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Synthesis of Biocompatible, BSA capped Fluorescent $CaCO_3$ Pre-Nucleation Nanoclusters for Cell Imaging Applications

Shivesh Sabbarwal,[1]Ashutosh Kumar Dubey,[3] Maneesha Pandey,[3]Manoj Kumar[1,2*]

[1]Nano& Micro System Fabrication and Design Lab, Department of Chemical Engineering and Technology, IIT (BHU) Varanasi-221005

[2]School of Biomedical Engineering, IIT (BHU) Varanasi-221005

[3]Department of Ceramic Engineering, IIT (BHU) Varanasi-221005

Corresponding author e-mail: *manojk.che@iitbhu.ac.in

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Section S 0.1: MALDI-MS. Bruker Daltonics (flexControl) Matrix assisted laser desorption ionization mechanism instrument was used with the matrix platform of sinapic acid. The sample was ionized with the pulsed nitrogen laser of 337 nm, followed by compilation of spectra in positive mode, with an average of 500 shots for each spectra. 1 ml of Matrix was prepared with 500 µl of 100% Acetonitrile, 500 µl of 100% Milli-Q Water (MQ), 1 µl of 0.1% Trifluoroacetic acid (TFA) and 10 mg of sinapic acid. The prepared matrix was stored for 7 days, 1 µl of the same matrix was used for spotting purpose. The ratio which was kept constant between the sample and the matrix was 1:1. 1 µl of matrix with 1 µl of sample was taken for spotting purpose.

Section S0.2: Lifetime. Fluorescence lifetimes of the FCPN, BSA and Moringa leaf extract were measured with Edinburgh FL920 Fluorescence Life Time Spectrometer by Time Resolved Fluorescence Spectrometer (TRFS) technique. The samples were excited by the Laser and LEDs at wavelength of 375 nm and 496 nm, 598 nm, respectively. Fluorescence Lifetime decay spectra were acquired as far as 10,000 counts were laid-back. External circulating water bath was put into the lifetime decay setup to maintain the sample temperature at 25°C.Tri- exponential fitting was performed on the lifetime-data to get the chi-square χ^2 (goodness of fitting) values close to 1.00 by GRG- Nonlinear solver.

Section S0.3: Corrected total cell fluorescence (CTCF) analysis

Corrected total cell fluorescence (CTCF) analysis was performed on Z- stacked images or shots of MG-63 cells acquired by confocal microscopy. The shots were acquired from bottom (0 µm) to top of the cell (22 µm) with total 12 number of slices (each slice of thickness ~1.8 µm). The details of CTCF analysis can be found elsewhere.[1]

Section S0.4: Quantum Yield

The quantum yield of FCPN for green emission in water was calculated relative to the Rhodamine 6G, which is used as a standard fluorophore, its quantum yield is 0.92 in water. The quantum yield of FCPN was evaluated by utilizing the following mathematical expression.

$$\varphi_{nc} = \varphi_r \frac{F_{nc} A_r \eta_{nc}^2}{F_r A_{nc} \eta_r^2} \quad (1)$$

In the above equation, φ = quantum yield, F = Integrated fluorescence intensity, A = Absorbance, nc = Nanocluster (FCPN) , r = Reference and η = refractive-index.

From fluorescence graph, we found: F_{nc}=31610650.15944, F_r =367458024.88005

Absorbance: A_{nc}= 0.09, A_r=0.02,

Refractive index: η_{nc}=1.33, η_r=1.33 and

Reference quantum yield φ_{std}=0.92.

Solvent: Water

Thus, The quantum yield of FCPN for green emission was calculated to be 0.0175 by eq. (1) with reference to quantum yield of Rhodamine 6G (0.92). (Fig. S20, ESI†)

Section S0.5: Quantitative methodology for determination of Nanocluster Concentration:

Method A: Based on TEM Data

Assumption:

Metal cluster is assumed to be spherical. Thus, cluster volume $(V_{cluster})$ is calculated by equation 1:

$$V_{cluster} = V_{atom} \times N \quad (1)$$

Where, V_{atom} is volume of atom and N is number of atoms present in one nanocluster.

Equation 1can be rearranged to give equation 2:

$$\left(\frac{4}{3}\right) \times \pi \times r_{cluster}^{3} = N \times \left(\frac{4}{3}\right) \times \pi \times r_{atom}^{3} \quad (2)$$

Where, $r_{cluster}$ is cluster radius obtained from TEM ($1.3/2 \sim 0.65$ nm) and r_{atom} is radius of calcium atom (180 pm). Thus equation 2 can be rearranged to calculate N:

$$N = \left(\frac{r_{cluster}}{r_{atom}}\right)^3 = \left(\frac{0.65 \times 10^{-9}}{180 \times 10^{-12}}\right)^3 = 47 \text{ atoms of calcium present in one nanocluster.}$$

Single unit of FCPN contains 47 atoms of calcium.

Method B: Based on MALDI-MS Data

MALDI-MS analysis indicates that the maximum population of nanocluster (FCPN) constitutes of 50 calcium atoms. It can be noted that the numbers of atoms obtained from Method A and Method B are matching closely.

Calculation for concentration of nanocluster in solution:

50 ml of 10 mM aqueous $CaCl_2$ is used for nanocluster synthesis.

Number of moles of $CaCl_2$=$50 \times 10 \times 10^{-3} = 0.5$

Number of calcium atoms in the precursor=$0.5 \times 6.022 \times 10^{23} = 3.011 \times 10^{23}$

Thus, to calculate number of nanoclusters N_{NC} equation 3 is used, **Method A**:

$$N_{NC} = \frac{N_{atom}}{N} \qquad (3)$$

$$= \frac{3.011 \times 10^{23}}{47} = 6.406 \times 10^{21}$$

Thus, 6.406×10^{21} nanoclusters are formed per 50 ml of $CaCl_2$

Answer: Thus, 1.281×10^{20} ***nanoclusters are formed per 1 ml of*** $CaCl_2$

Thus, to calculate number of nanoclusters N_{NC} equation 3 is used, **Method B**:

$$N_{NC} = \frac{N_{atom}}{N} = \frac{3.011 \times 10^{23}}{50} = 6.022 \times 10^{21}$$

Thus, 6.022×10^{21} nanoclusters are formed per 50 ml of $CaCl_2$

Answer: Thus, 1.204×10^{20} ***nanoclusters are formed per 1 ml of*** $CaCl_2$

Hence the final concentration of the FCPN (i.e. ***amount of nanoclusters****), was calculated by dividing* N_{NC} *by Avagadro's number* N_A

$$C_{NC} = \frac{N_{NC}}{N_A} = \frac{1.204 \times 10^{20}}{6.022 \times 10^{23}} = 2 \times 10^{-4}\, moles/ml$$

In FCPN powder, the concentration of nanocluster is measured to be 22.1 mg/ml.

Thus, Number of moles=0.5525

Number of nanoclusters in powder= $0.5525 \times 6.022 \times 10^{23} = 3.327 \times 10^{23}$

$$C_{NC,\,powder} = \frac{N_{NC}}{N_A} \frac{3.327 \times 10^{23}}{=6.022 \times 10^{23}} = 0.55 \frac{moles}{ml\ of\ nanocluster\ solution}$$

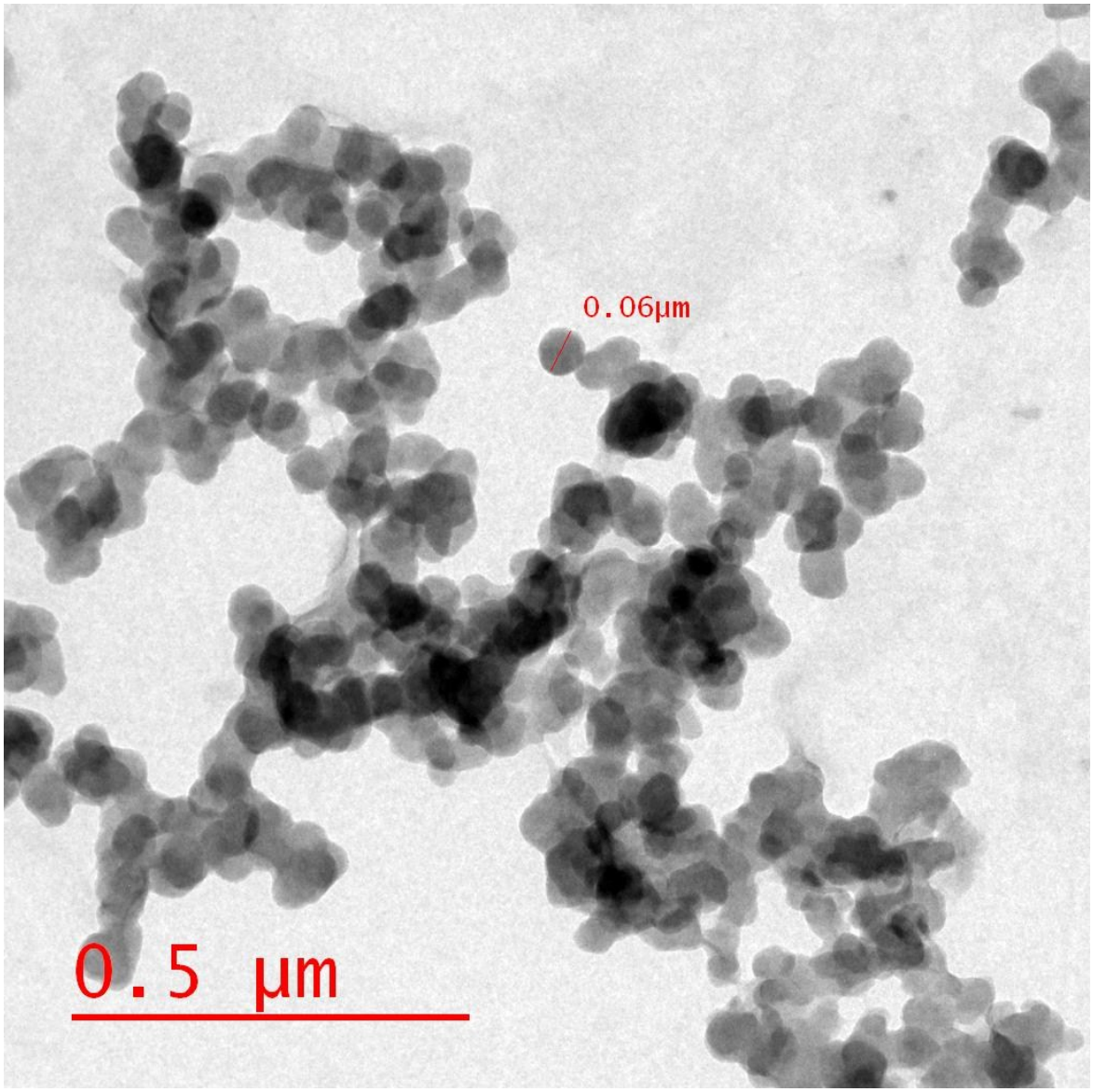


Fig. S1 TEM image of large size $CaCO_3$ Nanoparticles (NP'S) ~49 nm (Control A) synthesized (without BSA), using leaf extract of Moringa oleifera as reducing agent.

