

Multifunctional nanoadditives for the thermodynamic and kinetic stabilization of enzymes

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Supporting Information

Nanoparticle synthesis

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated: benzyl ether (99%), iron(III) acetylacetonate (97%), oleic acid (BDH, 92%), oleyl amine (70%), Pluronic F108, polyethylenimine (PEI 50% solution, *Mn* 1200, *Mw* 1300), poly(glycidyl methacrylate) (PGMA, 200 kDa, gift from IL and BZ, Clemson University, Clemson) rhodamine B (RhB, Kodak, 95%), and 1,2-tetradecanediol (90%) were used as received.

Preparation of Multimodal polymeric nanoparticles:

Magnetite synthesis

Magnetite was prepared in accordance with the method described by Sun *et al.*¹ Briefly, iron(III) acetylacetonate (2 mmol), 1,2-tetradecanediol (10 mmol), oleic acid (6 mmol), oleylamine (6 mmol), and benzyl ether (20 mL) were mixed with a magnetic stirrer and gradually heated under a constant flow of Nitrogen. The mixture was held at 100 °C for ≈1h before being ramped to 200 °C, held 2h and finally heated to reflux (300 °C) and held for 1 hour under a blanket of N₂. The sample was allowed to cool to

room temperature over night under N₂ flow. The sample was collected and purified through a series of precipitations with ethanol, collection via centrifugation and then resuspension in hexane.

PGMA modification with Rhodamine

PGMA (100 mg) and Rhodamine B (20 mg) were dissolved in ethyl methyl ketone (MEK) (30 mL) and heated to reflux under N₂ atmosphere for 18 h. The PGMA-Rhodamine modified polymer was precipitated with diethyl ether and dried before use in nanoparticle production.

Multimodal polymeric nanoparticle synthesis

Nanoparticles were prepared by an 'oil in water' emulsion process. The organic phase contained magnetite nanoparticles (20 mg), dissolved PGMA-Rhodamine B (80 mg) in a 1:3 mixture of CHCl₃ and MEK (6 mL). The organic phase was added drop wise to a vortexing aqueous solution of Pluronic F108 (1.25% w/v, 30 ml) with the resulting microemulsion homogenized with a probe-type ultrasonicator for 1 min. Organic solvents were allowed to evaporate under moderate stirring and N₂ flow overnight. Magnetite aggregates and unreacted polymer was removed via centrifugation (3000 x g, 45 min), with the supernatant being collected and incubated with PEI (50 wt% solution, 100 mg) at 70 °C for 20 h. The PEI modified magnetic polymeric nanoparticles were collected on a magnetic separation column (LS, Miltenyi Biotec), washed with milli-Q water to remove excess Pluronic and unattached dye before being collected, aliquoted and stored. The equivalent dry mass of samples was determined by freeze-drying.

Nanoparticle characterization

Transmission Electron Microscopy (TEM)

Nanoparticle samples were prepared by deposition onto carbon coated grids and imaged at 120kV on a JEOL JEM-2100.

Dynamic Light Scattering (DLS) and Zeta Potential measurements

Nanoparticle samples were thoroughly washed ($3 \times \approx 2\text{mL}$ milliQ water) while being held on a magnetic separating column before being resuspended in milli-Q water for analysis.

Superconducting quantum interference device (SQUID) Magnetometer measurements

Magnetic properties of the magnetite as well as the PGMA-magnetite-rhodamine B-PEI composite nanoparticles were measured using a Quantum Design MPMS SQUID magnetometer. For both samples hysteresis was measured at 5K and 300K; zero field cooled and field cooled (ZFC/FC) measurements were also collected.

Enzyme Stabilisation studies

Thermal Stabilisation studies

Enzyme samples prepared in phosphate-citrate buffer (pH 4.5) had either phosphate-citrate buffer added (naked enzyme) or polymeric nanoparticle additives suspended in phosphate-citrate buffer (enzyme + NPs) to a final nanoparticle concentration of $300 \mu\text{g mL}^{-1}$ unless stated otherwise. Samples were heat treated at the desired temperature (30, 50, 60, 70 °C) for 10 min before activity was determined (see activity determination). Each sample was measured in triplicate and results were analysed using ANOVA and Bonferroni/Dunn *post hoc* tests, requiring a significance of $p \leq 0.05$.

