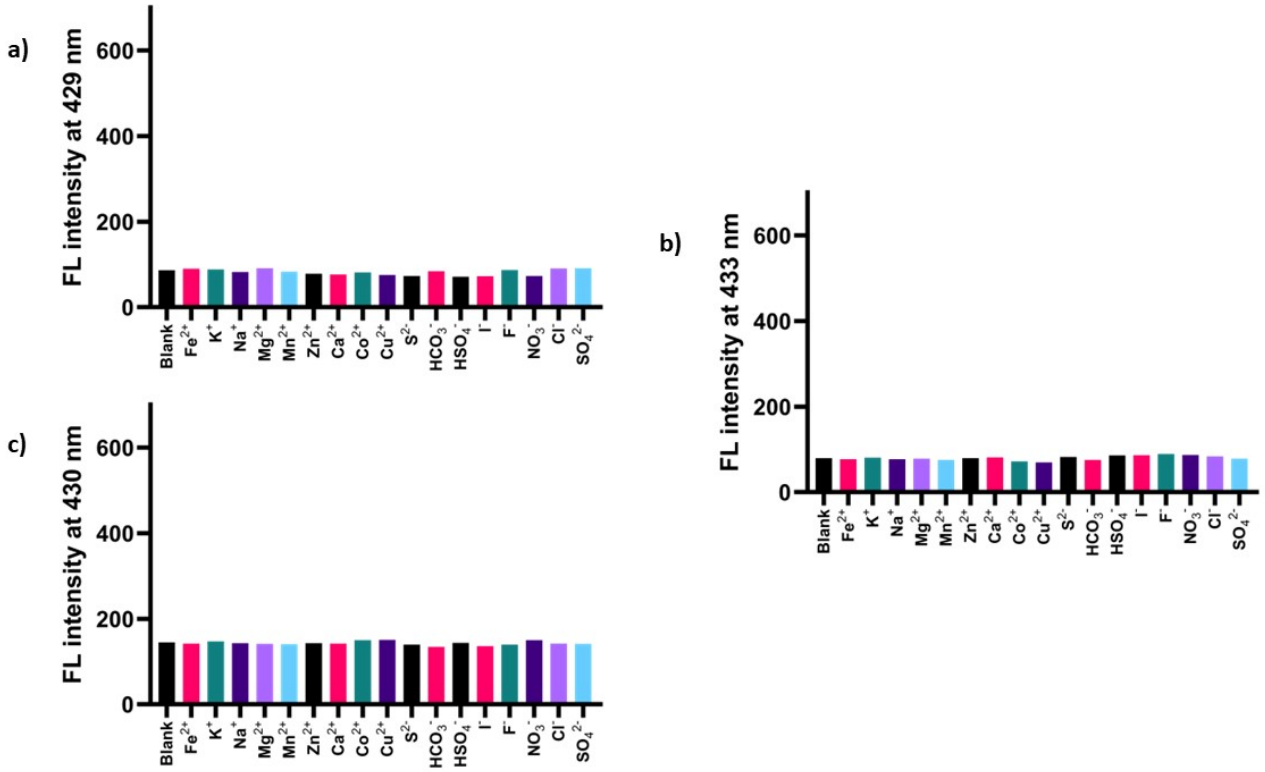

Fig. S14 Fluorescence response of 1 μM PBS solution of (a) **R-PEB**, (b) **S-PEB** and (c) **BB** in the presence of various cations and anions (5 eq); λ_{ex}= 360 nm.

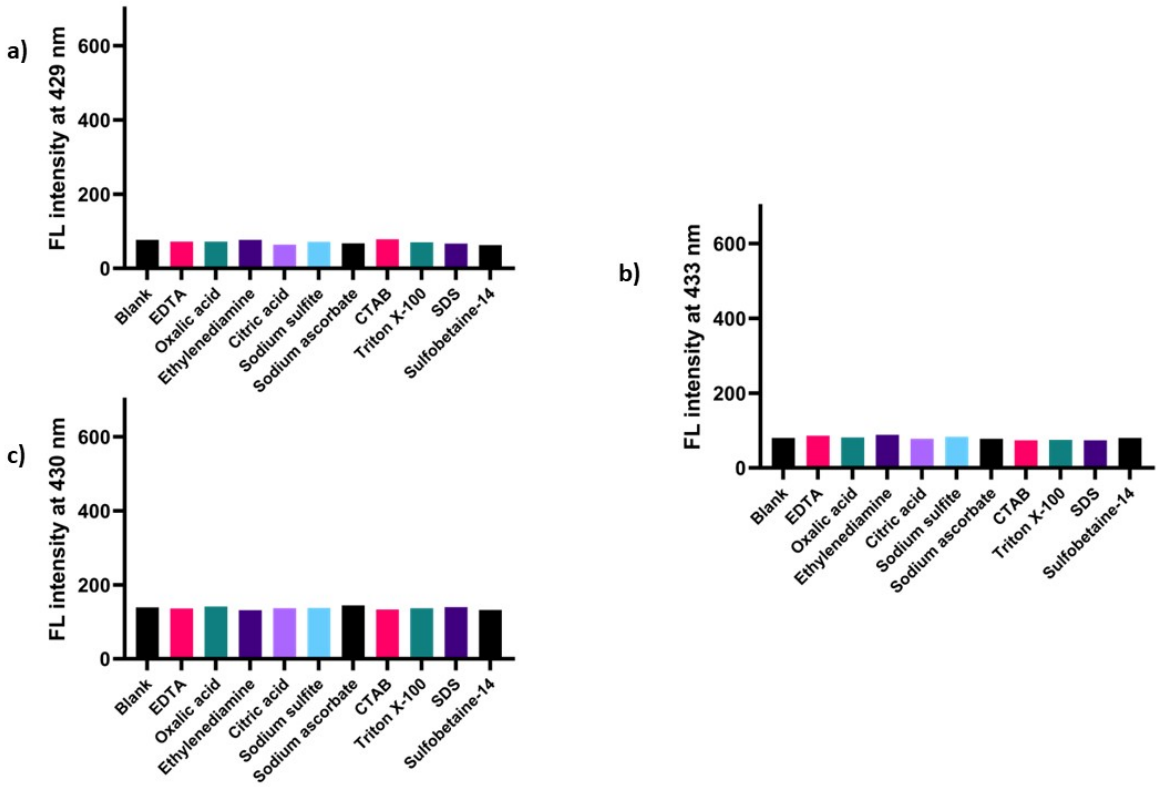

Fig. S15 Fluorescence response of 1 μM PBS solution of (a) **R-PEB**, (b) **S-PEB** and (c) **BB** in the presence of various chelates, reductants and surfactants (5 eq); λ_{ex}= 360 nm.

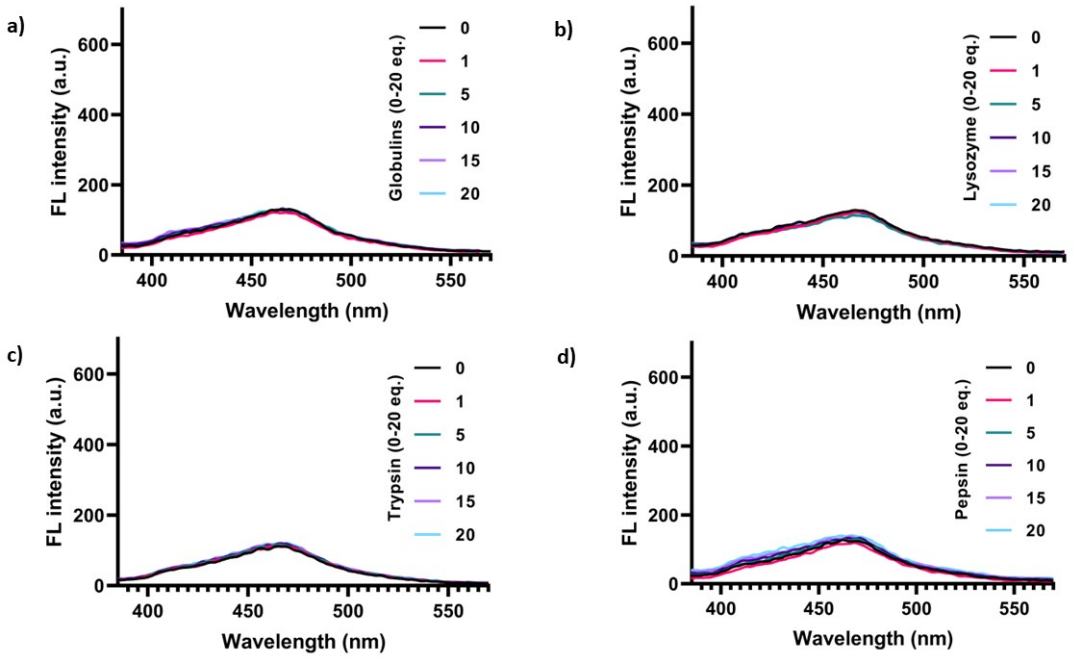

Fig. S16 Fluorescence spectra of **R-PEB** (1 μM) on addition of other representative proteins (0-20 eq.), (a) globulins, (b) lysozyme, (c) trypsin and (d) pepsin in PBS buffer (1 mM, pH 7.4), λ_{ex}= 360 nm.