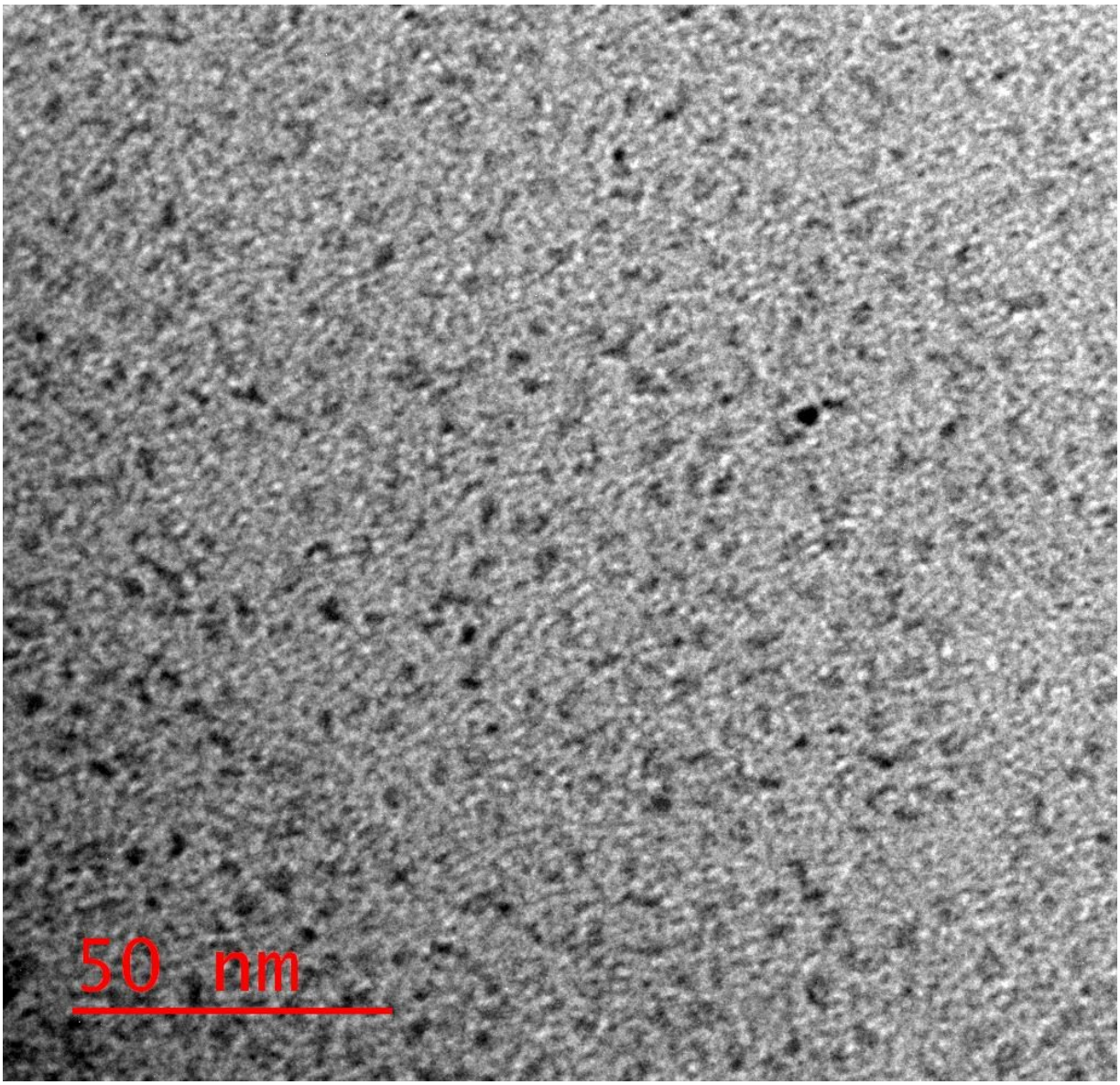


Fig. S2 TEM image $CaCO_3$ nanoclusters (FCPN) ~1.3 nm synthesized with BSA, using Moringa oleifera leaf extract as reducing agent.

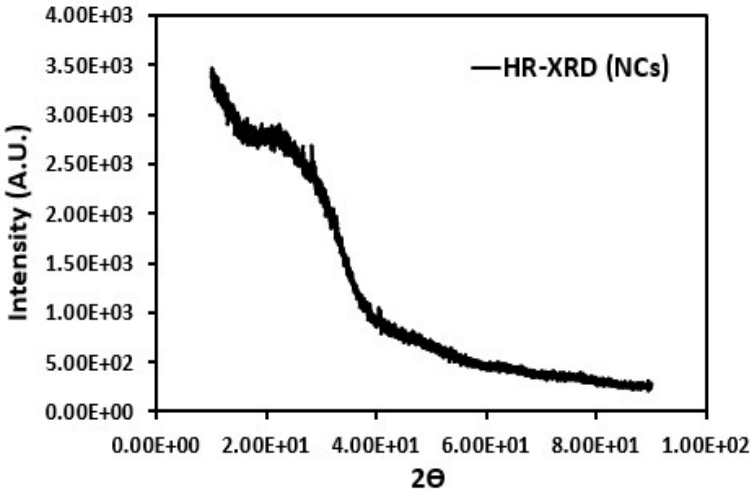


Fig. S3 XRD spectra of FCPN

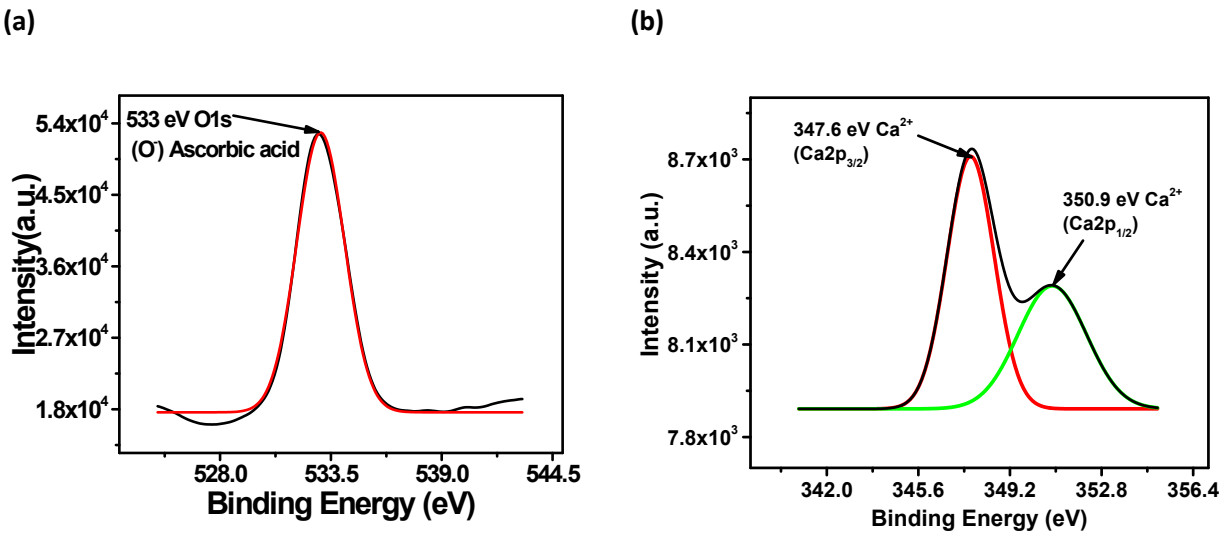


Fig. S4 XPS spectra: M.oleifera leaf extract (a) O1s, peak at 533eV (b) $Ca2p_{3/2}$ and $Ca2p_{1/2}$ of free Ca^{2+} in M.oleifera leaf extract, peak at 347.6 eV and 350.9 eV.