Longevity experiment

β -glucosidase samples were prepared with either phosphate citrate buffer (pH 4.5) added (naked enzyme) or nanoparticles suspended in phosphate citrate buffer (pH 4.5) (enzyme + NPs) to create a final nanoparticle concentration of $300 \mu\text{g mL}^{-1}$. Samples were incubated constantly at 37 °C in a water bath with aliquots removed at days 1, 3, 5, 7 and 9 days. Aliquots were immediately transferred to -20 °C until completion of the time course. Samples for all enzymes were thawed together and their activity determined (see activity determination). All samples were collected and measured in triplicate for statis-

tical analysis as described above.

Activity Determination

Enzyme aliquots (10 μL) both naked and with nanoparticles present were incubated with 90 μL of the appropriate *p*-nitrophenol substrate for the enzyme (4 mg mL^{-1} , phosphate citrate buffer pH 4.5, 30 $^{\circ}\text{C}$) and incubated for 30 min at the specified temperatures. Samples were quenched with 300 μL of glycine (200 mM, pH 10.75) and centrifuged (5 min, 17000 x g) to remove nanoparticles. For control purposes, the naked enzyme samples were also centrifuged. Samples of the supernatants were aliquoted (100 μL) into a 96-well plate and absorbance measured at 405 nm (BMG Fluorstar).

SDS-PAGE

A denaturing SDS-PAGE was used to quantify enzyme attachment to nanoparticles. β -Glucosidase (0.8 mg mL^{-1} final concentration) was incubated with nanoparticles at increasing concentrations (0, 1, 10, 50, 100 mg mL^{-1}) at room temperature for 30 min. Samples were centrifuged (20 min, 17000 x g) to remove all nanoparticles and aliquots of the supernatant were collected and electrophoresed using an SDS-PAGE denaturing gel.

Elemental analysis of PGMA nanoparticles by mass

- Nanoparticles before PEI modification: C 56.67%, H 7.04%, N <0.30%.
- PEI-modified nanoparticles: C 50.84%, H 6.56%, N 1.10%.

Assuming the contribution to the nitrogen content from the rhodamine B is negligible (below detection limits in sample without PEI) then we can calculate the percentage of PEI attached by attributing the nitrogen content of the PEI modified nanoparticles solely to the addition of PEI.

By mass, PEI is 32.53% N (PEI: $\text{C}_2\text{H}_5\text{N}$, $M_w = 43.07 \text{ g mol}^{-1}$ depending on extent of branching).

Hence if 1.1% of the composite nanoparticles is nitrogen, this equates to 3.38% of PEI incorporated with the nanoparticles by mass.