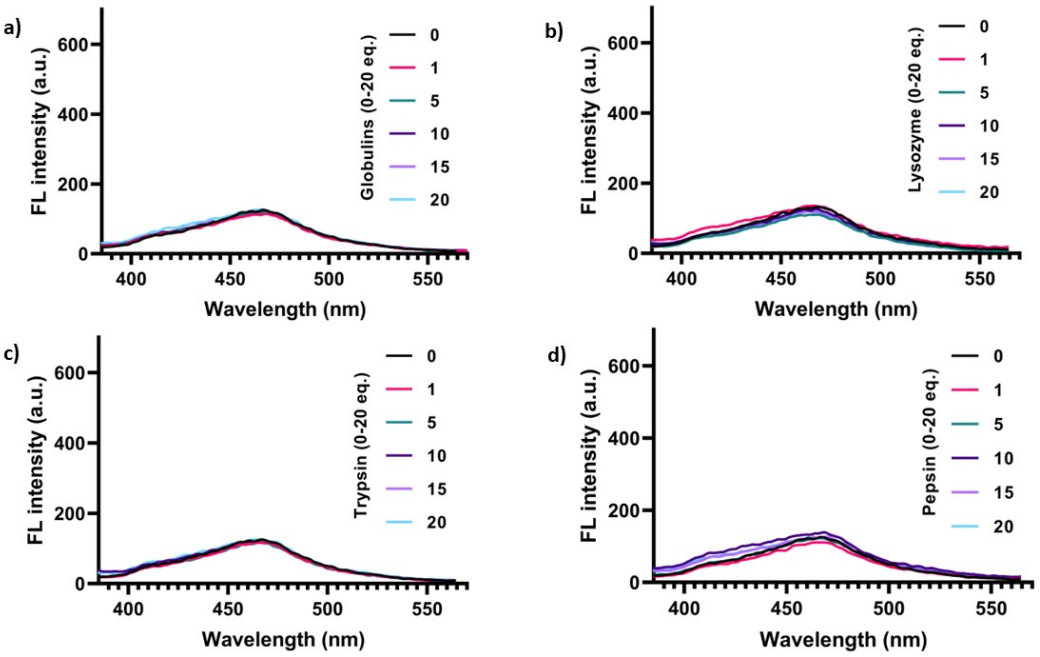

Fig. S17 Fluorescence spectra of **S-PEB** (1 μM) on addition of other representative proteins (0-20 eq.), (a) globulins, (b) lysozyme, (c) trypsin and (d) pepsin in PBS buffer (1 mM, pH 7.4), λ_{ex}= 360 nm.

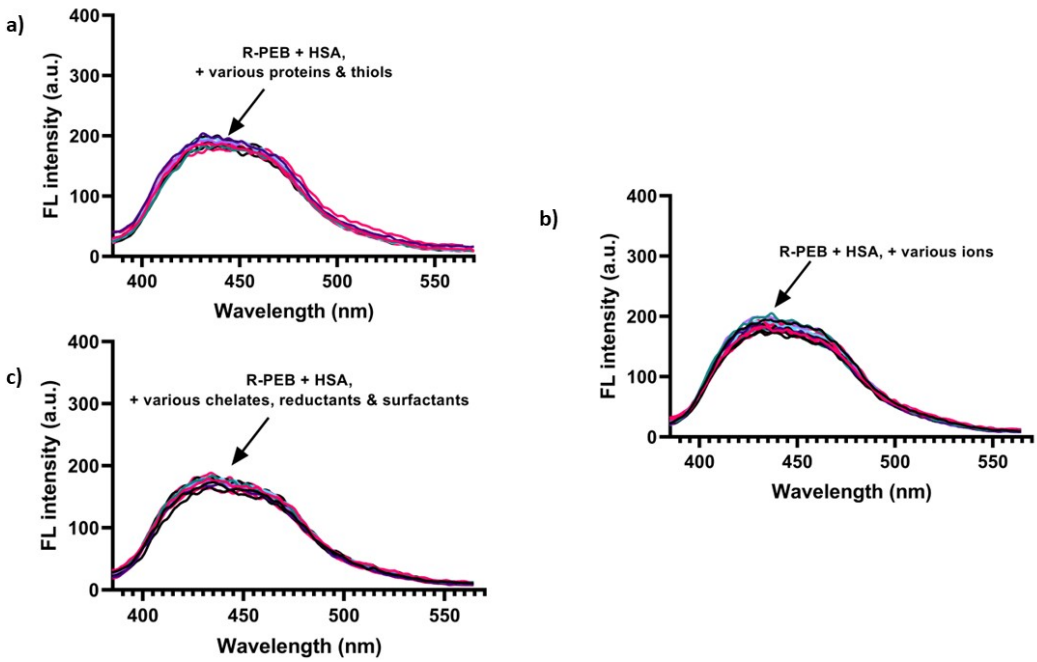

Fig. S18 Fluorescence spectra of **R-PEB** in the presence of various interfering species [proteins & thiols (globulins, transferrin, IgG, lysozyme, pepsin, trypsin, DTT, GSH, HCy, cysteine), cations & anions (Fe^{2+}, K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, S^{2-}, HCO_3^{-}, HSO_4^{-}, I^{-}, F^{-}, NO_3^{-}, Cl^{-}, SO_4^{2-}), chelates, reductants & surfactants (EDTA, oxalic acid, ethylenediamine, citric acid, sodium sulfite, sodium ascorbate, CTAB, SDS, triton X-100, sulfobetaine-14)] (1 eq) and HSA (1 eq) in PBS buffer (1 mM, pH 7.4); λ_{ex}= 360 nm.

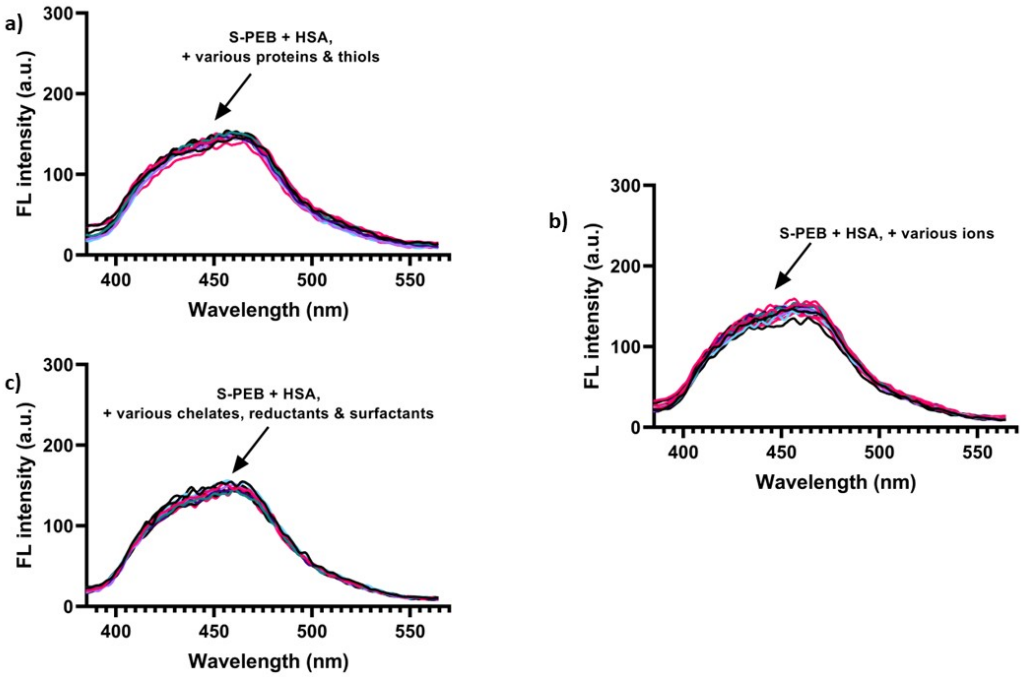

Fig. S19 Fluorescence spectra of **S-PEB** in the presence of various interfering species [proteins & thiols (globulins, transferrin, IgG, lysozyme, pepsin, trypsin, DTT, GSH, HCy, cysteine), cations & anions (Fe^{2+}, K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, S^{2-}, HCO_3^{-}, HSO_4^{-}, I^{-}, F^{-}, NO_3^{-}, Cl^{-}, SO_4^{2-}), chelates, reductants & surfactants (EDTA, oxalic acid, ethylenediamine, citric acid, sodium sulfite, sodium ascorbate, CTAB, SDS, triton X-100, sulfobetaine-14)] (1 eq) and HSA (1 eq) in PBS buffer (1 mM, pH 7.4); λ_{ex}= 360 nm.