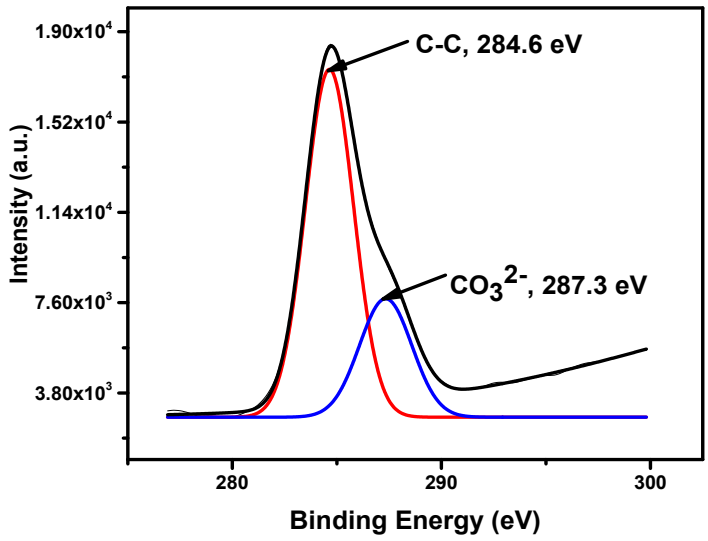
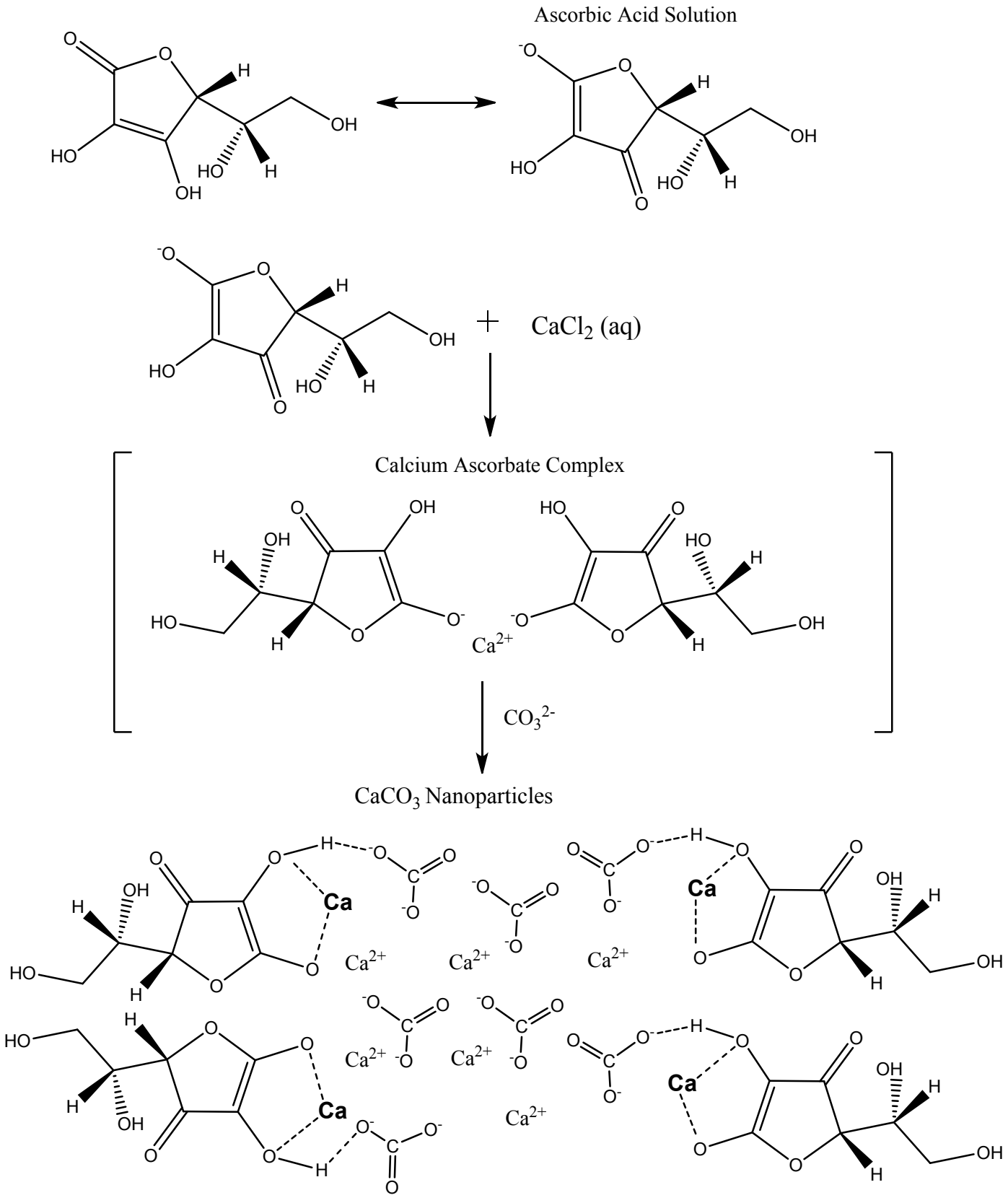


Fig. S5 XPS spectra: FCPN (a) C1s of C-C, peak at 284.6 eV. (b) C1s of CO_3^{2-}, peak at 287.3eV.


Ascorbic Acid Solution
CaCl2 (aq)
Calcium Ascorbate Complex
CO3 2-
CaCO3 Nanoparticles

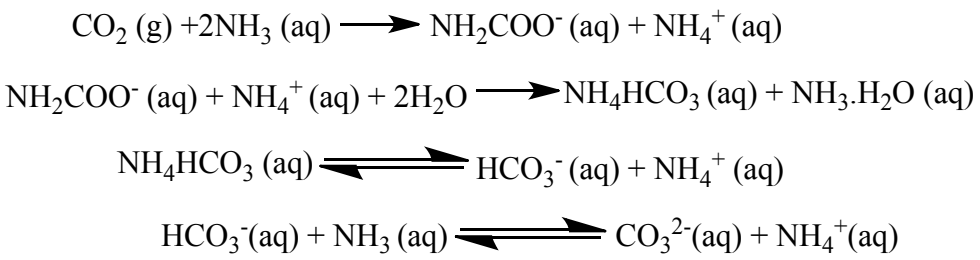


Fig. S6 Reaction scheme.

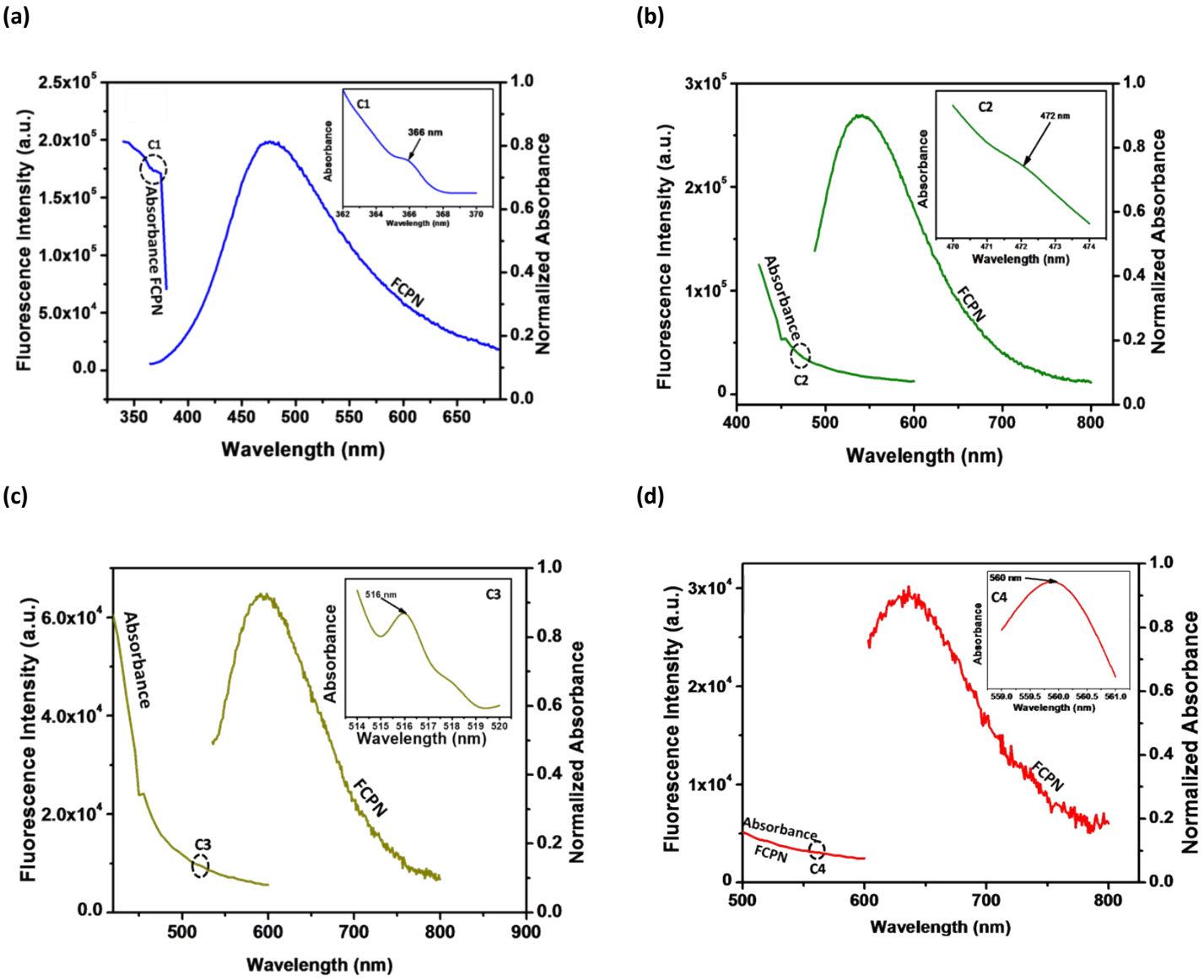


Fig. S7 Fluorescence Spectra-FCPN: (a)-(Absorbance, C1) = 366 nm, λ_{exc}= 366 nm, λ_{em}=476 nm-blue). (b)-(Absorbance, C2 = 472 nm, λ_{exc} = 472 nm, λ_{em}=541nm-green). (c)-(Absorbance, C3 = 516 nm, λ_{exc} = 516 nm, λ_{em}=595 nm-yellow). (d)-(Absorbance, C4 = 560 nm, λ_{exc} = 560 nm, λ_{em}=636 nm-red).

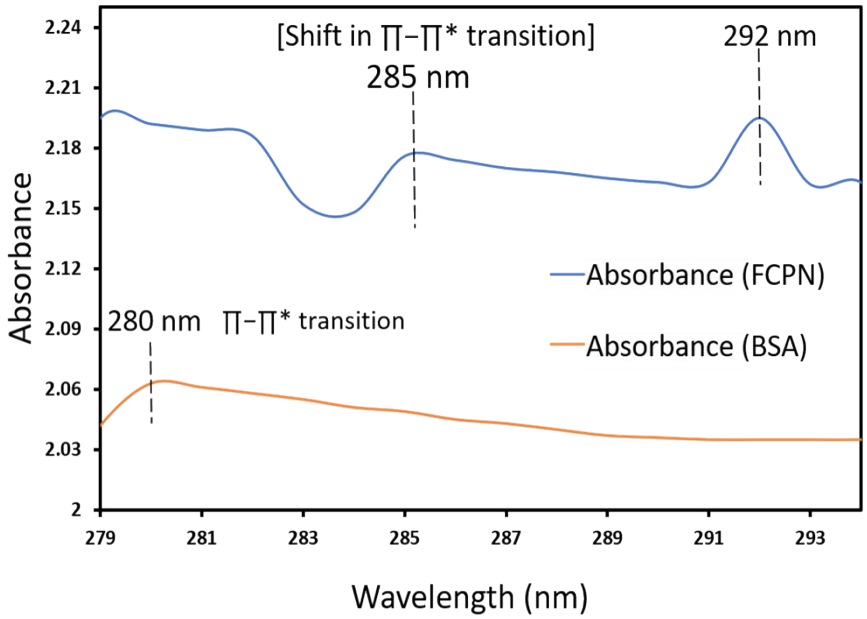
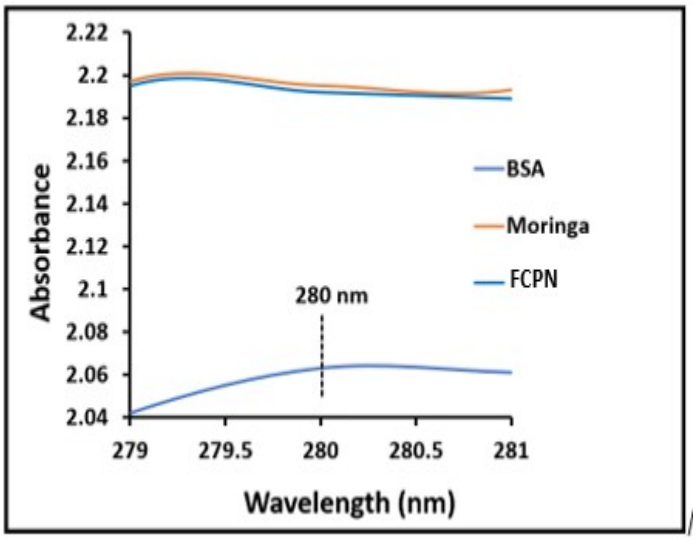


Fig. S8 Absorption spectra: FCPN and BSA in the range from 279 to 293 nm.

