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Supplementary Information:

Alendronate as robust anchor for ceria nanoparticle surface coating: facile binding and improved biological properties

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UPDATED 19th January 2021: The original version of this ESI was first published on 6th November 2014. This version of the ESI replaces the previous copy in which Figure S2b was incorrectly shown.

Chemicals

Poeusate sodium salt (96%), Oleylamine (technical grade, 70%), 1-Octadecene (technical grade, 90%), Cerium (III) nitrate hexahydrate (trace metals basis, 99%), N-Hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide (EDC), PEG diacid (MW600), Rhodamine B (95%), Nitrotetrazolium Blue chloride (98%), Riboflavin (98%), Succinic acid (99%) were purchased from Sigma-Aldrich. PEG diacid (MW2000) was purchased from Biomatrik. Other chemicals were all analytical reagents and were used as received.

Nanoparticles Synthesis.

Microemulsion method: Typically, the AOT (1.5 g) was dissolved in toluene (50 mL) and mixed with cerium nitrate solution (2.5 mL, 0.1 mol/L). The mixture was stirred for 45 min followed by adding H_2O_2 (5 mL, 30%). The solution was stirred for

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1 h and allowed to separate into two layers. The organic layer containing CNPs were collected for surface modification. Thermal decompositon method: The cerium (III) nitrate hexahydrate (0.434 g) was dissolved in oleylamine (0.802 g) and 1-octadecene (4 g). The mixture was stirred at 80 ºC for 30 min and then heated to 260 ºC to react for 2 h. After cooling to room temperature, the solution was added 80 mL acetone and the nanoparticles were collected by centrifugation. The nanoparticles were washed for three times and finally dispersed in 10 mL hexane.

CNPs-AL: Alendronate (20 mg) and Na_2CO_3 (200 mg) was dissolved in H_2O (4 mL). The mixture was added the prepared CNPs (8 mL) and heated to 80 ºC under magnetic stir. The reaction was kept under this condition for 12 h. After cooling to room temperature, the water layer containing nanoparticles was separated by centrifugation. The nanoparticles were precipitate out by adding acetone (20 mL). Finally, the nanoparticles were dialyzed in water with molecular weight cut off of 10 k for 24 h.

CNPs-AL-Rhodamine B: EDC (4 mg), NHS (2 mg) and Rhodamine B (5 mg) were mixed in 200 μL H_2O and shook at room temperature for 15 min. The mixture was added into 2.5 mL CNPs-AL solution and shook at room temperature for 24 h. After that the nanoparticles were dialyzed in phosphate buffer (5 mM, pH 7.4) with molecular cut off of 10 k for 48 h. The fluorescent spectrum of the CNPs-AL-Rhodamine B was determined by Synergy H4 microplate reader.

CNPs-AL-SA: Succinic acid (28 mg), EDC (30 mg), NHS (15 mg), Alendronate (20 mg) and Na_2CO_3 (50 mg)were dissolved in H_2O (4 mL). The reaction was allowed to carry out for 24 h. Then the prepared CNPs (8 mL) was added to the mixture and heated to 80 ºC under magnetic stir. The reaction was kept under this condition for 12 h. After cooling to room temperature, the water layer containing nanoparticles was separated by centrifugation. The nanoparticles were precipitated out by adding acetone (20 mL). Finally, the nanoparticles were dialyzed in water with molecular weight cut off of 10 k for 24 h.

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CNPs-AL-PEG600: PEG diacid 600 (147 mg), EDC (30 mg) and NHS (15 mg) were mixed in dichloromethane (2 mL) for 8 h at room temperature under magnetic stir. The solvent was removed by rotate evaporation. Alendronate (20 mg) and Na_2CO_3 were dissolved in H_2O (4 mL) and added to the above product. The reaction was allowed to carry out for 12 h. The other procedures were the same as that for CNPs-AL-SA synthesis. The surface coating process for CNPs synthesized by thermal decomposition method were similar as that for CNPs synthesized by microemulsion method. Except that the nanoparticles were precipitate out from hexane by adding ethanol and redispersed in tetrahydrofuran, and the surface coating was carried out in tetrahydrofuran /water system.

CNPs-AL-PEG2000: The procedures for CNPs-AL-PEG2000 synthesis were the same as that for CNPs-AL-PEG600 except using same molar PEG diacid 2000 instead of PEG diacid 600.