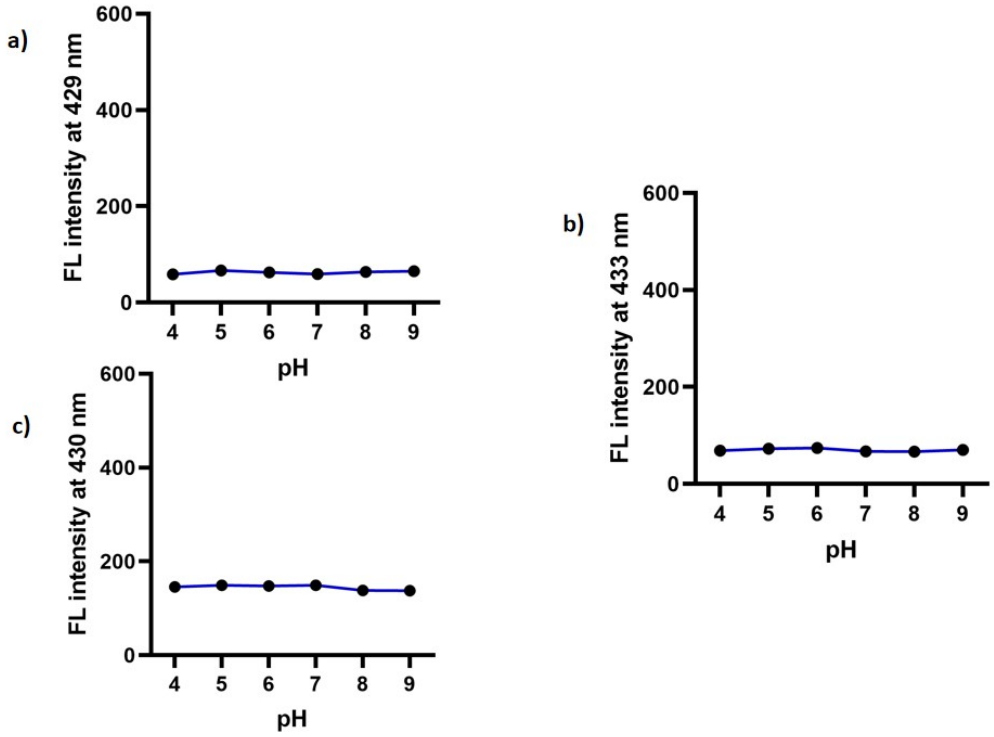

Fig. S20 Fluorescence response of 1 μM solution of (a) **R-PEB**, (b) **S-PEB**, (c) **BB** at different pH in PBS buffer (1 mM); λ_{ex}= 360 nm.

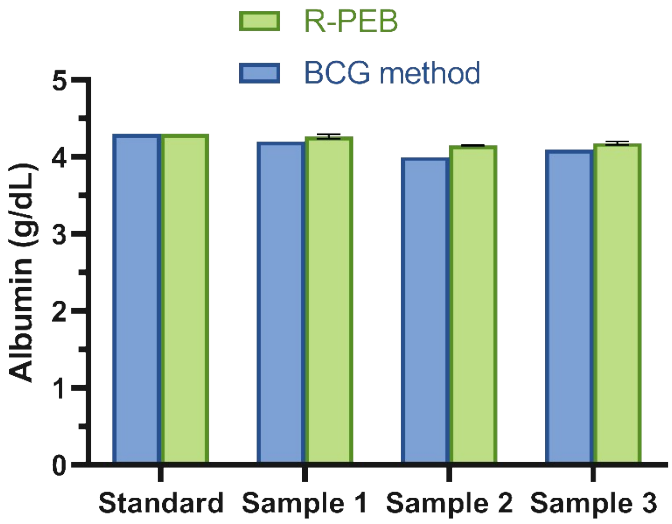

Fig. S21 Albumin evaluation results of three unknown serum samples (values are presented as mean ± SD, n = 3) using **R-PEB** [2 μM] in PBS buffer (1 mM , pH 7.4) and clinical BCG test. (Concentration of HSA in standard serum sample based on **R-PEB** was set as same as that of BCG method.)

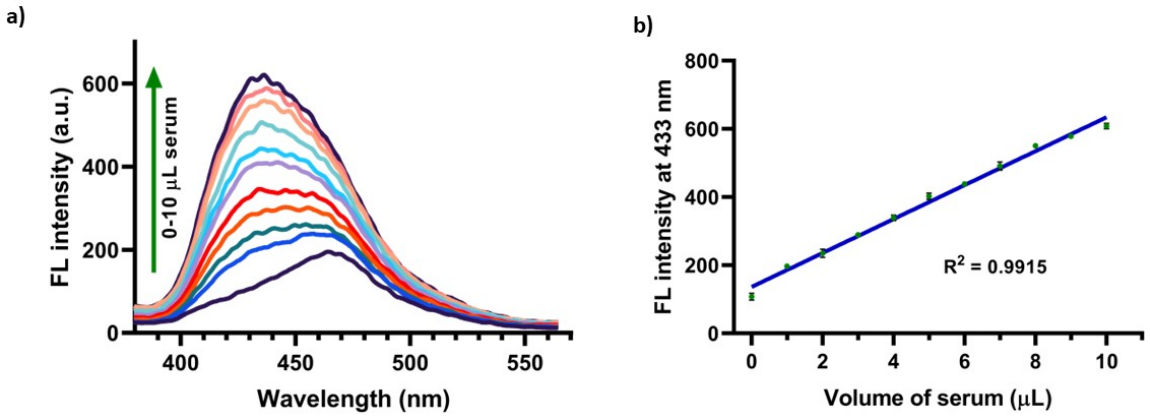

Fig. S22 (a) Fluorescence spectra of **S-PEB** (2 µM in 1 mM PBS buffer at pH 7 on adding increasing amounts of standard serum (0-10 µL); (b) the corresponding calibration curve (values are presented as mean ± SD, n = 3); λ_{ex} = 360 nm.

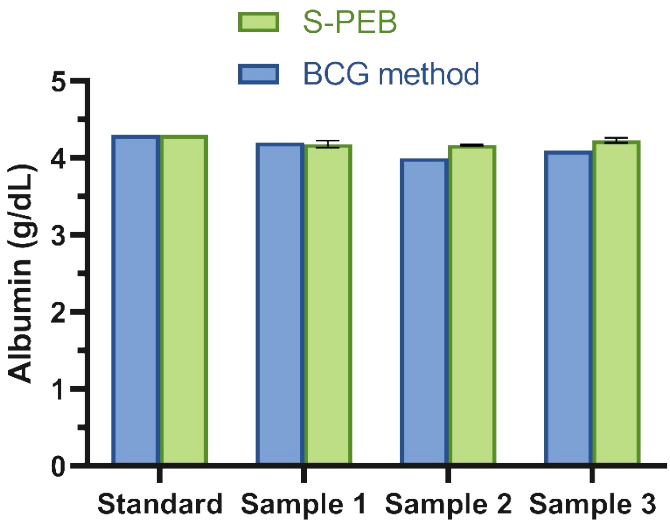

Fig. S23 Albumin evaluation results of three unknown serum samples (values are presented as mean ± SD, n = 3) using **S-PEB** [2 µM] in PBS buffer (1 mM , pH 7.4) and clinical BCG test. (Concentration of HSA in standard serum sample based on **S-PEB** was set as same as that of BCG method.)

HSA quantification in serum

Concentration of HSA in serum samples were determined based on the equation $\mathbf{C_{HSA}/C_{STD} = F/F_{STD}}$, where $\mathbf{C_{HSA}}$ and $\mathbf{C_{STD}}$ are the concentration of HSA in unknown and standard samples, respectively**. F** and $\mathbf{F_{STD}}$ are the corresponding fluorescence intensities of **R-PEB/S-PEB** on the addition of a particular volume of serum (here 5µL) from the unknown and standard samples. A representative example for calculation is given in the following table.