(a)



(b)

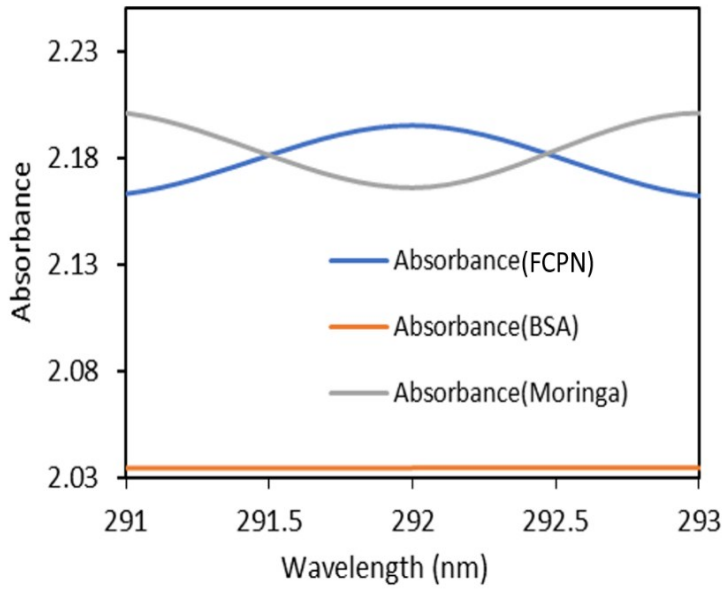
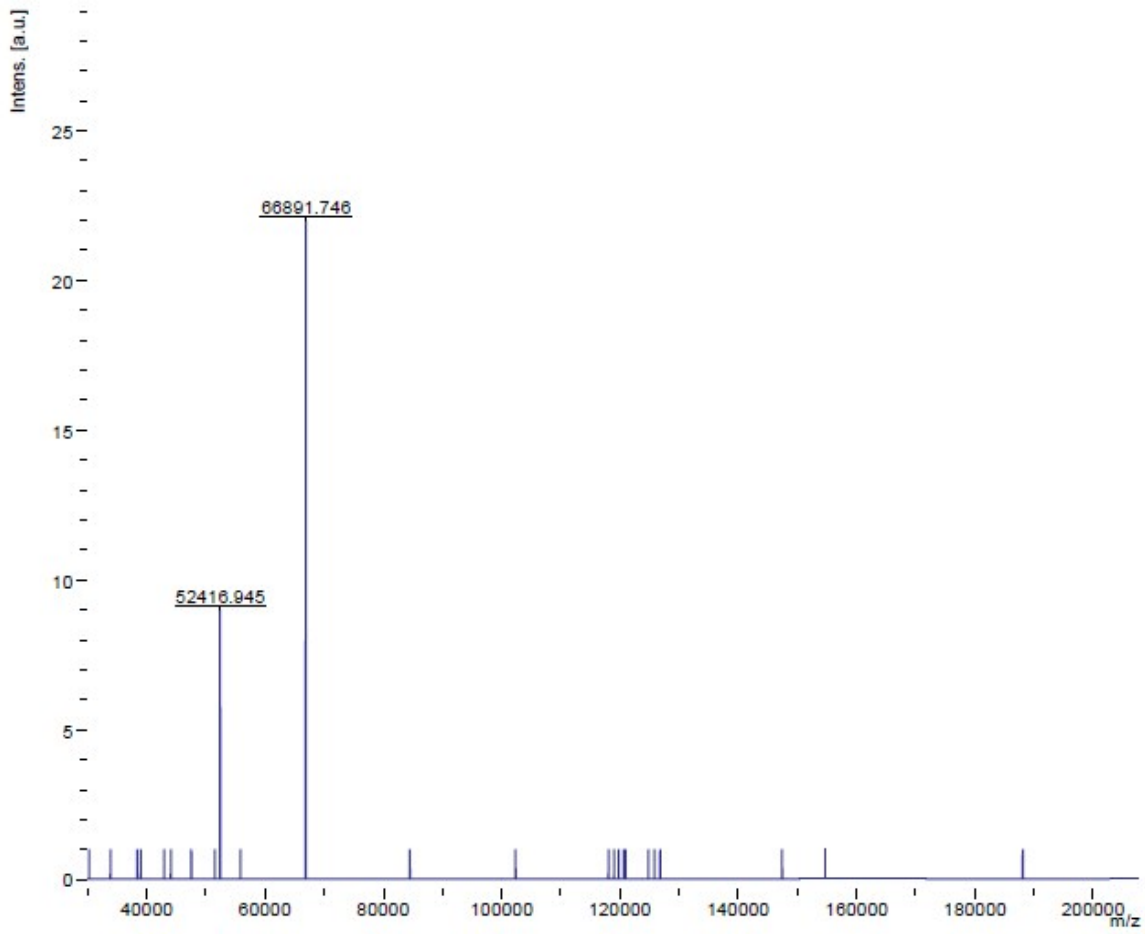


Fig. S9 (a) FCPN, BSA and Moringa leaf extract in the range from 279 to 281 nm. (b) FCPN, BSA and Moringa leaf extract in the range from 291 to 293 nm.

(a)


Intens. [a.u.]
25
20
15
10
5
0
66891.746
52416.945
40000
60000
80000
100000
120000
140000
160000
180000
200000
m/z

(b)

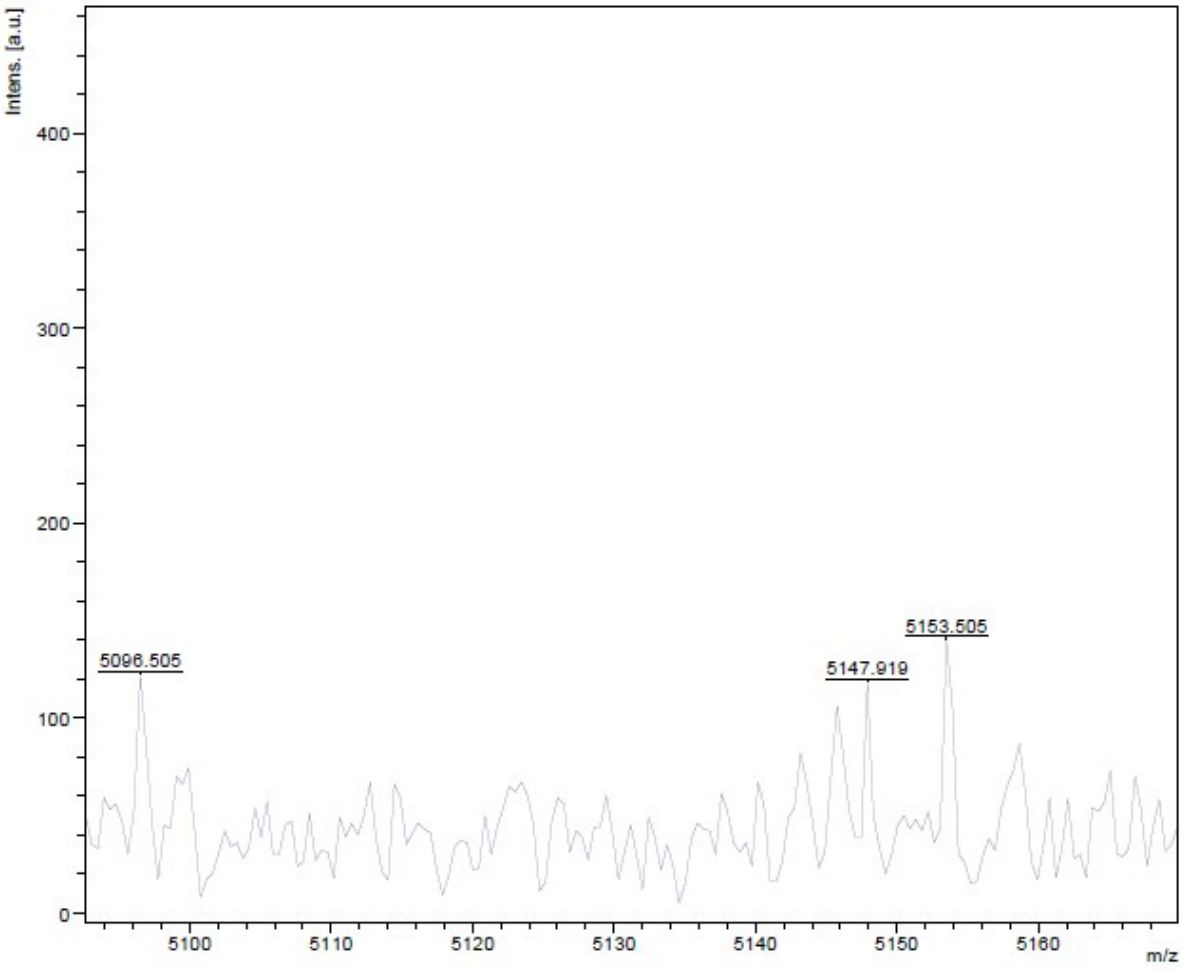
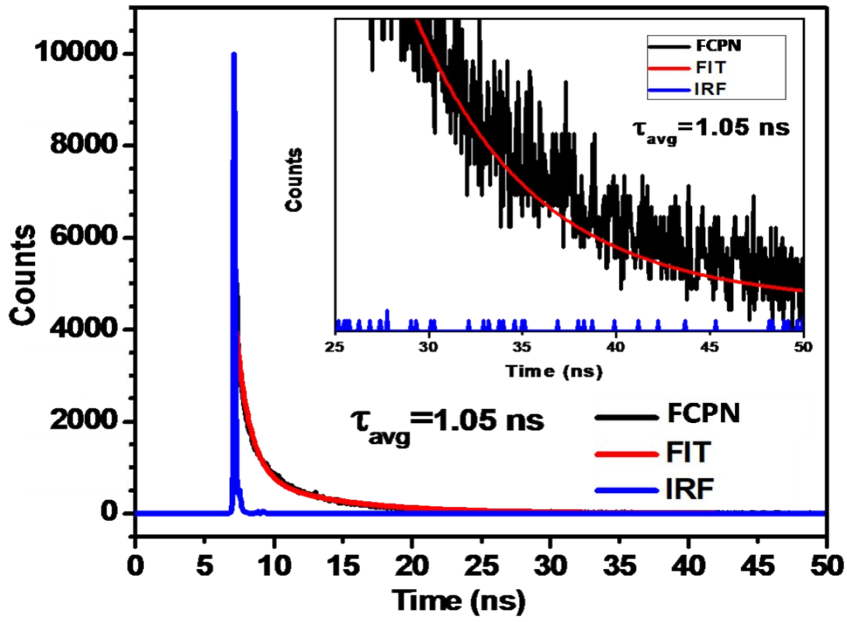
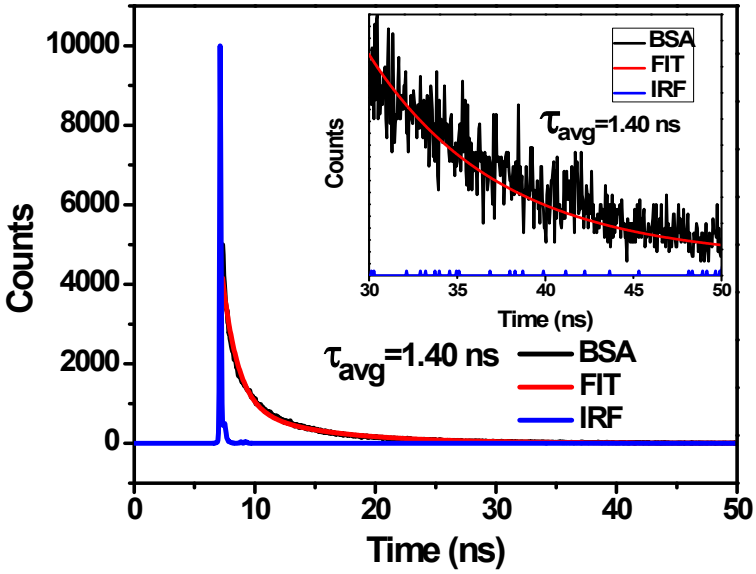