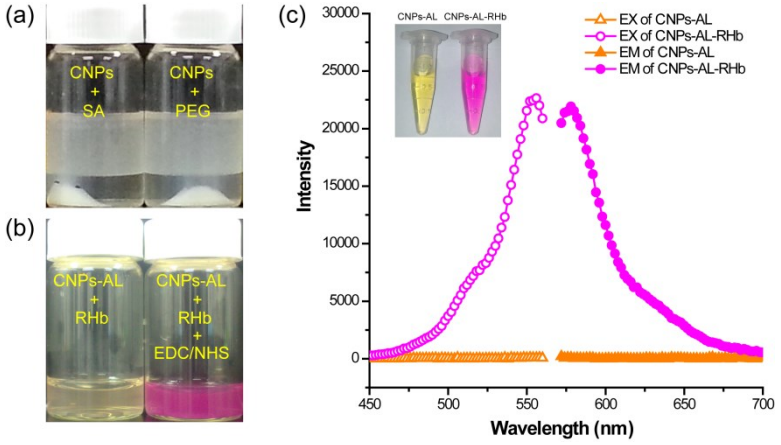

Fig. S1 (a) The reaction of SA (left) and PEG (right) with CNPs under alkaline condition at 80 ºC. (b) The conjugation of Rhodamine B to CNPs-AL catalyzed with and without EDC/NHS. (c) Fluorescent spectrum of CNPs-AL and CNPs-AL-RHb.

Characterization.

TEM measurement: TEM analysis was conducted using a Tecnai G2 F20 S-TWIN TEM operating at 200 KV. Samples were prepared by casting a drop of

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nanoparticle dispersion onto a carbon-coated copper grid. The nanoparticles were dispersed in water.

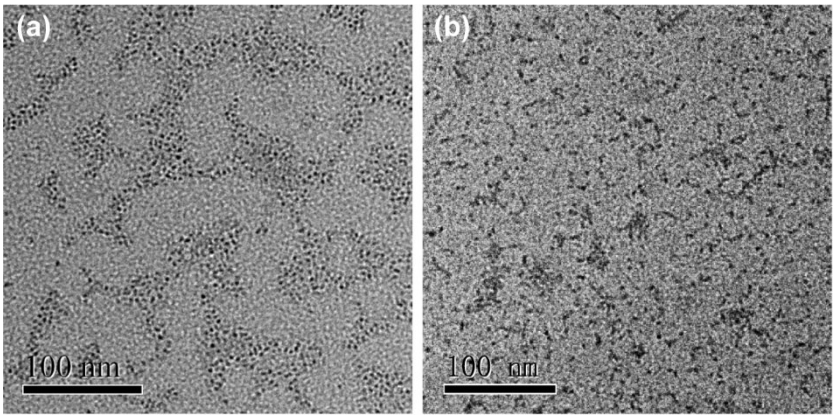

Fig. S2 The TEM images of CNPs-AL-SA (a) and CNPs-AL-PEG2000 (b) dispersed in water.

NMR measurement: NMR spectrum was measured using a DD2 600 MHz NMR spectrometer (Agilent). The nanoparticles were dried on EYELA FDU-2100 freeze drier for 48 h. Samples were dissolved in D_2O.

TGA measurement: TGA experiments were performed on TA Q50. Samples of the surface-modified nanoparticles were dried on EYELA FDU-2100 for 24 h before analysis. The dried nanoparticles were tested with temperature increase rate of 30 ºC/min.

XPS measurement and spectrum integration: XPS experiments were performed using a multipurpose surface analysis system (SCIENTIFIC ESCALAB 250, Thermo, UK).The photoelectron spectra were excited by an Al Ka (1486.6 eV) anode operating at 100 W. The base pressure during XPS analysis was maintained at less than10^{-9} mbar, and the binding energy scale was calibrated from the C1s peak at 284.8 eV. The 3d peak positions of cerium were fitted using XPSPEAK41 software with binding energy from 875 to 920 ev.[1]