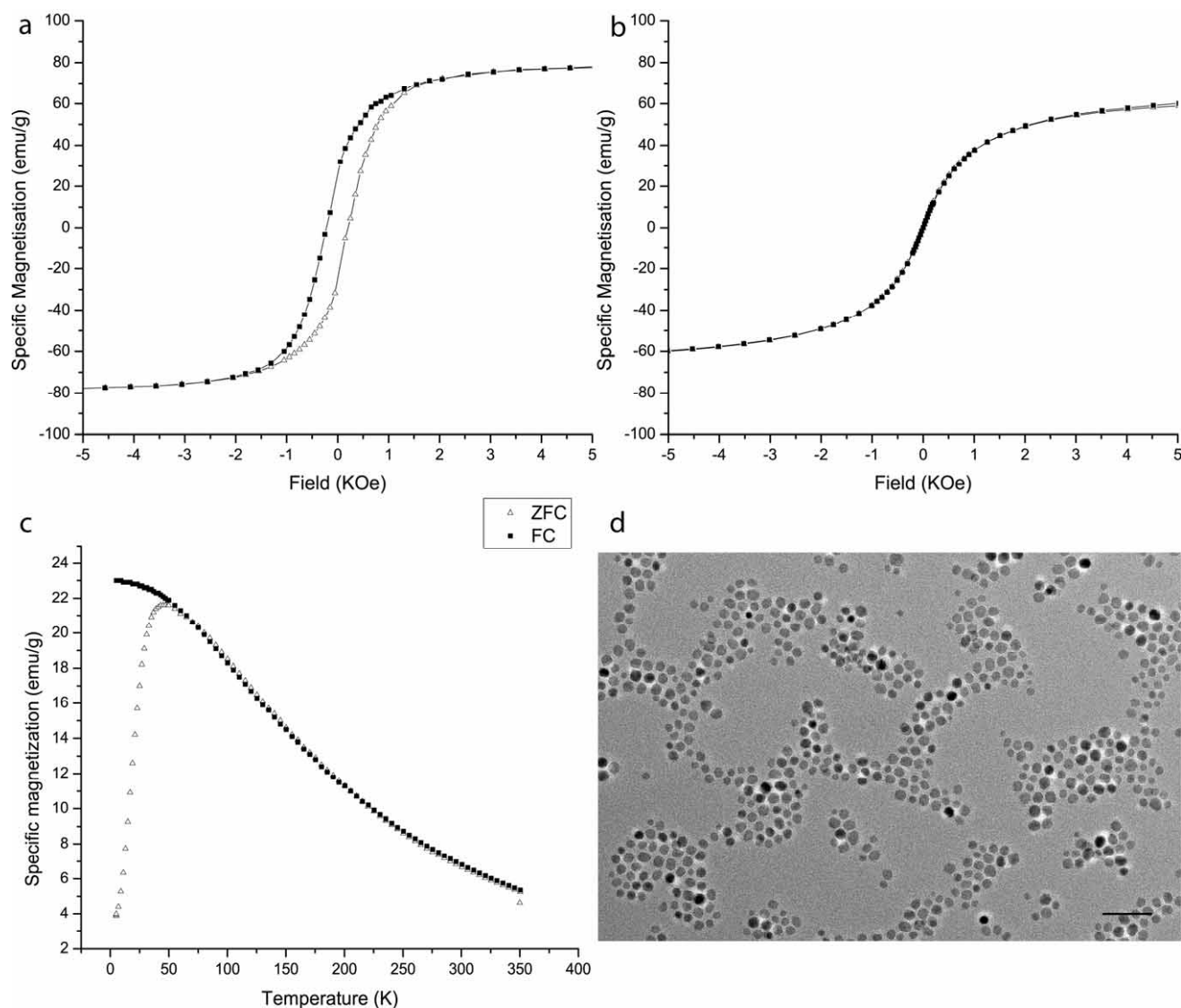


Figure S1 – Magnetite characterization. SQUID magnetometry of magnetite particles portraying superparamagnetic behavior. **a**, Hysteresis loop at 5 K. **b**, Hysteresis loop at 300 K, displaying no hysteresis. **c**, Zero field cooled/field cooled (ZFC/FC) curves are coincident at temperatures above 50 K. **d**, TEM image of magnetite sample, scale bar 50 nm.