Fig. S10 MALDI-MS spectra of (a) BSA, and (b) Moringa leaf Extract.

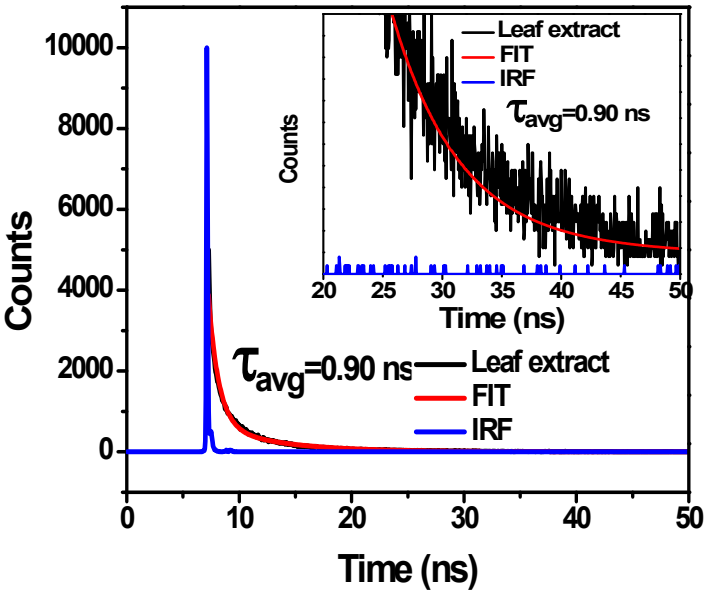
(a)


Counts
Time (ns)
FCPN
FIT
IRF
τavg=1.05 ns

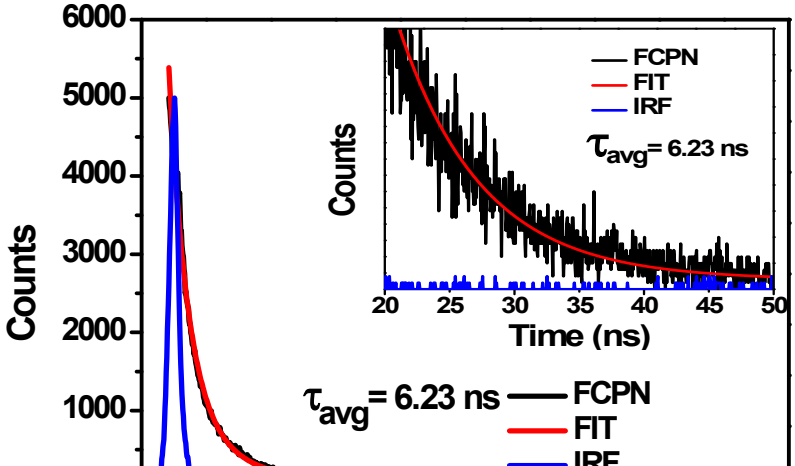
(b)


Counts
Time (ns)
BSA
FIT
IRF
τavg=1.40 ns

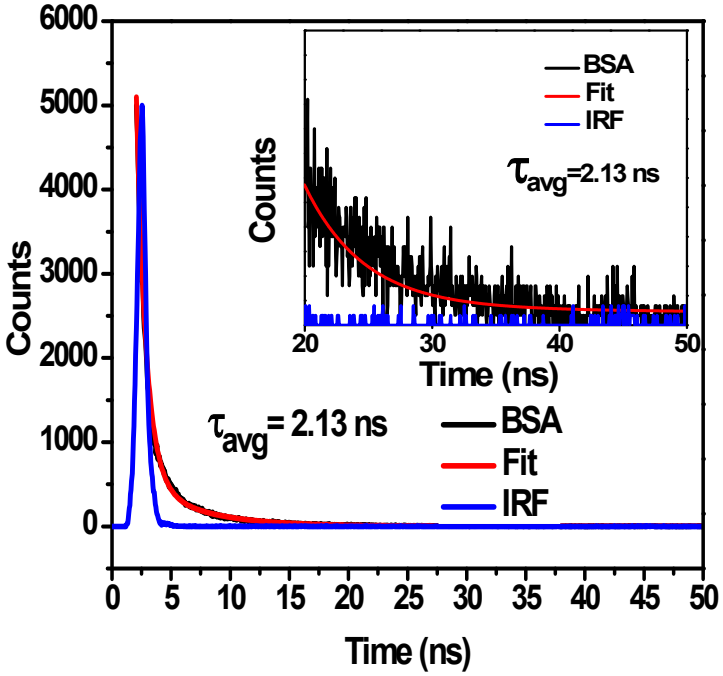
(c)


Counts
Time (ns)
Leaf extract
FIT
IRF
τavg=0.90 ns

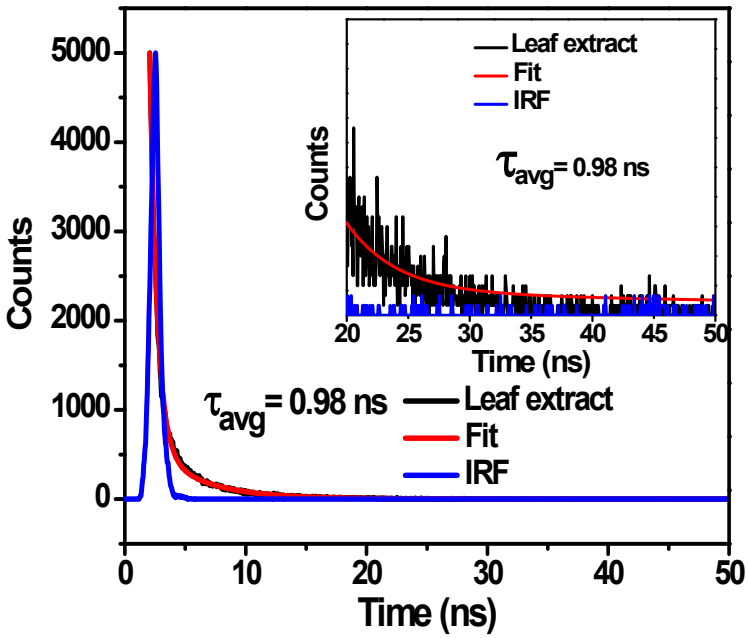
(d)


Counts
Time (ns)
FCPN
FIT
IRF
τavg= 6.23 ns

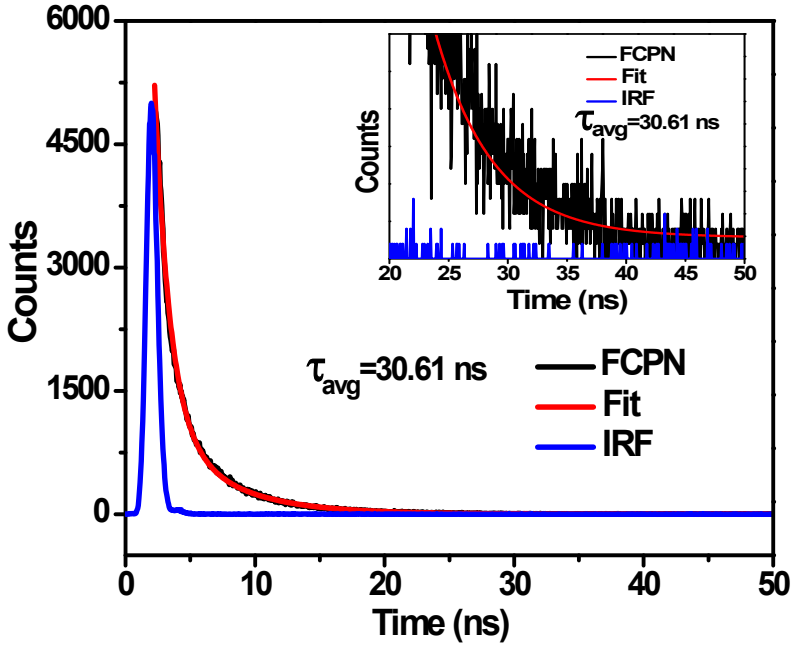
(e)