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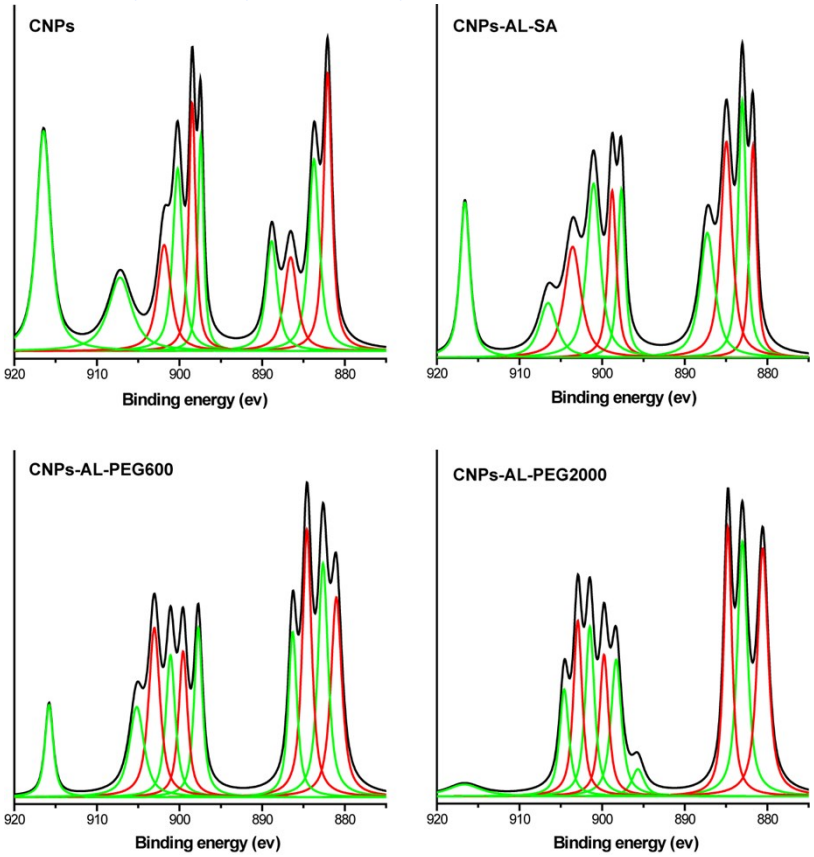

Fig. S3 Integrated XPS spectrum related to the valence state of cerium ions and corresponding binding energy peaks for Ce^{3+} (880.20, 885.00, 899.50 and 903.50 ev) and Ce^{4+} (882.10, 888.10, 898.00, 900.90, 906.40 and 916.35 ev). Red colored peaks represent the peak area of Ce^{3+} and the green colored peaks represent the peak area of Ce^{4+}.

Ligand/CNPs ratio of CNPs-AL-PEG600

Weight of CeO_2 in 1.0 g CNPs-AL-PEG600:

$$m_{CeO2} = 0.483 \times 1.0 = 0.483g$$

Weight of a single CNP:

$$m_{CNP} = \rho v = \rho\frac{4}{3}\pi r^3 = 7.132 \times \frac{4}{3}\pi\left(1.5 \times 10^{-7}\right)^3 = 1.0 \times 10^{-19}g$$

Number of CNPs in 1.0 g of CNPs-AL-PEG600:

$$n_{CNPs} = \frac{m_{CeO2}}{m_{CNP}} = \frac{0.483}{1.0 \times 10^{-19}} = 0.483 \times 10^{19}$$

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Weight of alendronate conjugated PEG in 1.0 g CNPs-AL-PEG600:

$$m_{AL-PEG} = 0.517 \times 1.0 = 0.517\ g$$

Number of CNPs in 1.0 g of CNPs-AL-PEG600:

$$n_{AL-PEG} = \frac{0.517}{600 + 324 - 18} \times 6.02 \times 10^{23} = 3.435 \times 10^{20}$$

Ratio of ligand to CNPs:

$$R = \frac{n_{AL-PEG}}{n_{CNPs}} = \frac{3.435 \times 10^{20}}{0.483 \times 10^{19}} = 71$$

Ligand/CNPs ratio of CNPs-AL-PEG2000

Weight of CeO_2 in 1.0 g CNPs-AL-PEG2000:

$$m_{CeO2} = 0.327 \times 1.0 = 0.327g$$

Weight of a single CNP:

$$m_{CNP} = \rho v = \rho\frac{4}{3}\pi r^3 = 7.132 \times \frac{4}{3}\pi\left(1.5 \times 10^{-7}\right)^3 = 1.0 \times 10^{-19}g$$