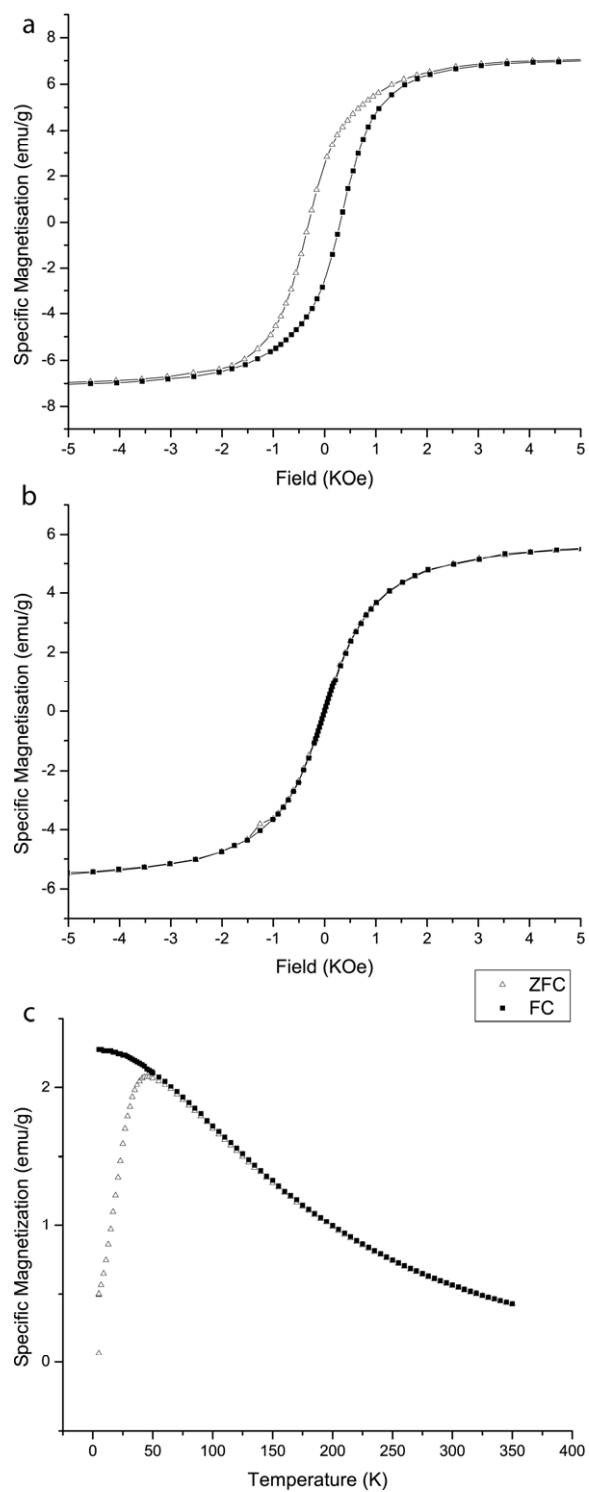


Figure S2 – Magnetic characterization of PGMA-magnetite-Rhodamine-PEI composite nanoparticles.

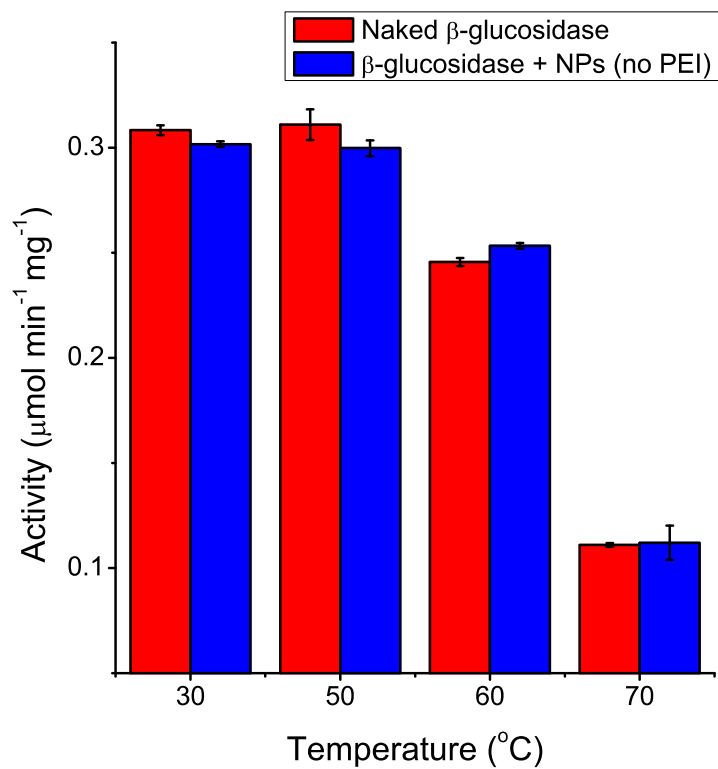


Figure S3 – Polymeric nanoparticles without PEI functionalized surface ($300 \mu\text{g mL}^{-1}$) were unable to stabilize β -glucosidase at a range of measured temperatures.