Counts
Time (ns)
BSA
Fit
IRF
τavg=2.13 ns
τavg= 2.13 ns

(f)


Counts
Time (ns)
Leaf extract
Fit
IRF
τavg= 0.98 ns
τavg= 0.98 ns

(g)


Counts
Time (ns)
FCPN
Fit
IRF
τavg=30.61 ns
τavg=30.61 ns

(h)

(i)

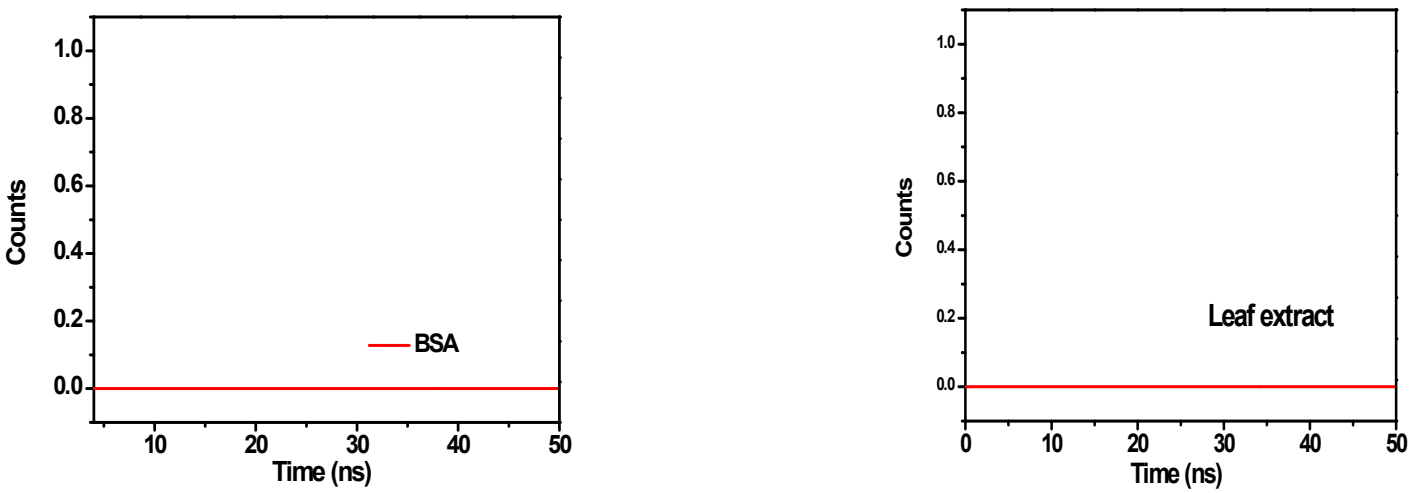


Fig. S11 Fluorescence lifetime (FL) spectra of (a) FCPN (b) BSA Protein (c) leaf extract, with ex/em 375/485 nm; FL spectra of (d) FCPN (e) BSA protein (f) leaf extract with ex/em of 496/565 nm, using LED lamp as excitation source; FL Spectra of (g) FCPN (h) BSA Protein (i) leaf extract, with ex/em of 598/674 nm, using LED lamp as excitation source; All of the above images contains inset figure showing the zoomed images of the same curve.

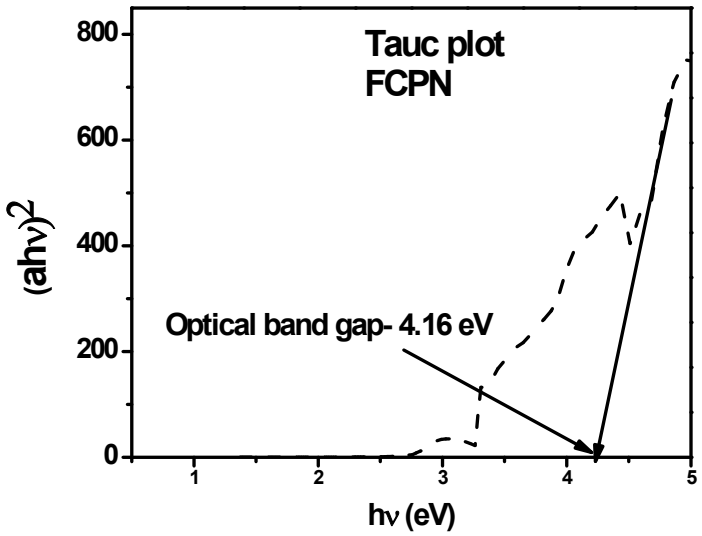


Fig. S12 Band energy gap for FCPN.

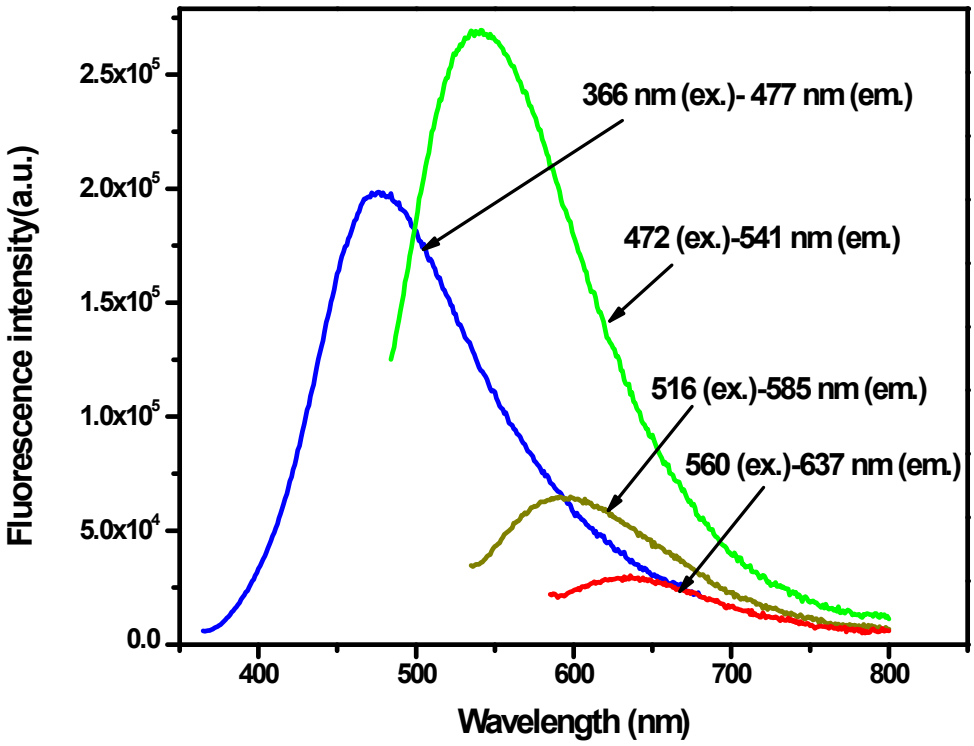


Fig. S13 Fluorescence spectra of FCPN under different excitations (ex.) and different emissions (em.).

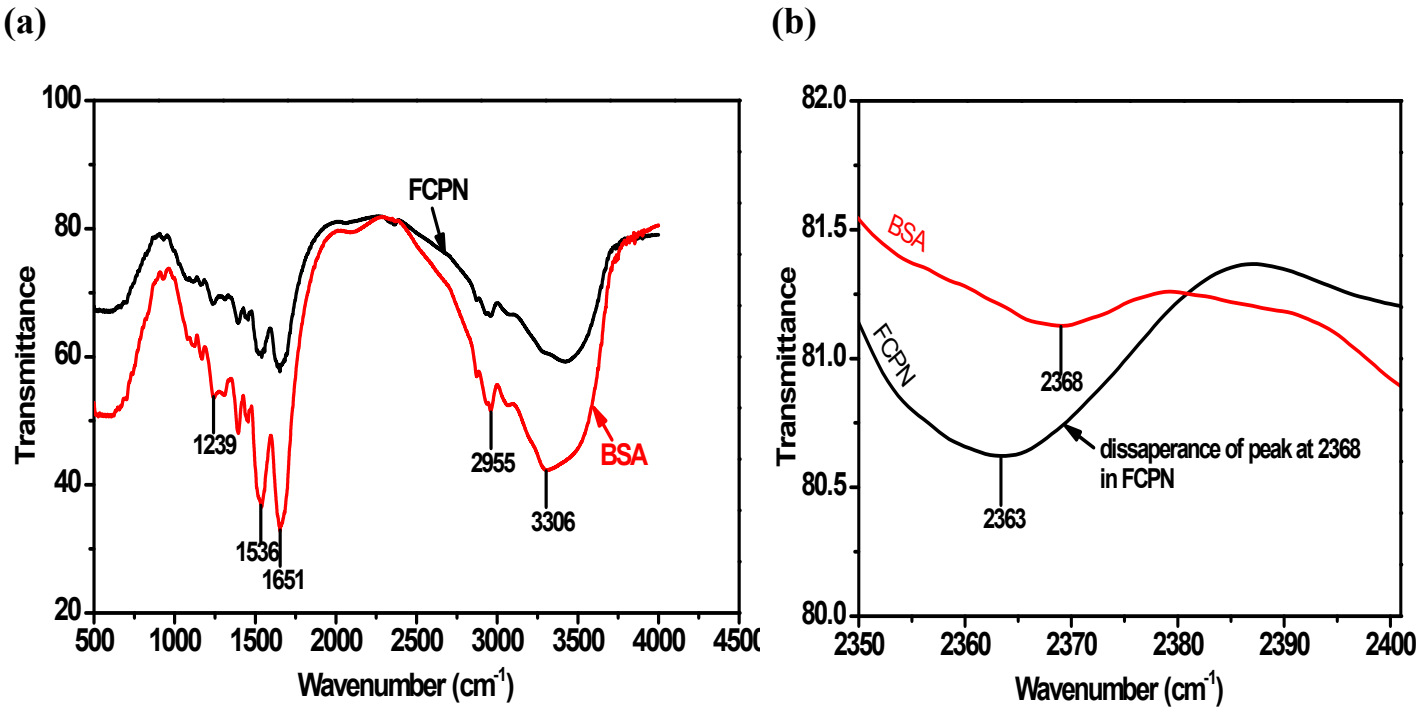


Fig. S14 FTIR spectra: (a) FCPN and BSA, (b) zoomed image of FCPN and BSA in the range varying from 2300 cm^{-1} to 2400 cm^{-1}.