Method	C_{STD} (g/dL)	F_{STD} (nm)	F (nm)	C_{HSA} (g/dL)
R-PEB	4.3	501	493 (at 429 nm)	4.23
S-PEB	4.3	386	374 (at 433nm)	4.16

Details of accuracy, robustness & precision

Accuracy and recovery

Accuracy was determined by collecting data for three different serum samples (n = 3) and the value is expressed as percentage of recovery between the mean concentrations of HSA recovered and that of the original. The average recoveries and percentage relative error for **R-PEB** and **S-PEB** based measurements of three independent samples are presented in Table S2.

Table S2 Determination of accuracy and percentage recovery.

Sample	[HSA] as obtained using			% Average recovery (r)		% Relative error (δ)	
	BCG method (g/dL), (C_{BCG})	R-PEB (g/dL) (C_{HSA}) (Mean ± SD, n=3)	S-PEB (gdL) (C_{HSA}) (Mean ± SD, n=3)	R-PEB	S-PEB	R-PEB	S-PEB
1	4.2	4.27 ± 0.0286	4.18 ± 0.0464	101.6	99.5	1.6	0.5
2	4.0	4.15 ± 0.0047	4.17 ± 0.0125	103.7	104.2	3.7	4.2
3	4.1	4.18 ± 0.0245	4.23 ± 0.0339	101.9	103.1	1.9	3.1

$$\% \text{ Average recovery (r)} = 100 * C_{HSA} / C_{BCG}$$

$$\% \text{ Relative error } (\delta) = 100 * (C_{HSA} - C_{BCG}) / C_{BCG}$$

Robustness

Robustness of the method was validated by performing measurements at slightly different emission wavelengths for detection and quantification. All parameters except the wavelength were made constant during the process. Seven independent measurements (n = 7) of a selected serum sample was done at each of these wavelengths. The statistical comparison was done with Friedman analysis and no significant difference was found between the results ($p = 0.1017 > p = 0.05$ in the case of **R-PEB** and $p = 0.8668 > p = 0.05$ in the case of **S-PEB**) (Table S3).

Table S3 Robustness data of the method.

[HSA] (g/dL) BCG method	**Wavelength (nm)**	**Found, [HSA] (g/dL)**	**% RSD**
		RPEB (Mean ± SD, n=7)	
4.2	429 427 431	4.27 ± 0.0228 4.24 ± 0.0246 4.28 ± 0.0094	0.53 0.58 0.22
	Friedman analysis: p = 0.1017 > p = 0.05		
[HSA] (g/dL) BCG method	**Wavelength (nm)**	**Found, [HSA] (g/dL)**	**% RSD**
		SPEB (Mean ± SD, n=7)	
4.2	433 431 435	4.20 ± 0.0194 4.22 ± 0.0275 4.21 ± 0.0185	0.46 0.65 0.44
	Friedman analysis: p = 0.8668 > p = 0.05		

Precision

In order to find the precision of the method, three different serum samples were analysed in three independent runs in the same day (intra-day precision) and on three consecutive days (inter-day precision) The precision of the analysis method was determined by calculating the relative standard deviation (RSD %). The RSD values obtained are presented in Table S4.

Table S4 Determination of intra-day and inter-day precision of the method

R-PEB	**Intra-day precision**			**Inter-day precision**		
[HSA] (g/dL) BCG method	**Found, [HSA] (g/dL)** (Mean ± SD, n=3)	% RSD	± SE	**Found, [HSA] (g/dL)** (Mean ± SD, n=3)	% RSD	± SE
4.2	4.23 ± 0.0208	0.49	0.012	4.25 ± 0.0199	0.47	0.011
4.0	4.12 ± 0.0264	0.64	0.015	4.17 ± 0.0351	0.84	0.020
4.1	4.16 ± 0.0351	0.84	0.020	4.14 ± 0.0208	0.50	0.012
S-PEB	**Intra-day precision**			**Inter-day precision**		
[HSA] (g /dL) BCG method	**Found, [HSA] (g/dL)** (Mean ± SD, n=3)	% RSD	± SE	**Found, [HSA] (g/dL)** (Mean ± SD, n=3)	% RSD	± SE
4.2	4.21 ± 0.0451	1.07	0.026	4.21 ± 0.0251	0.60	0.014
4.0	4.18 ± 0.0251	0.60	0.014	4.16 ± 0.0451	1.08	0.026
4.1	4.17 ± 0.0416	1.00	0.024	4.17 ± 0.0351	0.84	0.020

Standard deviation (SD) = square root of $\sum (m-i)^2/n-1$ (m is the mean)

Percentage relative standard deviation (% RSD) = $100*(SD /m)$

Standard error (SE) = $SD/\sqrt{n}$

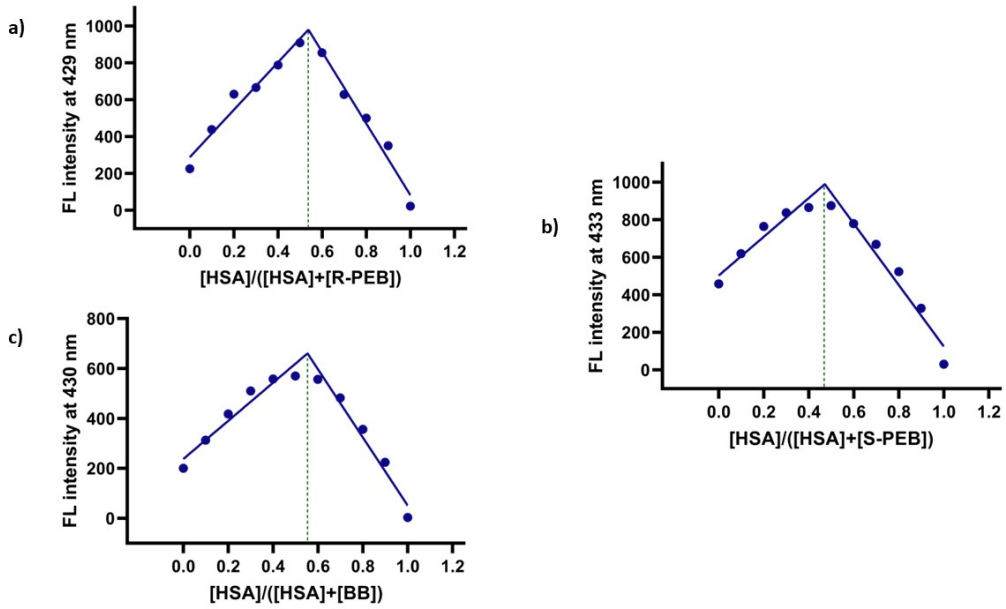

Fig. S24 Job's plot of (a) **R-PEB**, (b) **S-PEB** and (c) **BB** with HSA at varying ratios of probe and HSA (0-1). Total concentration ([HSA]+[PROBE]) maintained at 10 µM in PBS buffer (1 mM , pH 7.4); λ_{ex}= 360 nm (**R-PEB**, **S-PEB**), 385 nm (**BB**).

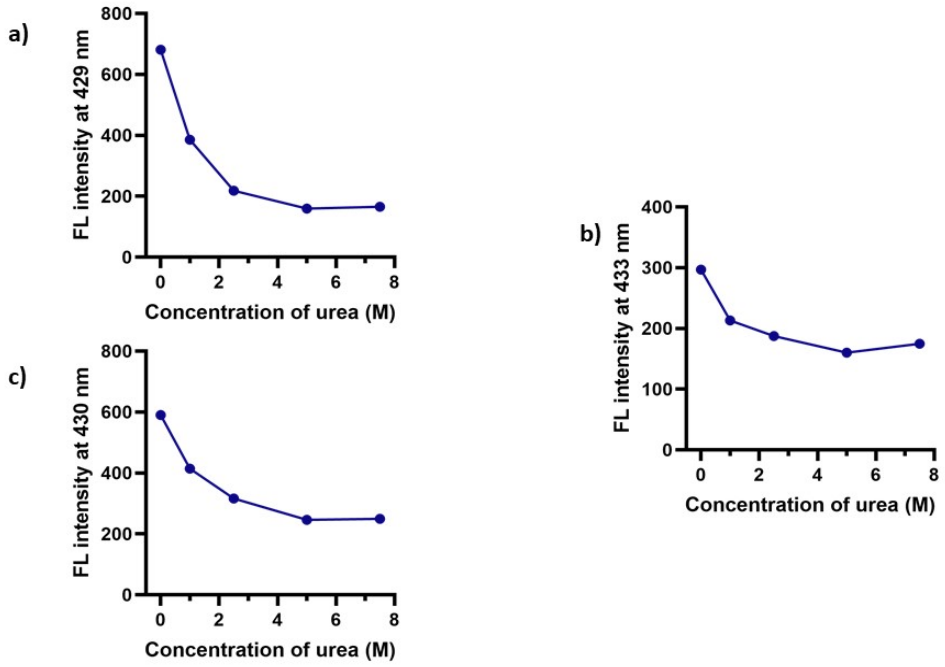

Fig. S25 Variation in the fluorescence intensity of HSA complex of (a) **R-PEB** [1 µM], (b) **S-PEB** [1 µM] and (c) **BB** [1 µM] in the presence of urea (0-7.5 M) in PBS buffer (1 mM, pH 7.4); λ_{ex} = 360 nm.