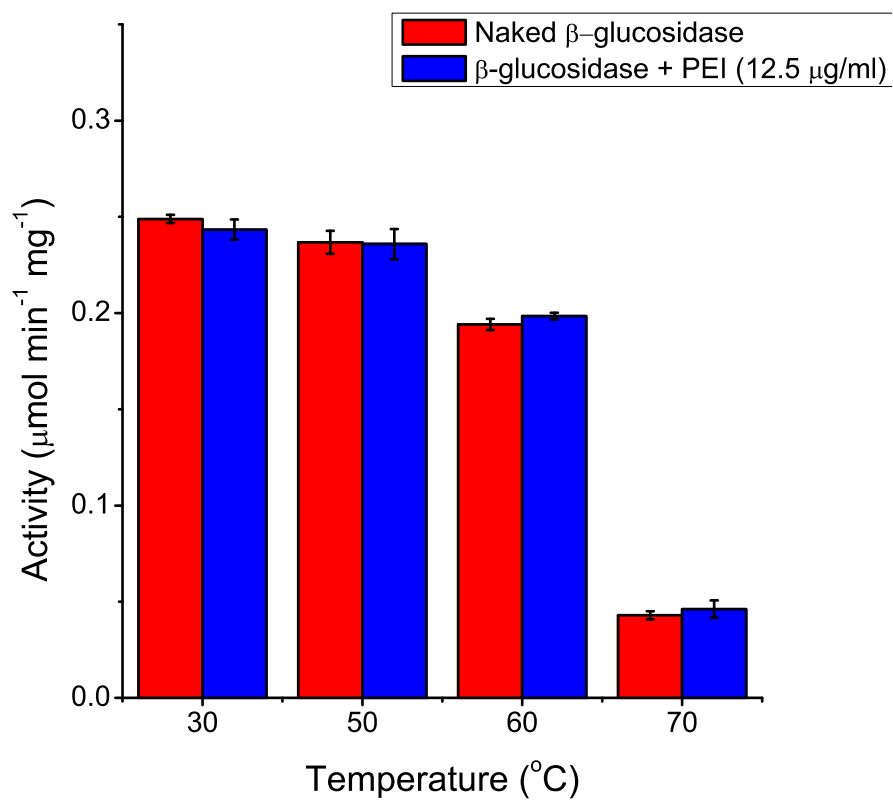


Figure S4 – β -glucosidase incubated with free PEI (final concentration 12.5 $\mu\text{g mL}^{-1}$). No significant stabilization evident across temperatures assayed (30 $^{\circ}\text{C}$ – 70 $^{\circ}\text{C}$) (* $P \leq 0.05$).

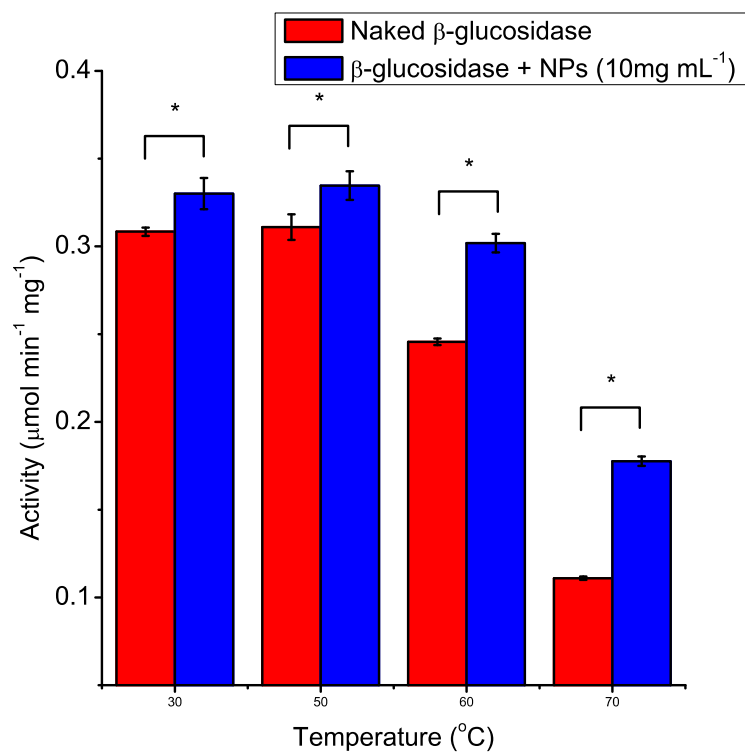


Figure S5 – Higher nanoparticle concentration (10mg mL^{-1}) significantly enhanced the degree of enzyme stabilization observed with β -glucosidase ($*P \leq 0.05$).