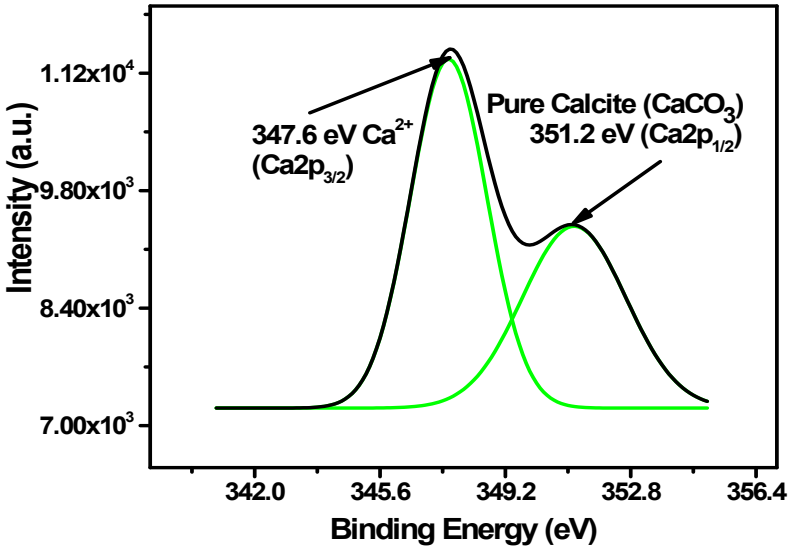


Fig. S15 XPS spectra: large size $CaCO_3$ Nanoparticles (NP'S) ~49 nm (Control A). (a) $Ca2p_{3/2}$ of Ca^{2+}, peak at 347.6 eV. (b) $Ca2p_{1/2}$ of pure calcite crystal, peak at 351.2 eV.

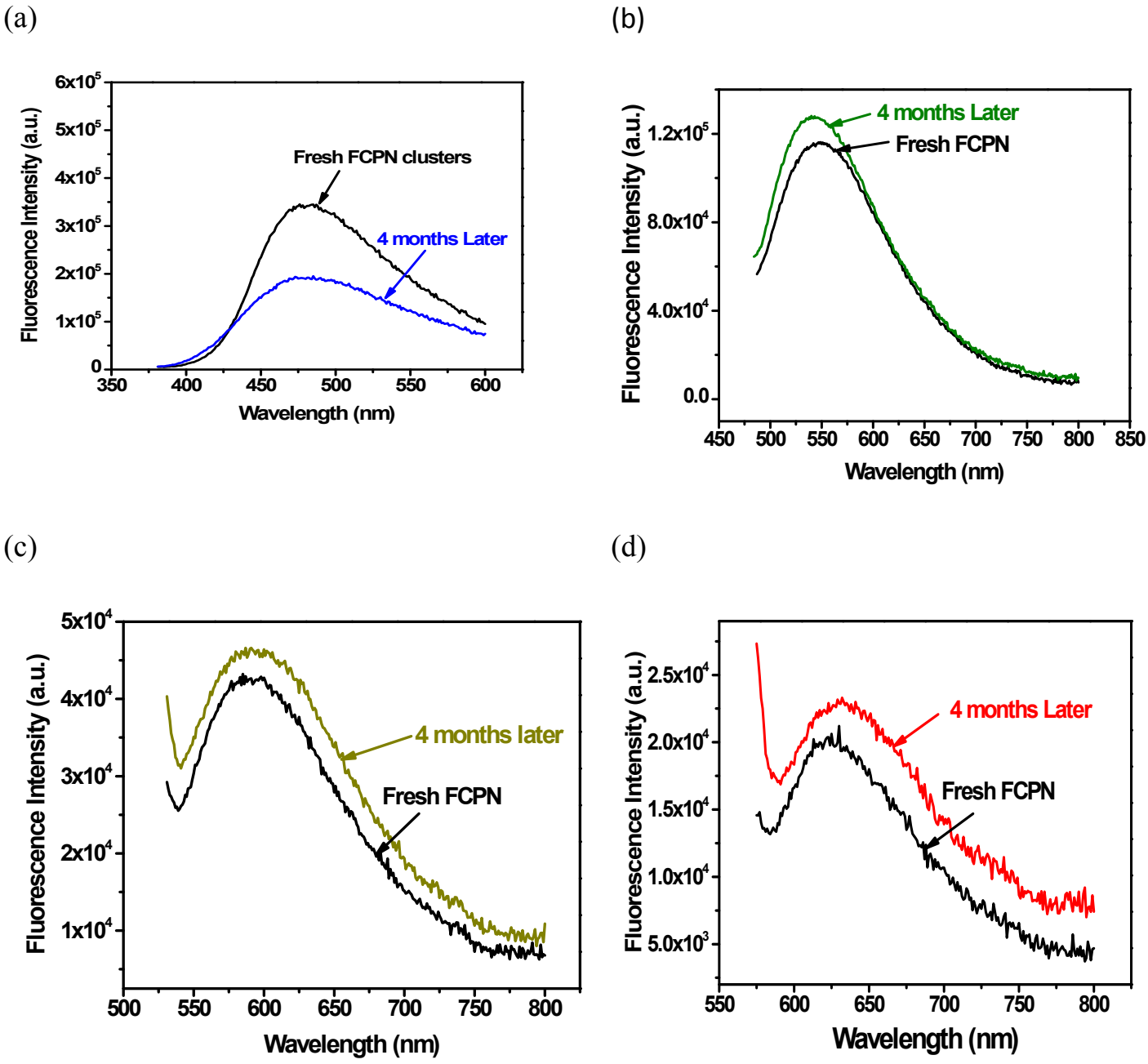


Fig. S16 Fluorescence stability of FCPN over a period of time (4 months) at: (a) Ex. 366 nm, Em. 477 nm, (b) Ex. 472 nm, Em. 541 nm, (c) Ex. 516, Em. 595 nm (d) Ex. 560 nm, Em. 636 nm.

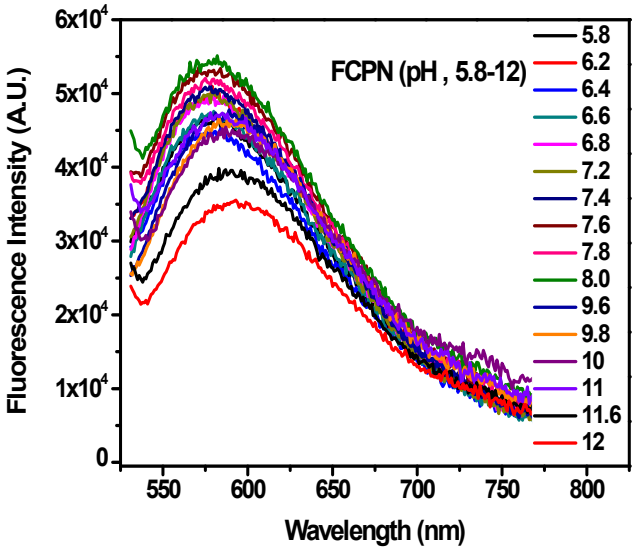


Fig. S17 pH stability of FCPN at different pH, ranging from 5.8 to 12.