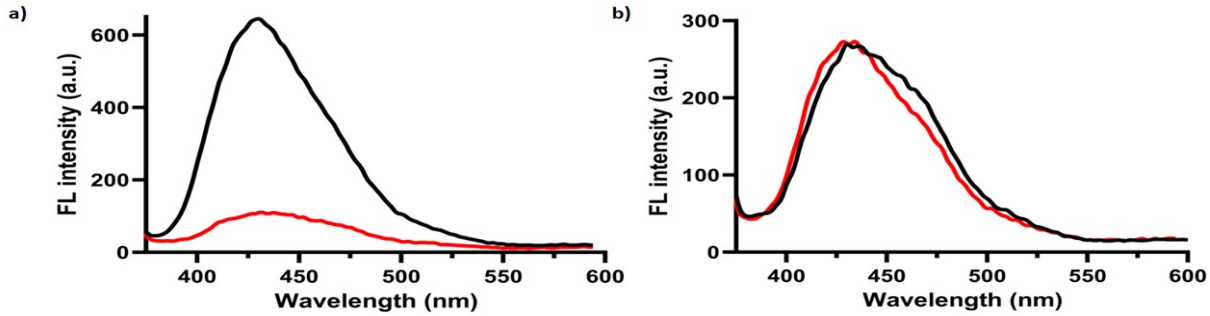

Fig. S26 Fluorescence spectra of complexes of HSA (5 eq) and (a) **R-PEB** [1 µM] and (b) **S-PEB** [1 µM] before (black) and after (red) addition excess stearic acid (25 eq) in PBS buffer (1 mM, pH 7.4) ; λ_{ex} = 360 nm.

Binding Site	Ligand Name	BE	Ligand Efficiency	H-bonding AAs	Grid Center	Grid Dimensions
Site II	Ibuprofen	-6.73	-0.45	ARG410, TYR411	(7.771, 3.136, -14.041)	(34, 16, 26)
Site II	**R-PEB**	-6.47	-0.28	ARG410	(7.771, 3.136, -14.041)	(34, 16 ,26)
Site II	**S-PEB**	-6.60	-0.29	LEU430	(7.771, 3.136, -14.041)	(34, 16, 26)
Site I	Warfarin	-8.46	-0.37	TYR150, ARG222	(2.931, -9.920, 7.847)	(30, 30, 36)
Site I	**R-PEB**	-5.09	-0.22	NIL	(2.931, -9.920, 7.847)	(30, 30,36)
Site I	**S-PEB**	-5.64	-0.25	NIL	(2.931, -9.920, 7.847)	(30, 30,36)

Table S5 Molecular docking calculation results and associated parameters.

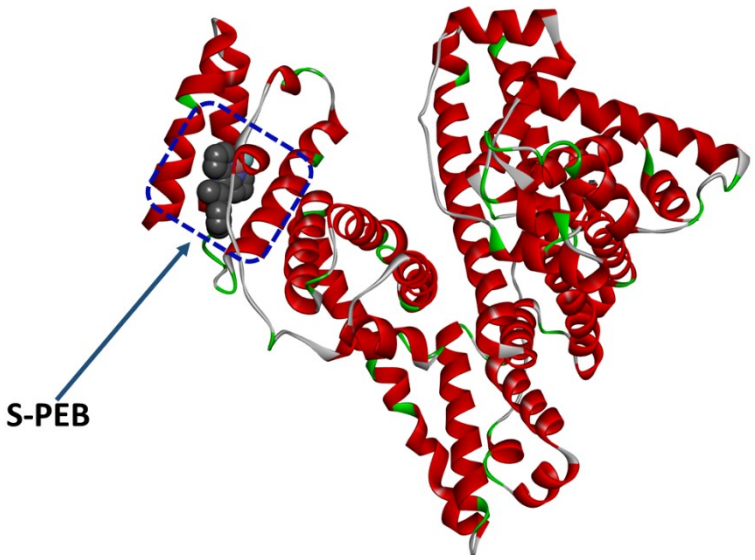

Fig. S27 Molecular docking for the binding of **S-PEB** with HSA (blind docking).