Number of CNPs in 1.0 g of CNPs-AL-PEG2000:

$$n_{CNPs} = \frac{m_{CeO2}}{m_{CNP}} = \frac{0.327}{1.0 \times 10^{-19}} = 0.327 \times 10^{19}$$

Weight of alendronate conjugated PEG in 1.0 g CNPs-AL-PEG2000:

$$m_{AL-PEG} = 0.673 \times 1.0 = 0.673\ g$$

Number of CNPs in 1.0 g of CNPs-AL-PEG2000:

$$n_{AL-PEG} = \frac{0.673}{2000 + 324 - 18} \times 6.02 \times 10^{23} = 1.757 \times 10^{20}$$

Ratio of ligand to CNPs:

$$R = \frac{n_{AL-PEG}}{n_{CNPs}} = \frac{1.757 \times 10^{20}}{0.327 \times 10^{19}} = 54$$

Superoxide Dismutase Mimetic Activity

The superoxide dismutase mimetic activity of CNPs was tested by inhibiting the superoxide free radical generated by riboflavin under light illumination.[2] The solution containing 200 μL EDTA (0.1 mol/L), 75 μL nitrotetrazolium blue chloride (2 mmol/L)

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and 2.9 mL sodium phosphate buffer (10.0 mmol/L, pH 7.8) was incubated at 37 ºC for 5 min. The solution was added 50 μL riboflavin (1.2 mmol/L) and the mixture was used as detection solution. The CNPs with different Ce concentration in 50 μL water was miexed with 100 μL detection solution. The mixture was shook at 37 ºC for 5 min followed by illumination for 2 min using a 27 W light tube. The excessive superoxide free radical which could not be inhibited by CNPs reacted with nitrotetrazolium blue chloride to generate blue product which could be detected at 560 nm. The inhibition was calculated by the decrease of absorbance at 560 nm.

Nanoparticle Stability

The stability of CNPs-AL-PEG600 and CNPs-AL-PEG2000 in PBS plus 10% FBS were tested by DLS using a Brookheaven Zeta PALS analyzer. Nanoparticles were incubated at 37 ºC throughout the test process. Every test took three runs with data collecting time of 2 min for each run.

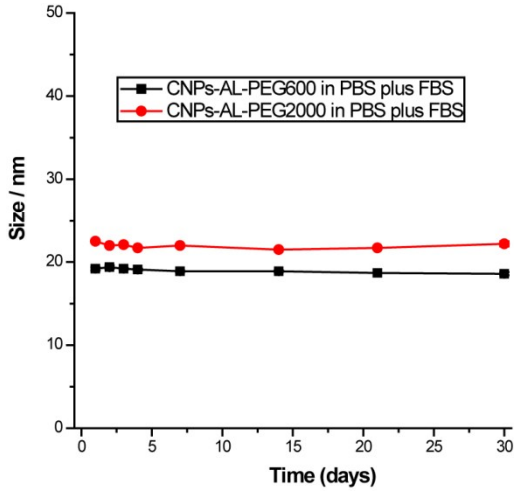

Fig. S4 Stability of CNPs coated with PEG in PBS plus FBS

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Table S1. DLS data of CNPs coated with PEG dispersed in PBS plus 10% FBS

Time (days)	CNPS-AL-PEG600 (nm)	CNPS-AL-PEG2000 (nm)
1	19.2 ± 0.2	22.5±0.1
2	19.4 ± 0.2	22.0±0.1
3	19.2 ± 0.1	22.1±0.2
4	19.1 ± 0.1	21.7±0.0
7	18.9 ± 0.1	22.0±0.2
14	18.9 ± 0.1	21.5±0.1
21	18.7 ± 0.0	21.7±0.0
30	18.6 ± 0.2	22.2±0.3

Cell viability

The HL7702 liver cells were cultured in a humidified atmosphere (5% CO2) at 37 ºC, and grown in RPMI-1640 medium supplemented with 10% FBS. For cell viability assay, the cells were seeded in 96 well plate at 5000 cells in 0.1 mL medium 24 h prior to the experiment. The CNPs, CNPs-AL-SA, CNPs-AL-PEG600 and CNPs-AL-PEG2000 were added to the cell culture medium with cerium concentration of 5, 10, and 20 μg/mL. The cells were cultured for another 24 h followed by adding cell viability detection reagent CCK-8 (10 μL/well). After incubation for 2 h, the absorbance at 450 nm was detected for viability analysis.