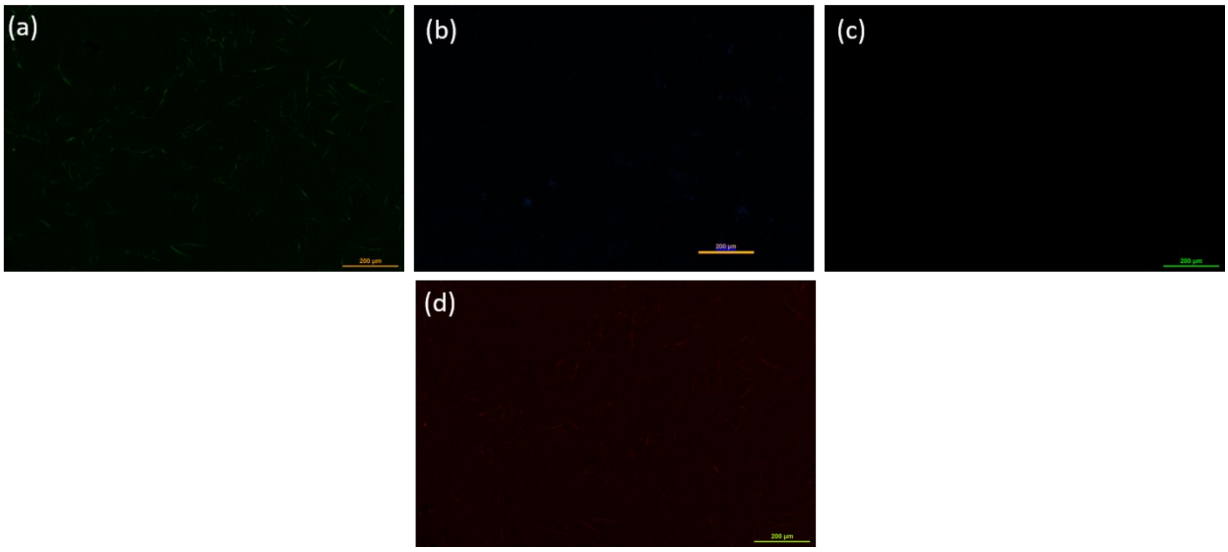
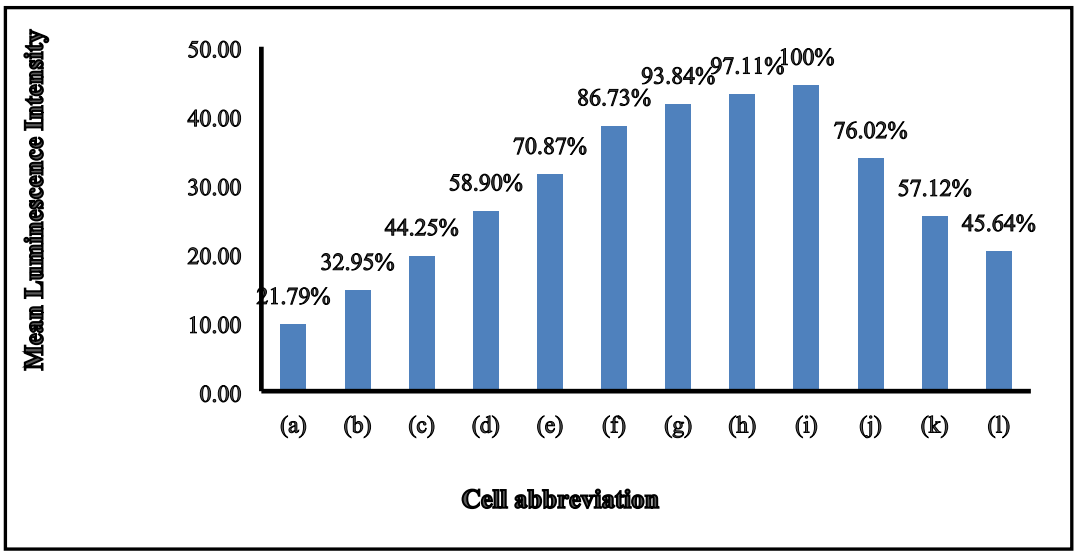
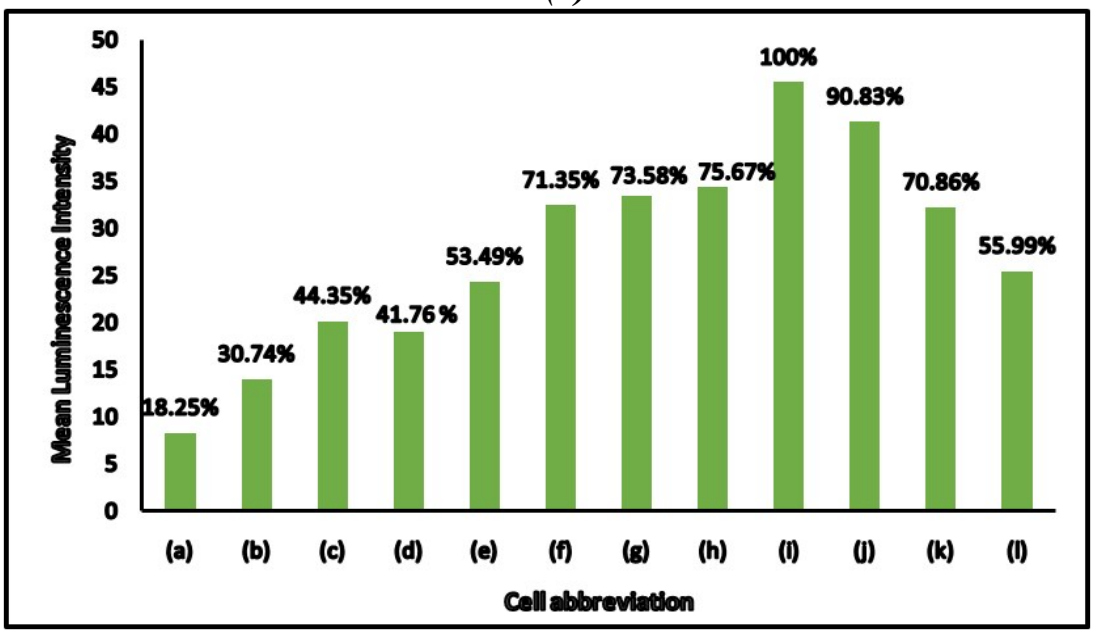


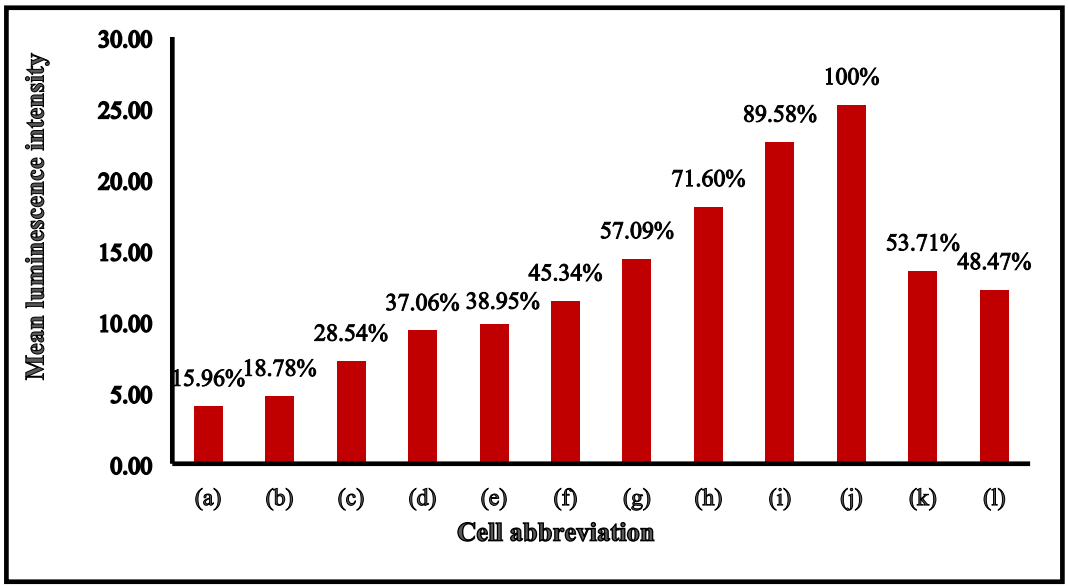
Fig. S18 Fluorescent microscopy images of MG-63 cells incubated with culture media (control) (a) (Ex. 472 nm), (b) (Ex. 366 nm), (c) (Ex. 516) (d) (Ex. 472 nm).The control images appear to be dark, due to lack of emission from the samples.


Mean Luminescence Intensity
50.00
40.00
30.00
20.00
10.00
0.00
21.79%
32.95%
44.25%
58.90%
70.87%
86.73%
93.84%
97.11%
100%
76.02%
57.12%
45.64%
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
Cell abbreviation

(1)


Mean Luminescence Intensity
50
45
40
35
30
25
20
15
10
5
0
18.25%
30.74%
44.35%
41.76 %
53.49%
71.35%
73.58%
75.67%
100%
90.83%
70.86%
55.99%
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
Cell abbreviation

(2)



(3)

Fig. S19 Mean Luminescence Intensity in MG-63 cells (1) FCPN [Ex. 366 nm, Em. Blue (477 nm)] (2) FCPN [Ex. 472 nm, Em. Green (541 nm)] (3) FCPN [Ex. 472 nm, Em. Red (636 nm)]: (a) = 0 µm, (b) = 2µm, (c) = 4µm, (d) = 6µm, (e) = 8µm, (f) = 10µm, (g) = 12µm, (h) = 14 µm, (i) = 16 µm, (j) = 18 µm, (k) = 20 µm, (l) = 22 µm.

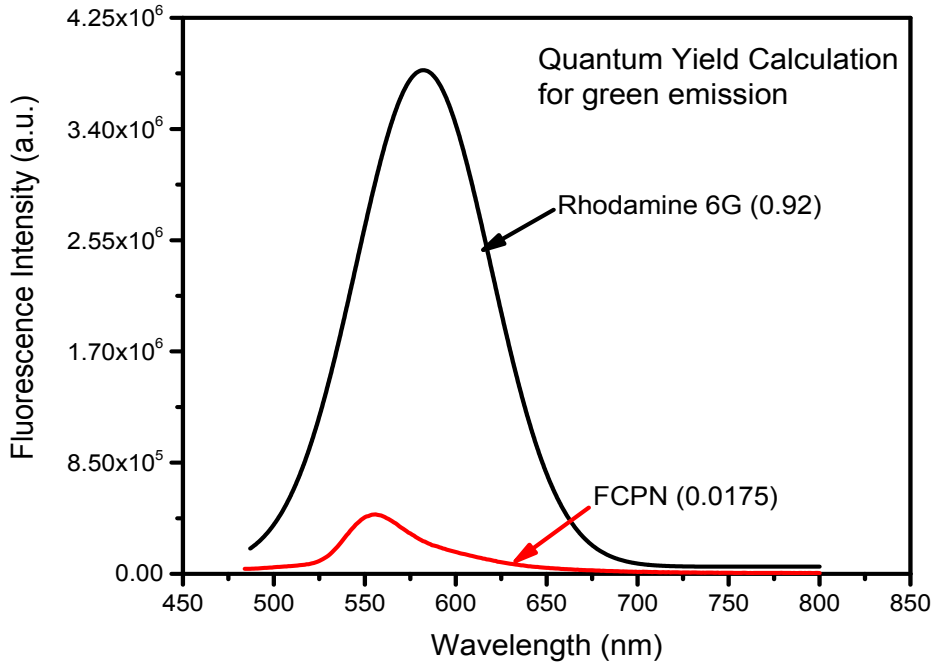


Fig. S20 Fluorescence Spectra of FCPN against Rhodamine 6G for calculation of Quantum Yield of FCPN (green emission).

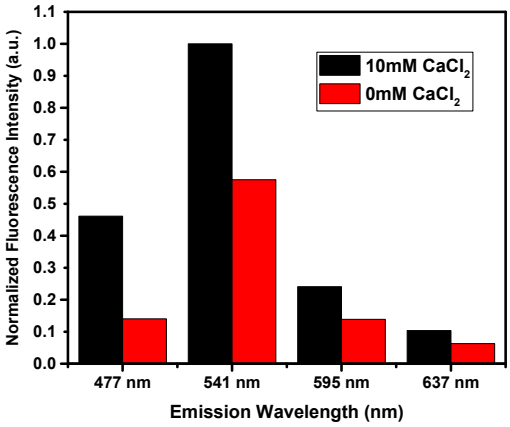


Fig.S21: The comparative fluorescence for FCPN synthesized using 10mM $CaCl_2$ and without $CaCl_2$ (negative Control-B)

Reference

1. M. Grossi, M. Morgunova, S. Cheung, D. Scholz, E. Conroy, M. Terrile, A. Panarella, J. C. Simpson, W. M. Gallagher and D. F. O'Shea, *Nature communications*, 2016, **7**, 1-13.