Supporting Information for:

Modulation of $\alpha$-Chymotrypsin Conjugated to Magnetic Nanoparticles by Non-Heating Low Frequency Magnetic Field: Molecular Dynamics, Reaction Kinetics and Spectroscopy Analysis

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Synthesis of magnetite MNPs. Six hundred fifty milligrams of FeCl$_3$ and 398 mg of FeCl$_2$·4H$_2$O were dissolved in 5 ml of 2M HCl, the obtained solution was added dropwise to 50 ml of 0.7M NH$_3$·H$_2$O solution, and then the mixture was magnetically stirred for 30 minutes. After that, magnetite nanoparticles were magnetically decanted and washed with 50 ml of 2 M HClO$_4$, then nanoparticles were magnetically decanted again and dissolved in 50 ml DI H$_2$O for 30 minutes via ultrasonication.

Synthesis of gold-coated magnetite MNPs (GM-MNPs). Twenty milliliters of HAuCl$_4$ water solution (35 mg of HAuCl$_4$·3H$_2$O) under vigorous stirring was heated under reflux to boiling state, and 5 ml of freshly prepared Fe$_3$O$_4$ MNP dispersion was quickly added to HAuCl$_4$ solution. After 10 min, 5 ml of sodium citrate (80 mM) was rapidly added to the reaction mixture. This mixture was boiled under reflux with vigorous stirring for 5 min; the heating was then turned off, and the mixture was cooled to room temperature.
Figure S1. The kinetic curve of p-nitrophenol formation in a reaction of p-nitrophenol trimethylacetate hydrolysis by CT (7.5 μM). Substrate concentration 6 μM. 20 mM TRIS-HCl (pH 8.2), 1% acetonitrile, temperature 25 °C. Enzyme’s active sites concentration is equal to the initial rapid burst of the product. \( \varepsilon = 18500 \text{ M}^{-1}\text{cm}^{-1} \).
**Description of LFMF generator.** The source of LFMF of original patented design was a pair of water-cooled solenoids in Helmholtz geometry with a gap between them of 60 mm. Coils created a homogenous magnetic field of adjustable amplitude and frequency in the gap. A sample suspension was placed in a thermostated cell located in the center of the gap between the solenoids. Thermostating guaranteed the absence of the sample heating from structural elements of LFMF generator. Inhomogenity of the field within the working cell did not exceed 1%, which prevented the transport of the MNPs along the magnetic field gradient at long exposures time. The solenoid were powered by regulated electronic generator with a power of 1.5 kW
**Figure S2.** The structure of CT molecule based on Protein Data Bank (code 1ACB). Atoms of catalytic triad Ser-195, His-57, and Asp-102 (green) and C\(_\alpha\)-atoms of Cys-191, Ser-217, and Ser-218 forming the entrance to substrate binding site (orange) are presented in spheres. Residues Lys-79, 90, 107, 175, 202 (blue) and Glu-21, 49, Asp-64, 129 (red) are corresponded to force application sites of NH\(_2\)-stretched and COOH-stretched protocols, respectively (as described in Figure 2).
Figure S3. Full curves of the evolution of the distance between $C_{\alpha}$ atoms in the amino acid residues forming the entrance into substrate binding site of the CT molecule: (A) Cys-191:$C_{\alpha}$-Ser-217:$C_{\alpha}$ and (B) Cys-191:$C_{\alpha}$-Ser-218:$C_{\alpha}$. The external forces of 80 pN were applied as described in Figure 2. The data for the NH$_2$-stretched protocol shown in the figure was previously published by us in $^{[14]}$. 

![Graph A](image1.png)  

![Graph B](image2.png)
**Figure S4.** TEM micrographs of (A) uncoated Fe$_3$O$_4$ nanoparticles and (B) core-shell Fe$_3$O$_4$-Au nanoparticles.
Mössbauer spectroscopy. Table S 1 summarizes the parameters extracted from the fitting of the Mössbauer spectra of iron oxide nanoparticles (Figure S 5). $\delta_s$ – isomeric shift, $\Delta$ - quadrupole splitting, $H_{\text{eff}}$ – effective magnetic field. The corresponding errors are 5 kOe for $H_{\text{eff}}$, 0.02 mm/s for $\delta_s$, 0.01 mm/s for $\Delta$, 0.6% for the component area S determination.

A feature of the Mössbauer spectra of nonstoichiometric magnetite is the distribution of the electron exchange between $\text{Fe}^{3+}$ and $\text{Fe}^{2+}$ ions in the octahedral positions in the presence of vacancies. At the same time, some of $\text{Fe}^{3+}$ ions that are not involved in such exchanges because of the $\text{Fe}^{2+}$ ions deficit forming a spectra which is not distinguishable from a Mössbauer spectra of $\text{Fe}^{3+}$ ions in the tetrahedral positions. The accuracy of Mössbauer spectroscopy for such fitting is about 3%. The parameter of vacancies $x$ can be calculated as $^1,^2$:

$$x = \frac{1.88S_A - 1}{4.7 + 5.64S_A S_B}$$

where $S_A$ and $S_B$ are the relative Mössbauer spectral weights of $\text{Fe}^{3+}$ ions in tetrahedral and octahedral sites, respectively. The fitting delivers $\frac{S_A}{S_B} = 1.39$ resulting in $x = 0.129$. Thus, the $\text{Fe}_{3-x}\text{O}_4$ stoichiometry can be written as $\text{Fe}^{2+}_{0.61}\text{Fe}^{3+}_{2.26}\text{O}_4$ with a corresponding ratio of $\text{Fe}^{2+}/\text{Fe}_{\text{total}} = 0.21$. 