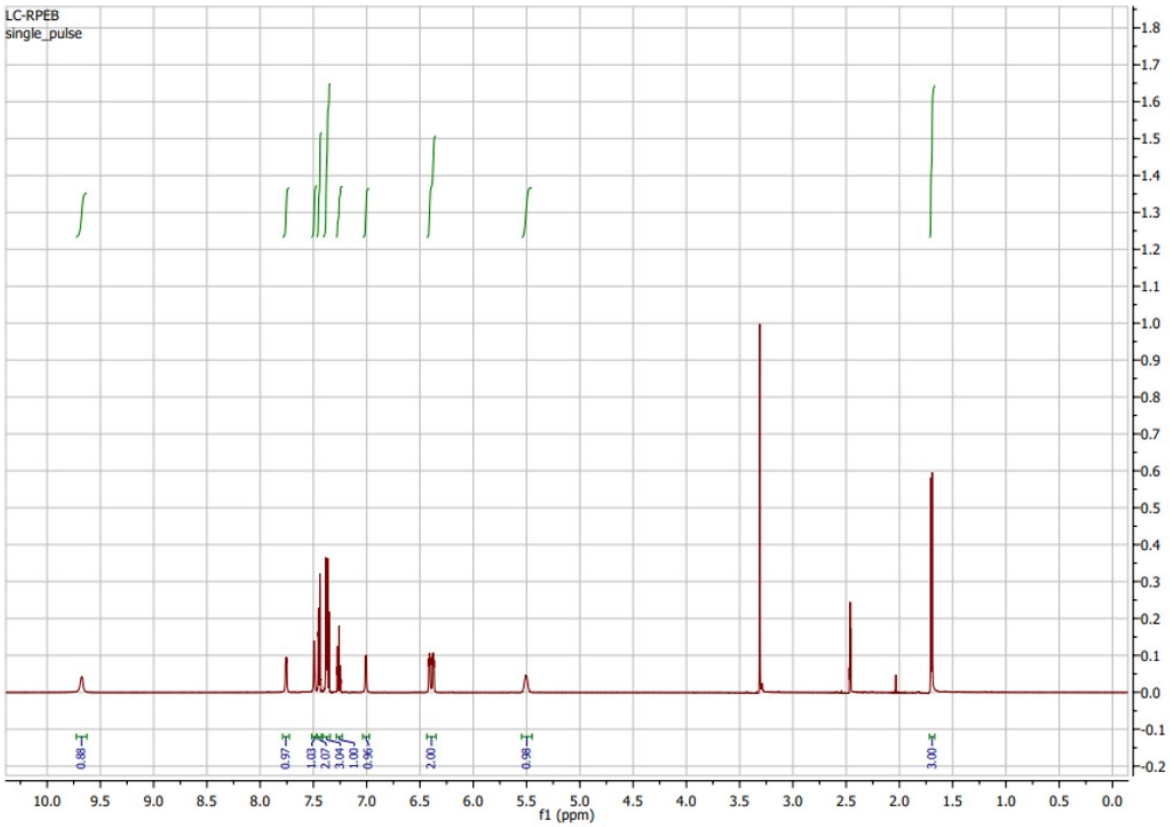

Fig. S28 500 MHz ^{1}H NMR (DMSO-d_6) spectra of **R-PEB.**

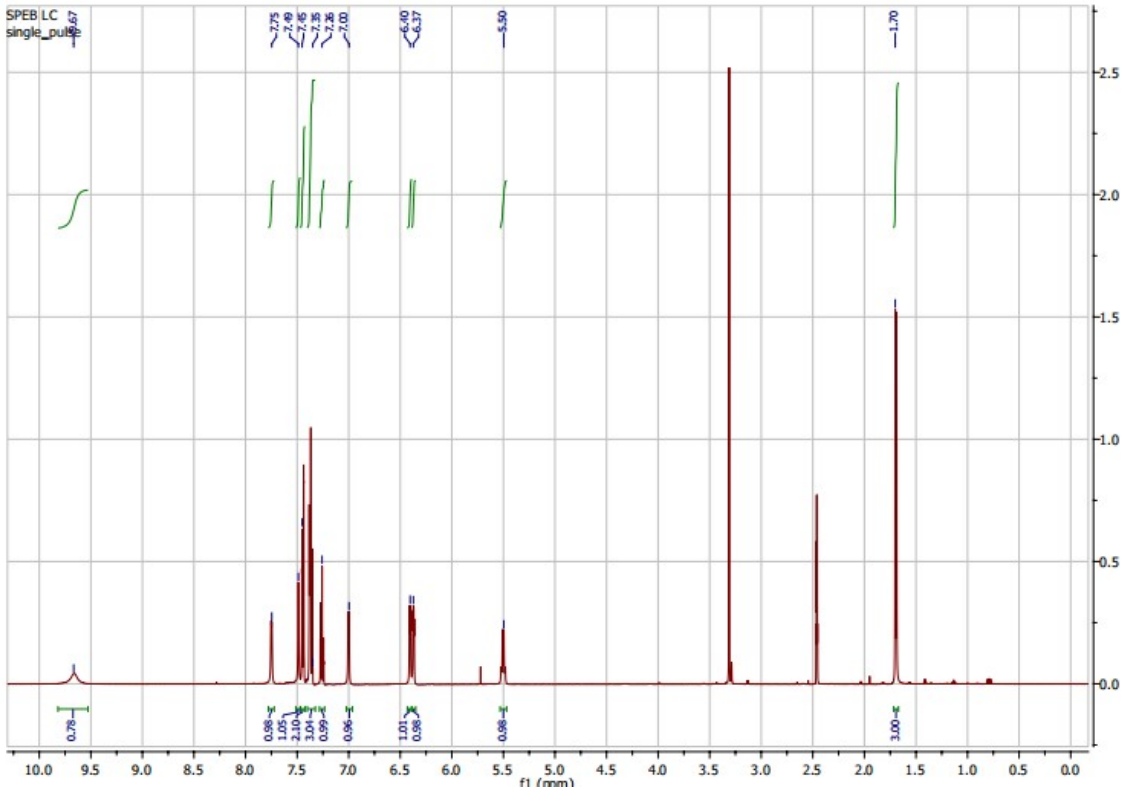

Fig. S29 500 MHz ^{1}H NMR (DMSO-d_6) spectra of **S-PEB.**

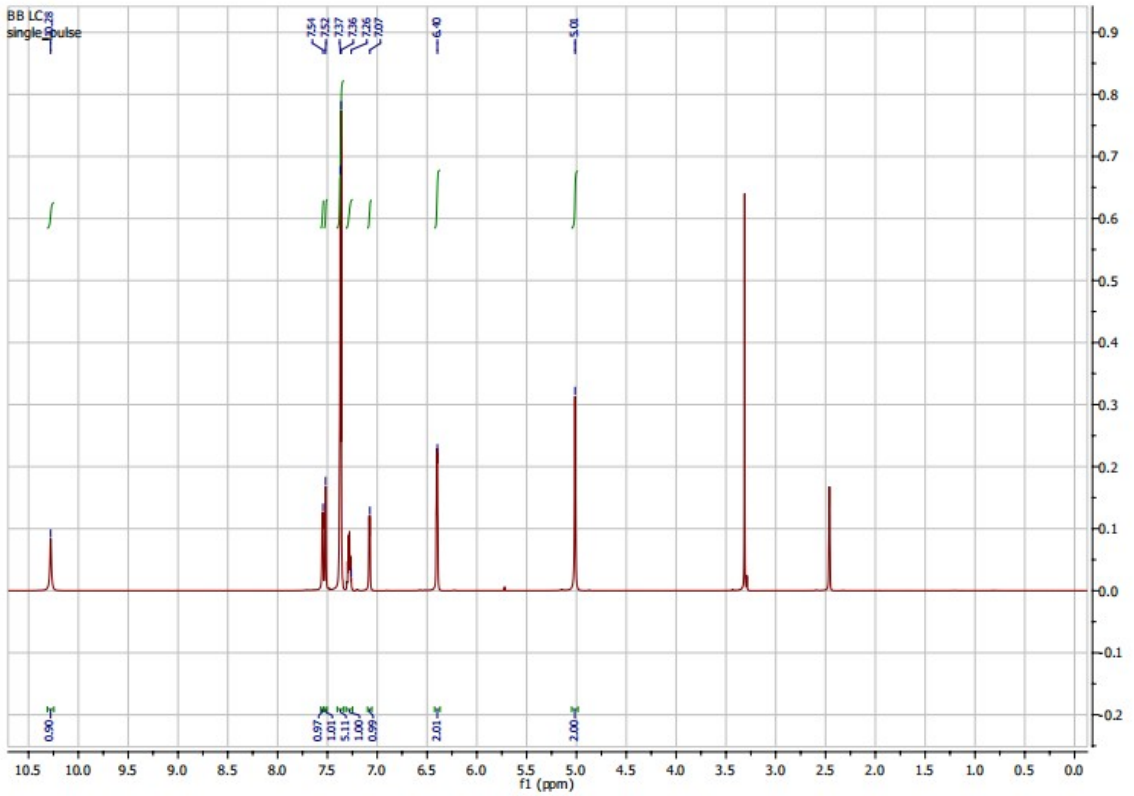

Fig. S30 500 MHz ^{1}H NMR (DMSO-d_6) spectra of **BB.**

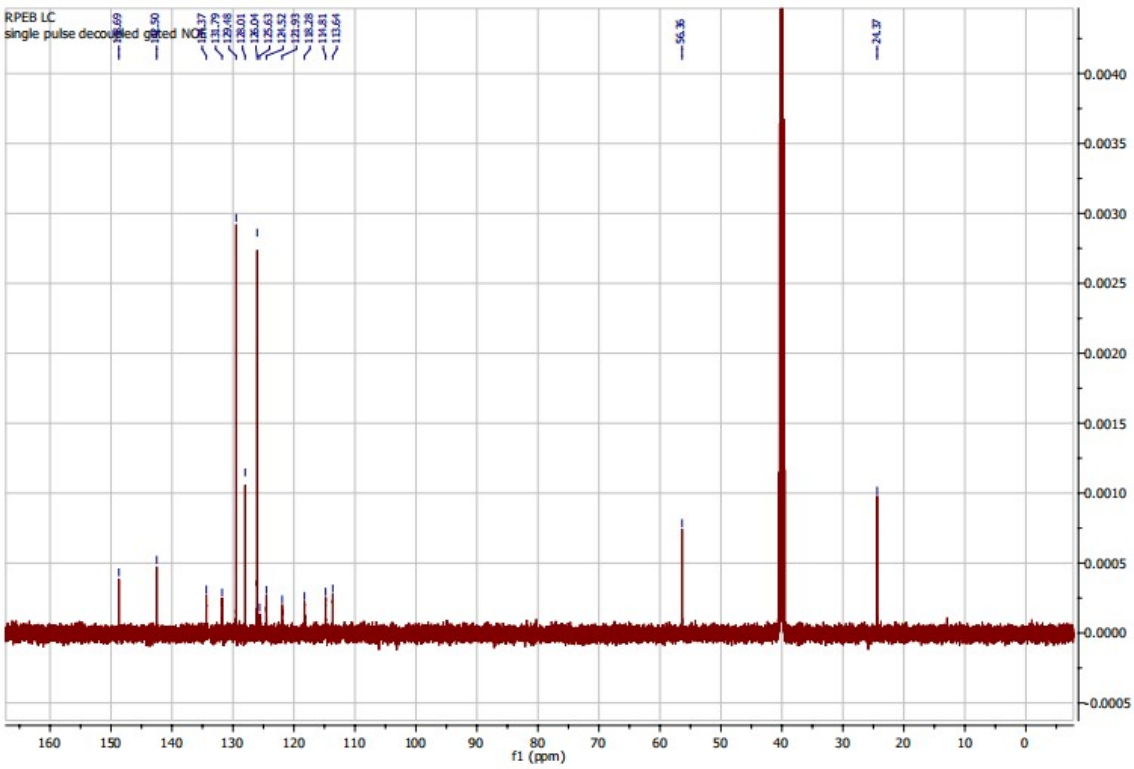