Blood circulation

Male MALB/c mice were purchased from the Third Military Medical University Animal Center, Chongqing, China. Animals were housed under controlled laboratory conditions with a 12: 12 h light: dark cycle at 20 ± 5 °C and 40-70% humidity. All animal protocols were reviewed and approved by the Animal Ethics Committee of the Third Military Medical University. The nanoparticles were injected via the tail ven with Ce dose of 10 mg/kg. The blood samples (10μL) were collected from angular vein 0.25, 1, 2, 4, 12, 24 and 48 h after the infusion. The samples were added concentrated nitric

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acid and digested at 70 ºC for 3 h. The cerium concentration was tested by ICP-MS (Agilent 7500ce).

Table S2 Original data of cerium concentration in mice blood (ppm)

Sample collection	0.25h	1h	2h	4h	12h	24h	48h
CNPs	1.98644	0.08814	0.08998	0.21142	0.2899	0.06679	0.04731
	1.63603	0.14996	0.07606	0.17258	0.299	0.08371	0.04607
	1.24785	0.10488	0.07017	0.14402	0.5868	0.17226	0.03789
Average	1.62344	0.11433	0.07874	0.17601	0.3919	0.10759	0.04376
STDEV	0.36945	0.03198	0.01017	0.03383	0.16885	0.05664	0.00512
CNPs-AL-SA	16.98465	6.09285	4.47435	1.5765	0.1551	0.08715	0.1254
	13.8459	5.6991	3.26955	1.40265	0.17115	0.09465	0.10965
	15.5889	6.2007	3.2853	1.46295	0.1734	0.07905	0.11895
Average	15.47315	5.99755	3.6764	1.4807	0.16655	0.08695	0.118
STDEV	1.57257	0.26403	0.69109	0.08827	0.00998	0.0078	0.00792
CNPs-AL-PEG600	47.62608	39.64829	41.47524	34.16954	9.5509	0.17867	0.04463
	41.30211	34.68718	39.28457	28.44561	8.5073	0.18375	0.05358
	46.73446	37.51387	35.98189	28.37366	9.9451	0.23885	0.18834
Average	45.22088	37.28311	38.9139	30.3296	9.33443	0.20042	0.09552
STDEV	3.42291	2.48859	2.76537	3.32568	0.74294	0.03337	0.08051
CNPs-AL-PEG2000	44.9132	35.49913	40.93582	36.44924	25.4163	11.57233	1.0994
	45.53551	43.17919	41.88061	40.05135	25.7165	12.99332	2.1837
	46.89	44.19926	41.06953	38.0493	27.6838	11.78848	0.77753
Average	45.77957	40.95919	41.29532	38.1833	26.2722	12.11804	1.35354
STDEV	1.01074	4.75598	0.51127	1.80479	1.23166	0.76568	0.73673

Organ distribution

After blood sample collection, the animals were sacrificed at day 7. The organs including brain, heart, lung, kidney, liver and spleen were collected and weighted. The tissues were rinsed with ultrapure water to remove any superficial cerium. After that the tissues were added 3.0 mL concentrated nitric acid and digested with a microwave reaction system (CEM MARS 6) at 150 °C for 20 min. Finally, the Ce concentration in the tissues were determined by ICP-MS.