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Table S 1. Mössbauer parameters obtained from fitting the experimental spectra.

| Name   | $\delta_s$, mm/s | $\Delta$, mm/s | $H_{\text{eff}}$, kOe | Area S, % | Component            | Total area of Fe$^{3+}$ ions in tetrahedral site $S_A$, % | Total area of Fe$^{3+}$ ions in octahedral sites $S_B$, % |
|--------|------------------|----------------|------------------------|-----------|-----------------------|----------------------------------------------------------|----------------------------------------------------------|
| Sextet 1 | 0.2933           | 0.2960         | 447.49                 | 11.23     | Fe$^{3+}$ (A)          |                                                          |                                                          |
| Sextet 2 | 0.4163           | 0.3192         | 446.41                 | 9.79      | Fe$^{3+}$ (B)          |                                                          |                                                          |
| Sextet 3 | 0.3193           | 0.0990         | 404.78                 | 10.68     | Fe$^{3+}$ (A)          |                                                          |                                                          |
| Sextet 4 | 0.2989           | 0.1502         | 373.31                 | 9.41      | Fe$^{3+}$ (A)          |                                                          |                                                          |
| Sextet 5 | 0.7604           | 0.7532         | 333.60                 | 9.24      | Fe$^{3+}$ + Fe$^{2+}$ (B) | 50.88                                                    | 36.50                                                    |
| Sextet 6 | 0.2694           | 0.2142         | 283.44                 | 7.84      | Fe$^{3+}$ (A)          |                                                          |                                                          |
| Sextet 7 | 0.3889           | 0.2778         | 191.79                 | 11.53     | Fe$^{3+}$ (B)          |                                                          |                                                          |
| Doublet 1 | 0.2314           | 0.9309         |                        | 11.72     | Fe$^{3+}$ (A)          |                                                          |                                                          |
| Doublet 2 | 0.8030           | 1.3255         |                        | 5.94      | Fe$^{3+}$ + Fe$^{2+}$ (B) |                                                          |                                                          |

**Figure S5.** Mössbauer spectrum of iron oxide MNP at 300 K. A bold line represents the experimental spectrum simulated with 7 sextets and 2 doublets (thin lines) corresponding to various combinations of Fe$^{2+}$ and Fe$^{3+}$ signals in tetrahedral and octahedral positions. The spectrum at the bottom corresponds to the difference between the experiment and the simulation.
Relaxation times of MNPs. In the absence of an external magnetic field, the orientations of the magnetic moment of all atoms in single domain MNPs coincide with the easy magnetization axis. When subjected to an external magnetic field, these MNPs realign their magnetic moment in the direction of the applied field to minimize the system’s energy. Such realignment can proceed through two distinct mechanisms – Neel’s or Brown’s relaxation.\(^3\) In the first case, reorientation of the magnetic moments proceeds while the whole particle remains immobile. In the second case, the MNPs rotate as a whole. Neel and Brown mechanisms are described by the respective characteristic times\(^1\), \(\tau_N\) and \(\tau_B\):

\[
\tau_N = \tau_0 \frac{\sqrt{\pi}}{2} \exp\left(\frac{K_{\text{eff}} V}{k_B T}\right)
\]

\[
\tau_N = \frac{3h V_H}{k_B T}
\]

where \(\eta\) is the viscosity of the solvent in which the particles are dispersed, \(k_B\) the Boltzmann constant, \(T\) the absolute temperature (K), \(V_H\) the hydrodynamic volume of the particle (including the nonmagnetic shell if it exists), \(V\) the magnetic volume of the core, and \(K_{\text{eff}}\) effective anisotropy constant. The relaxation time of each mechanism strongly depends on the size of MNP and on the material which the MNP is made of in the case of the Neel’s mechanism (Figure S 6). The crossover between two regimes (\(\tau_N = \tau_B\)), marked by squares in the figure, at some critical radius of MNP, \(R_c\), corresponds to the point of transition from Neel’s relaxation to Brown’s relaxation mechanisms. The critical radius estimates at 300°K in water (\(\eta=1\text{mPa*s}\)) are \(R_c \approx 4.1\) and 5.9 nm for magnetite and maghemite, respectively. This estimate used \(K_{\text{eff}}\) values for 10 nm nanoparticles\(^4,5\) and did not account for the dependency of \(K_{\text{eff}}\) on MNP size. As the MNP
size decreases, the $K_{\text{eff}}$ increases which should result in the increase of the $\tau_N$ and lowering of the $R_C$. Therefore, this method provides the higher estimates of the $R_C$ values. The estimate also does not account for a shell around the MNP core, which could increase $V_H$ and $\tau_B$. These assumptions can influence $R_C$ in a different fashion: either decrease its value for magnetite or increase its value for maghemite.