Fig. S31 125 MHz ^{13}C NMR (DMSO-d_6) spectra of **R-PEB.**

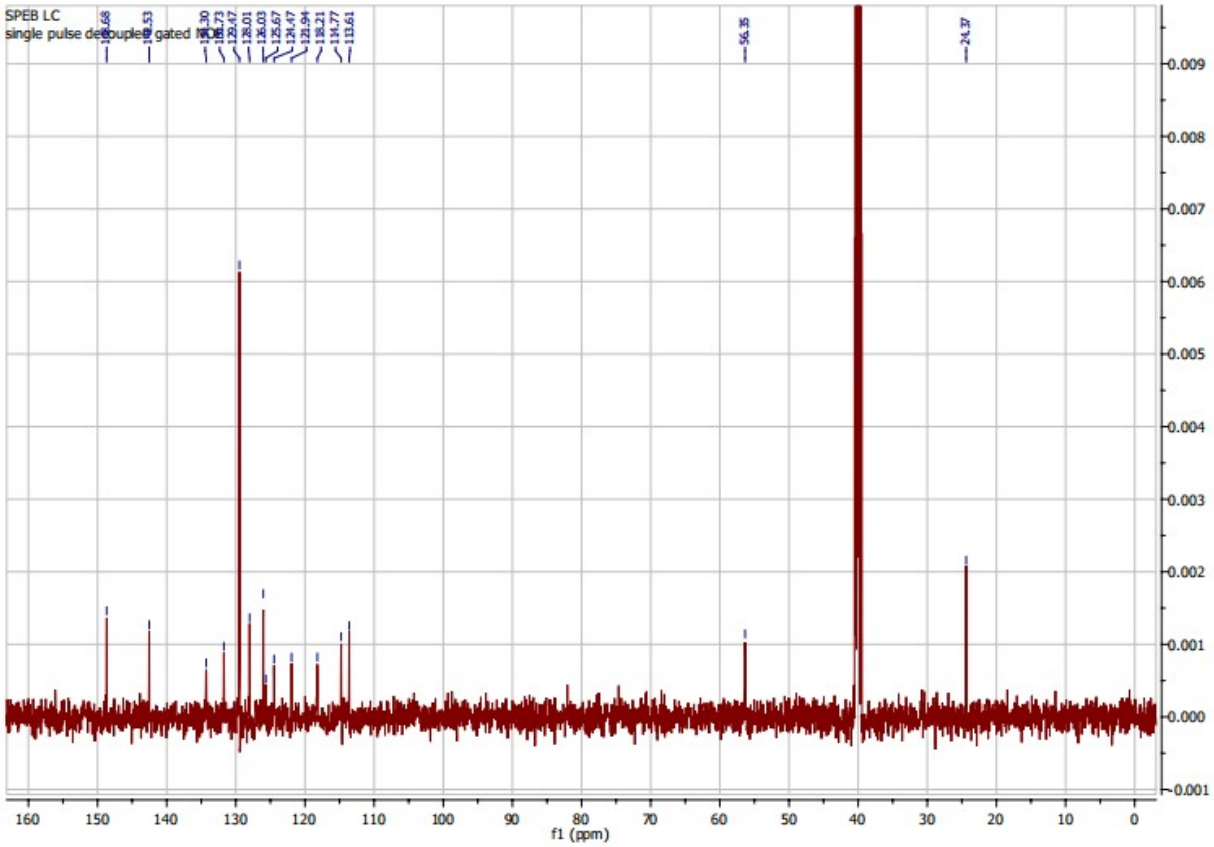

Fig. S32 125 MHz ^{13}C NMR (DMSO-d_6) spectra of **S-PEB.**

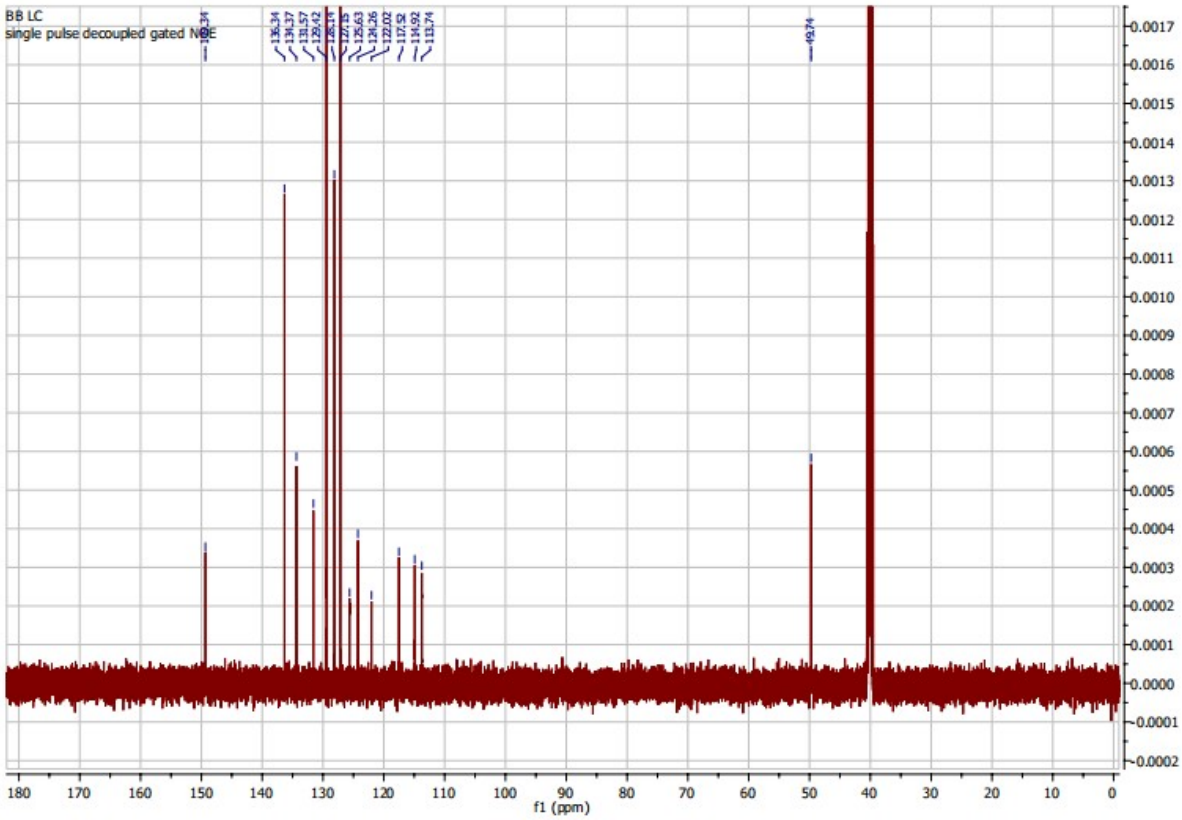

Fig. S33 125 MHz ^{13}C NMR (DMSO-d_6) spectra of **BB.**

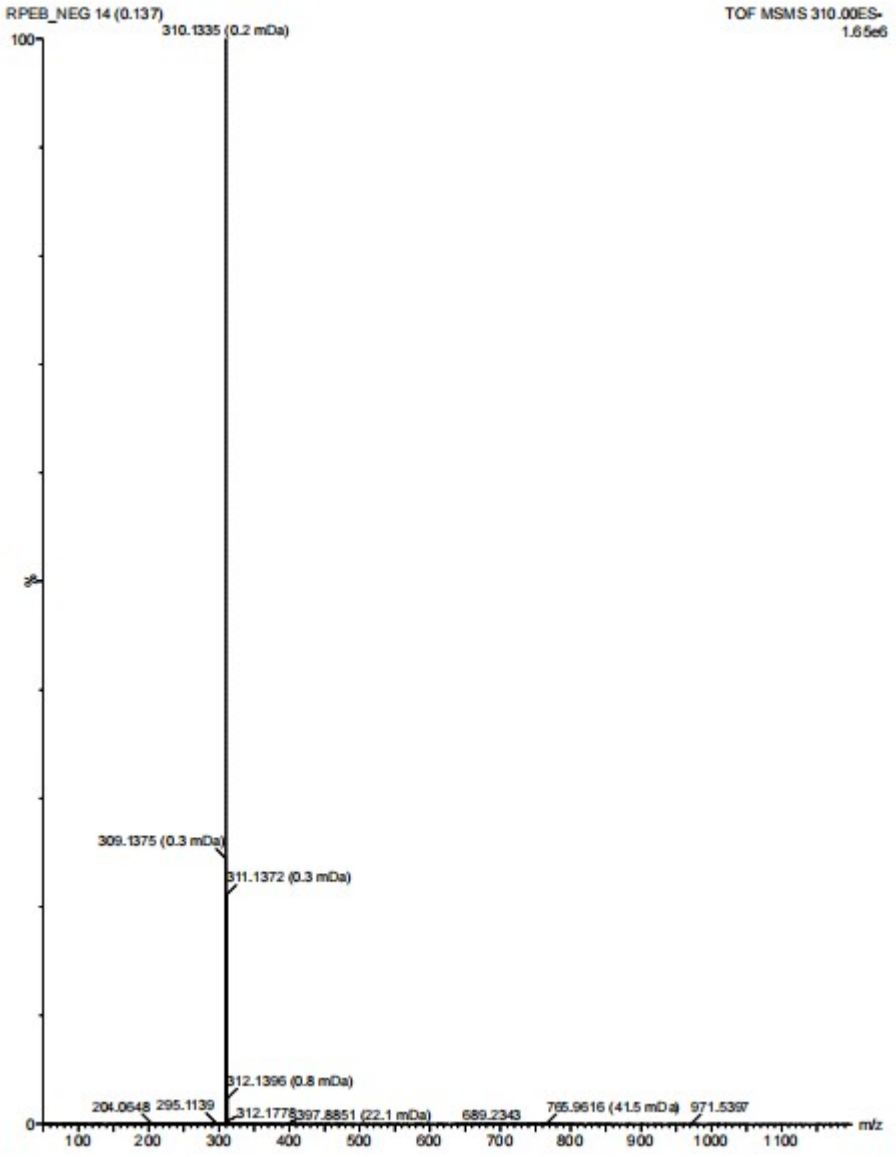

Fig. S34 HRMS of **R-PEB**.