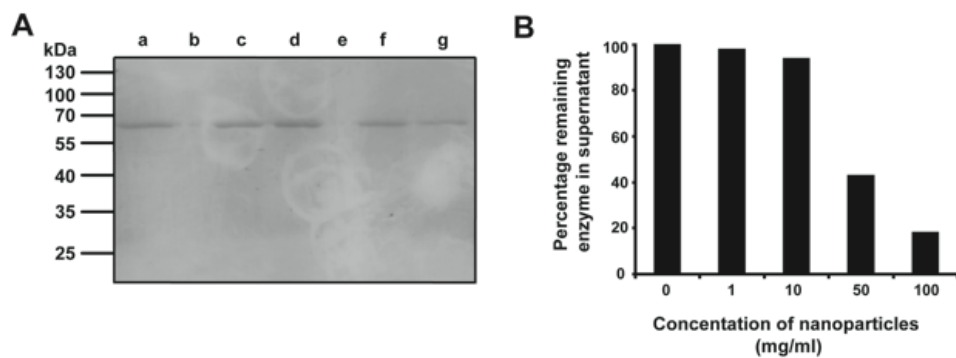


Figure S6 – SDS page investigation of enzyme attachment to nanoparticles. A) SDS page gel with nanoparticle concentrations of a) 0 mg mL⁻¹, b) empty, c) 1 mg mL⁻¹, d) 10 mg mL⁻¹, e) empty, f) 50 mg mL⁻¹ and g) 100 mg mL⁻¹ across the lanes. B) Densitometry plot of SDS page bands.

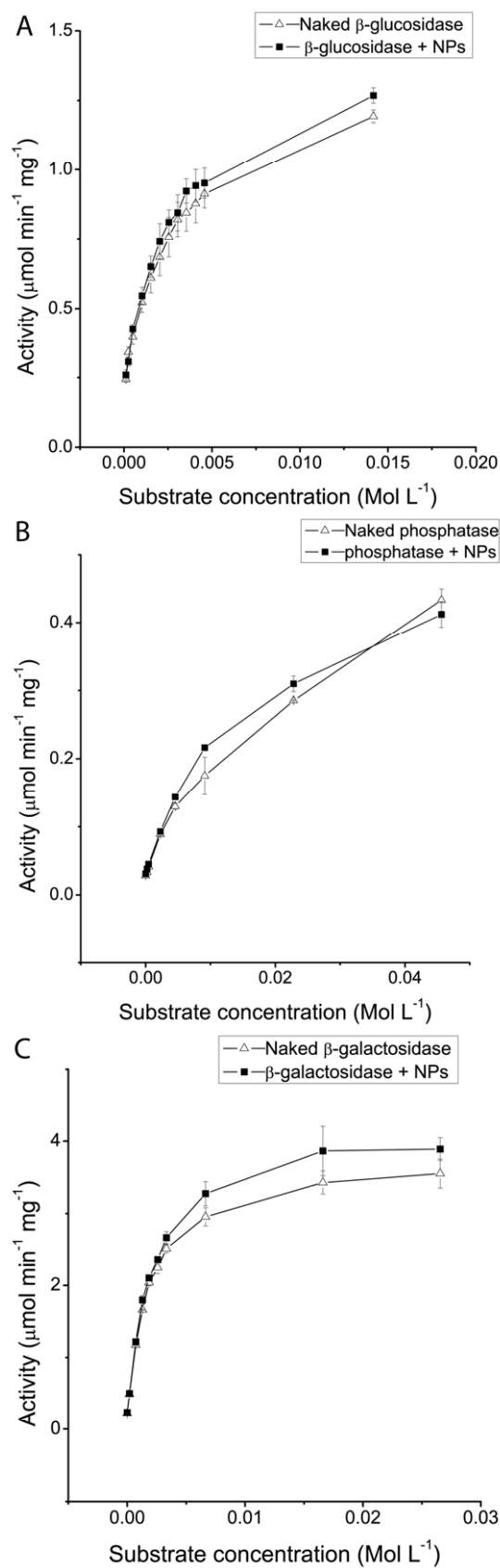


Figure S7 – Michaelis-Menten kinetics for naked enzyme and enzyme with nanoparticles for A) Almond β -glucosidase, B) acid phosphatase from potato C) β -galactosidase from *A. oryzae*.

References

1. Sun, S.; Zeng, H.; Robinson, D.; Raoux, S.; Rice, P.; Wang, S.; Li, G., Monodisperse MFe₂O₄ (M= Fe, Co, Mn) Nanoparticles. *J. Am. Chem. Soc.* 2004, *126*, 273-279.