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Table S3 Original data of cerium concentration in mice organs (μg/g)

Organs	Brain	Heart	Kidney	Lung	Liver	Spleen
	0.01618	0.65173	0.84193	2.52931	78.14646	166.4361
CNPs	0.05916	0.53761	0.78099	4.33784	51.94707	142.5082
	0.01972	0.38626	0.68723	5.21337	74.38813	144.7065
Average	0.03168	0.5252	0.77005	4.02684	68.16056	151.2169
STDEV	0.02386	0.13317	0.07792	1.36879	14.16648	13.22596
	0.05917	1.85512	5.22206	2.09857	60.58608	100.737
CNPs-AL-SA	0.41429	2.12208	4.13745	2.12755	49.24168	61.51611
	0.06191	1.3225	4.28636	1.56505	44.8148	105.6488
Average	0.17846	1.76657	4.54862	1.93039	51.54752	89.30061
STDEV	0.20424	0.40708	0.58795	0.31673	8.13455	24.18709
	0.03733	1.65094	4.14337	2.50851	55.00702	38.94745
CNPs-AL-PEG600	0.05336	1.89641	3.38332	1.9051	39.46317	26.74552
	0.02942	1.50091	3.34997	1.5382	48.752	28.99394
Average	0.04004	1.68275	3.62555	1.98394	47.74073	31.5623
STDEV	0.0122	0.19966	0.44875	0.48994	7.82111	6.49378
	0.04194	1.03377	2.54885	0.60497	33.696	25.89853
CNPs-AL-PEG2000	0.03372	1.30066	4.06459	2.0932	44.98762	24.226
	0.0308	1.39914	3.50613	1.43475	28.76485	20.23741
Average	0.03549	1.24452	3.37319	1.37764	35.81616	23.45398
STDEV	0.00578	0.18904	0.76656	0.74576	8.3166	2.90845

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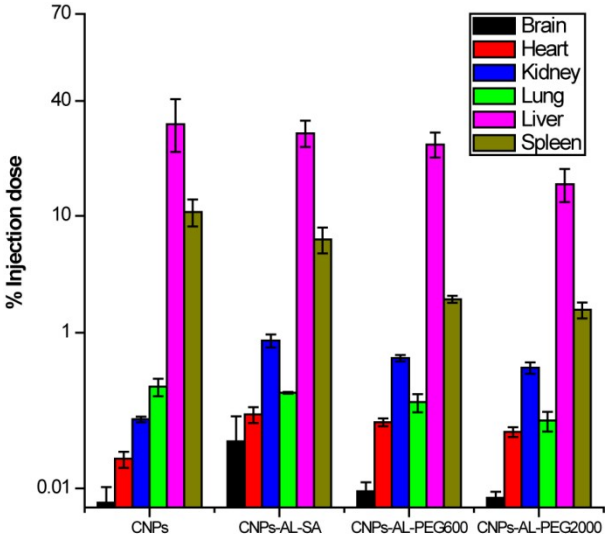

Fig. S5 Distribution of nanoparticles in organs

Table S4 Original data of Ce dose percentage in organs (%)

Organs	Brain	Heart	Kidney	Lung	Liver	Spleen
	0.00337	0.03446	0.10548	0.18316	38.5792	13.10445
CNPs	0.01114	0.02825	0.09244	0.26116	22.81961	9.01583
	0.00343	0.02005	0.09098	0.30039	35.28123	9.716
Average	0.00598	0.02759	0.0963	0.24823	32.22668	10.6121
STDEV	0.00447	0.00723	0.00799	0.05967	8.31197	2.18665
	0.016	0.11807	0.96757	0.20461	33.41322	6.97963
CNPs-AL-SA	0.11349	0.13465	0.80185	0.21365	29.30227	5.18122
	0.01572	0.08362	0.71071	0.2076	25.24085	8.17926
Average	0.0484	0.11211	0.82671	0.20862	29.31878	6.78004
STDEV	0.05637	0.02603	0.13023	0.00461	4.08621	1.50896
	0.00763	0.08614	0.54269	0.20177	23.94599	2.28605
CNPs-AL-PEG600	0.01285	0.0995	0.57015	0.15758	30.10894	2.14625
	0.00659	0.07921	0.48574	0.11964	23.83431	1.96514
Average	0.00902	0.08828	0.53286	0.15966	25.96308	2.13248
STDEV	0.00335	0.01031	0.04306	0.0411	3.59085	0.1609
	0.00917	0.05453	0.36272	0.06149	16.10743	1.93527
CNPs-AL-PEG2000	0.00623	0.06835	0.48319	0.1068	19.32632	1.76751
	0.00601	0.07346	0.39619	0.11119	12.25383	1.36603
Average	0.00714	0.06545	0.41403	0.09316	15.89586	1.6896
STDEV	0.00176	0.00979	0.06219	0.02751	3.54099	0.29251

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