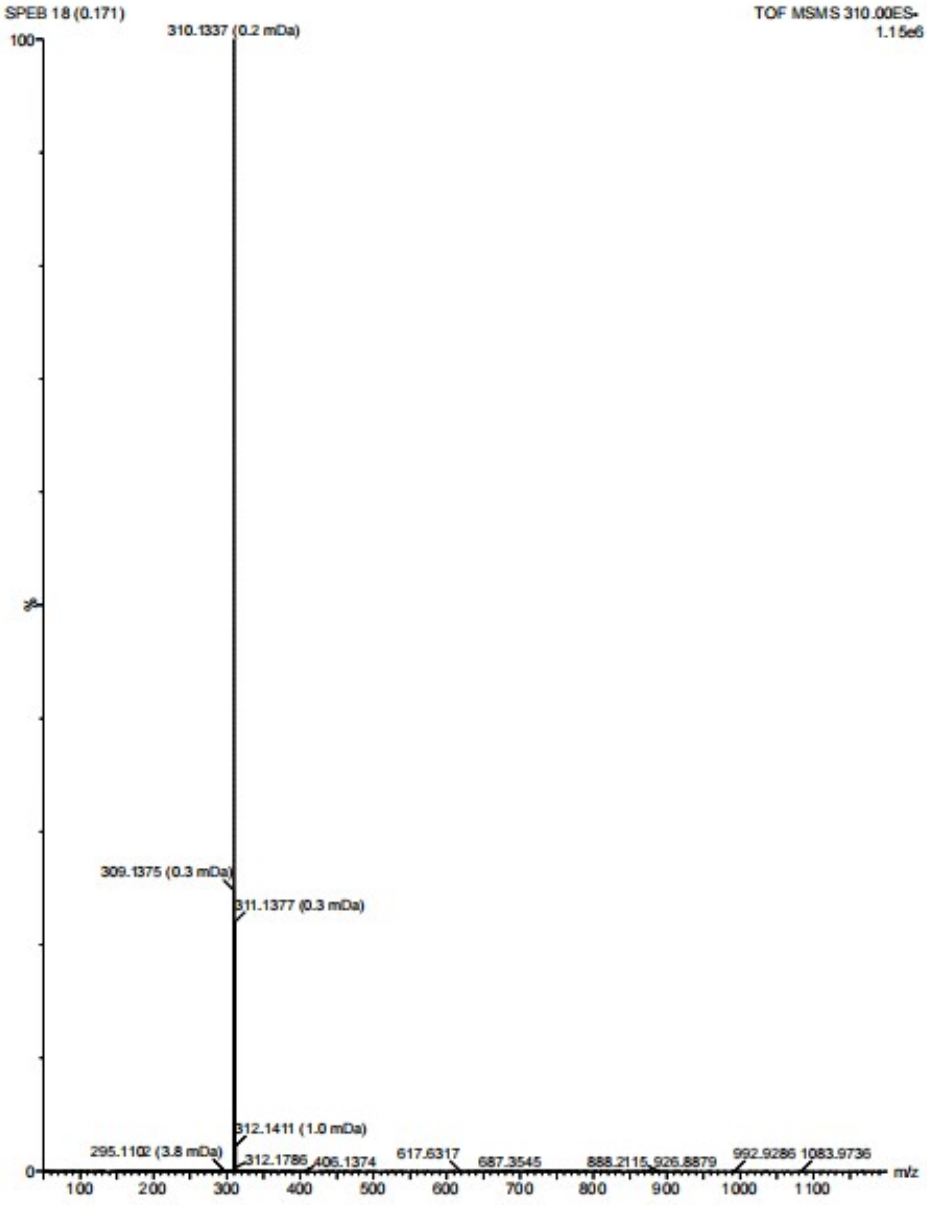

Fig. S35 HRMS of **S-PEB**.

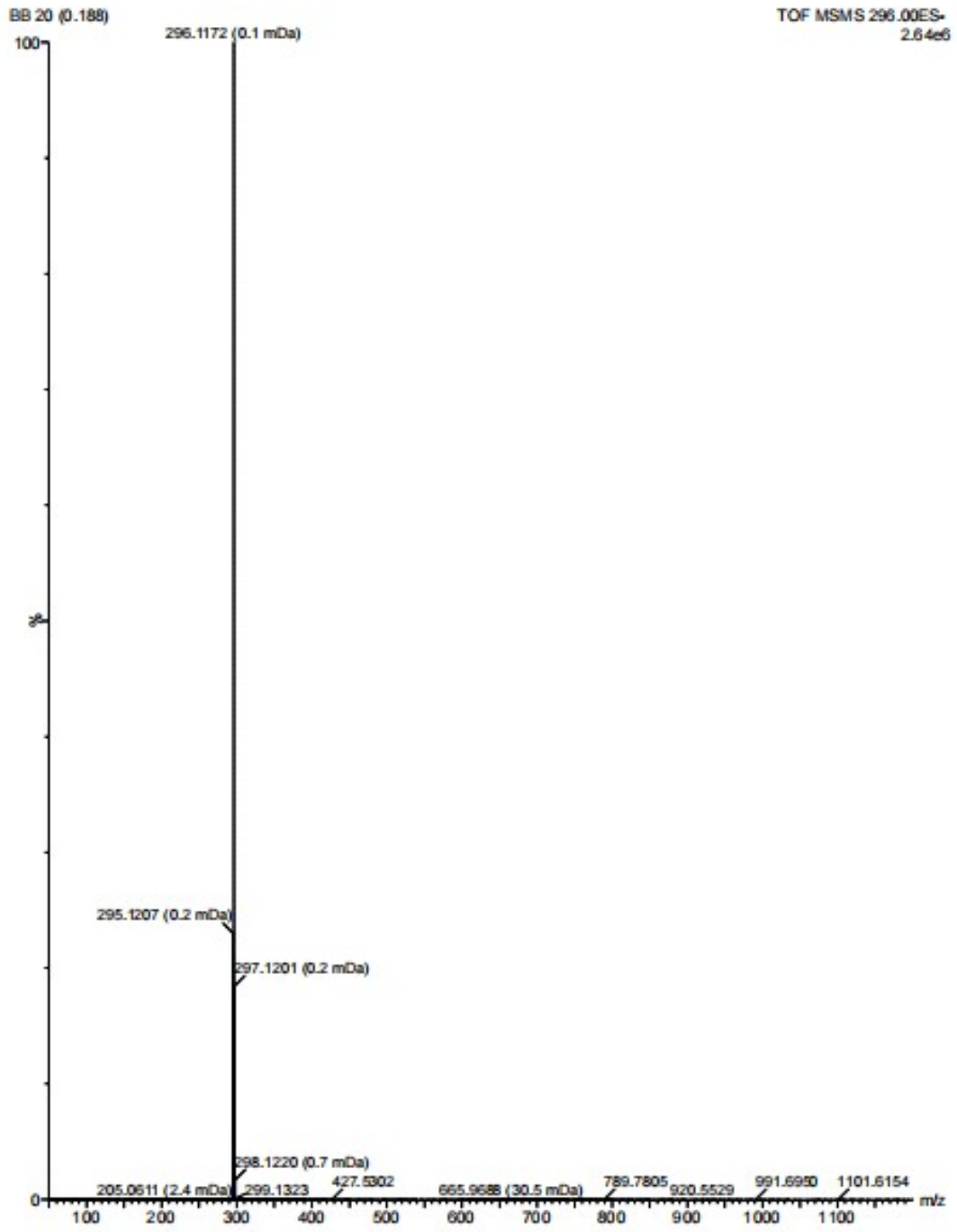

Fig. S36 HRMS of **BB**.