**Figure S6.** The dependency of characteristic relaxation time of Neel’s ($\tau_N$) and Brown’s ($\tau_B$) mechanisms for magnetite (Fe$_3$O$_4$) and maghemite (γ-Fe$_2$O$_3$). Critical radius ($R_C$) corresponds to the MNP radius where the transition from Neel’s to Brown’s relaxation mechanism occurs.
Figure S7. Characterization of functionalized MNPs. (A) Time dependence of functionalized MNPs hydrodynamic diameter while storage. The values are presented as Mean +/- Standard Error of Mean=SD/√N, where N is the value of completed nanoparticles tracks during measurements. (B, C) Zeta potential of (B) MNP-LA and (C) MNP-Cy. Negative zeta potential for MNP-Cy is a result of the presence of citrate ions left after MNPs functionalization.
Figure S 8. The example of the TEM micrographs for (A) MNP-LA, (B) MNP-LA-CT, (C) MNP-Cy and (D) MNP-Cy-CT.
Figure S 9. Kinetic curves of NSAAPFpNA hydrolysis observed with (Enzymatic hydrolysis) and without (Autohydrolysis) CT (0.6 μg/ml). NSAAPFpNA concentration was 7.5 μM, 20 mM TRIS-HCl buffer (pH 8.2), 0.5% acetonitrile, 0.5% 1,4-dioxane, temperature 25 °C.
Figure S 10. Kinetic curves of NSAAPFpNA hydrolysis by MNP-Cy-CT before or after pulsed (as described in Figure 6) LFMF treatment (50 Hz 140 mT). NSAAPFpNA concentration was 7.5 µM, 20 mM TRIS-HCl buffer (pH 8.2), 0.5% acetonitrile, 0.5% 1,4-dioxane, temperature 25 °C.
Figure S 11. Restoration of the relative activity of MNP-LA-CT and MNP-Cy-CT in time after pulsed (as described in Figure 6) LFMF (50 Hz, 140 mT) treatment. NSAAPFpNA concentration was 0.5 μM for MNP-LA-CT and 7.5 μM for MNP-Cy-CT, 20 mM TRIS-HCl (pH 8.2), 0.5% acetonitrile, 0.5% 1,4-dioxane, temperature 25 °C. Relative activity was computed as the ratio of the initial rates of reaction catalyzed by MNP-CT conjugates after magnetic field exposure ($V_0(LFMF)$) to those catalyzed by the respective conjugates that were not subjected to the magnetic field ($V_0$):

$$\text{Relative activity} = \frac{V_0(LFMF)}{V_0}$$
Figure S 12. Deconvolution of Amide I area of ATR-FTIR spectra of native CT (A) before and (B) immediately after pulsed (as described in Figure 6) LFMF (50 Hz, 140 mT) treatment. The spectra are normalized. CT concentration 0.5 mg/ml, 20 mM citrate buffer (pH 4.5), 22 °C. Experimental spectra presented in black. The color coding of the fitting curves corresponded to α-helixes (red), β-sheets presented (blue and green), β-turns (magenta and orange), random structures (yellow). Here and below (Figures S 9, S 10), we set a 5% limit for maximal difference between the combined areas of the fitting curves and the actual spectra curve area.
**Figure S 13.** Deconvolution of amide I band area in ATR-FTIR spectra of MNP-LA-CT (A) before, (B) immediately after, and (C) in 3 hours after pulsed (as described in Figure 6) LFMF (50 Hz, 140 mT) treatment. Samples obtained after synthesis of the conjugate were 20x concentrated. The other experimental conditions, deconvolution requirements, and colors codes of the curves are the same as in Figure S 8.
Figure S 14. Deconvolution of Amid I area of ATR-FTIR spectra of MNP-Cy-CT (A) before, (B) immediately after, and (C) in 3 hours after pulsed (as described in Figure 6) LFMF (50 Hz, 140 mT) treatment. Samples obtained after synthesis of the conjugate were 20x concentrated. The other experimental conditions, deconvolution requirements, and colors codes of the curves are the same as in Figure S 8.
**Movie S1.** Visualization of CT molecule behavior through MD simulation in Non-stretching conditions. Here and below (**Movie S2-S3**), atoms of catalytic triad are presented in spheres mode.

**Movie S2.** Visualization of CT molecule behavior through MD simulation in NH$_2$-stretching conditions. Lys residues to which the force was applied are presented in sticks mode.

**Movie S3.** Visualization of CT molecule behavior through MD simulation in COOH-stretching conditions. The Glu and Asp residues to which the force was applied are presented in sticks mode.
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