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Adsorption Reaction Dynamics of Systems Lysozyme and Nanodiamond/Nanosilica at pH=7−13

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Adsorption reactions between surfaces of nanodiamond and nanosilica with diameter of 100 nm prepared as suspension solutions of 0.25 μg/μL and lysozyme molecule with different concentrations of 7 mmol/L PPBS at pH=7, 9, 11, and 13 have been investigated by fluorescence spectroscopy. Adsorption reaction constants and coverages of lysozyme with different concentrations of 0−1000 nmol/L under the influences of different pH values have been obtained. Helicities and conformations of the adsorbed lysozyme molecules, free spaces of every adsorbed lysozyme molecule on the surfaces of nanoparticles at different concentrations and pH values have been deduced and discussed. The highest adsorption capabilities for both systems and conformational efficiency of the adsorbed lysozyme molecule at pH=13 have been obtained. Lysozyme molecules can be prepared, adsorbed and carried with optimal activity and helicity, with 2 and 10 mg/m^2 on unit nanosurface, 130 and 150 mg/g with respect to the weight of nanoparticle, within the linear regions of the coverages at around 150−250 nmol/L and four pH values for nanodiamond and nanosilica, respectively. They can be prepared in the tightest packed form, with 20 and 55 mg/m^2, 810−1680 and 580−1100 mg/g at threshold concentrations and four pH values for nanodiamond and nanosilica, respectively.

Key words: Protein adsorption, Interfacial reaction dynamics, Fluorescence spectroscopy, Single molecular spectroscopy, Biochip, Proteomics

I. INTRODUCTION

Biomolecular chain reaction as well as stereospecific oligomerization on a cell membrane has been pionieered by Jovin *et al.* in 1995 [1]. Biomolecular interface research was initiated and keeps growing as one of the important branches of biomolecular researches [2−12]. Immobilization of proteins on solid surfaces constitutes a research field of considerable importance in emerging technologies employing biocatalytic and biorecognition events, *e.g.* biosensors for pathogen detection, investigation of conformational reaction dynamics on membrane, and the following chain reactions, micro arrays for proteomic analysis, *etc.* Ability to quantitatively immobilize functionally stable proteins is paramount importance in achieving high sensitivity and sustains operating efficiency in these applications.

Lysozyme is one of the proteins, which has been mostly investigated and reported. It contains 6 tryptophanes, which are useful for the spectroscopic detection. UV-absorbance combined with FTIR (Fourier transformed infrared spectroscopy) or/and TIR (Total internal reflection) method by use of a prism [13, 14] has usually been applied in the recent 10 years, whatever can resolve the concentration of lysozyme up to ca. 50−100 μmol/L, via Sore band at 409 nm, even the molecular orientation of the adsorbed protein on the surface can be deduced. The adsorption capabilities on the surfaces of nanosilica [4−6, 11, 12], nanodiamond [11−15], and other materials, as well as their adsorption mechanisms of proteins, *e.g.* lysozyme on these surfaces can not be well differentiated by use of these methods.

The unimaginable adsorption capabilities of nanosilica (NS) and nanodiamond (ND) with diameter 100 nmol/L nearly all kinds of proteins in a solution can be completely adsorbed [11, 12, 15], and the detectability of extremely diluted lysozyme solution down to 10 nmol/L are demonstrated by use of the fluorescence method [11, 12, 15−20].

Fluorescences for free lysozyme of 0−1000 nmol/L in 7 mmol/L PPBS (potassium phosphate buffer solution) at pH=11.0 after adsorption reactions on the surfaces of nanodiamond and nanosilica with diameter 100 nmol/L, and with Xe lamp as light source monochromated at 285 nm of ca. 0.6 mW and PMA-11 (Hamamatsu, Japan) as fluorescence spectrometer, have been measured before [11, 12, 19, 20]. 20 μL suspension solution containing

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5 μg nanoparticle was put into the 2.0 mL lysozyme solutions with different concentrations for the fluorescence measurement. Coverages as well as adsorption reaction constants 1.6×10^8 $(\text{nmol/L})^{-1}$ for the system of nanodiamond and lysozyme, and 4.5×10^7 $(\text{nmol/L})^{-1}$ for the system of nanosilica and lysozyme have been obtained [11, 12, 19, 20]. Helicity of every adsorbed lysozyme molecule, free space on the surface of the nanoparticle for itself, and loading-capability−optimal loading of lysozyme molecules on a unit surface of nanoparticle dependent upon the lysozyme concentration have been estimated. The interaction strength between lysozyme molecule and nanoparticle surface dependent upon different concentrations of lysozyme can be conjectured. It should be more significant, if the obtained informations mentioned above can be compared with those obtained at other pH values.

In order to differentiate the adsorption behaviors, compared with the results at pH=11.0, measurements of coverages and adsorption reaction constants of both nanoparticles with same suspension solution concentration 0.25 μg/μL at pH=7.0, 9.0, and 13.0 have been followed and completed (Fig.1).

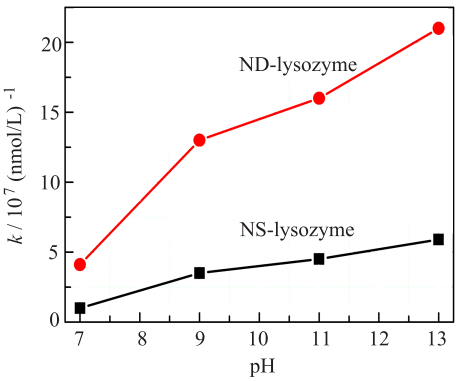

FIG. 1 Interfacial adsorption reaction constants k at pH=7.0−13.0 of 7.0 mmol/L PPBS solutions of both systems lysozyme-nanodiamond (ND) and lysozyme-nanosilica (NS).

TABLE I Interfacial adsorption reaction constants k at pH=7.0−13.0 of 7.0 mmol/L PPBS solution for both systems lysozyme-nanodiamond and lysozyme-nanosilica.

pH	$k/10^7$ $(\text{nmol/L})^{-1}$		Ratio
	ND-lysozyme	NS-lysozyme	
7.0	4.1	1.0	4.05
9.0	13	3.5	3.7
11.0	16	4.5	3.6
13.0	21	5.9	3.6

II. EXPERIMENTAL PROCEDURE AND EVALUATION

The experimental setup for measurement, evaluation procedure of coverages and adsorption reaction constants at pH=7.0, 9.0, and 13.0 were kept the same for the experiment at pH=11.0 [11, 12, 19, 20]. The pH values were measured with pH-meter−Beckman, Φ390, USA. Fluorescence measurement with Xe lamp (ORC, XM 2500H/VC, 2.5 kW, Model: 8532/8506 8-85; LH 152 N of L. P. Associates, Inc., USA) as light source at 285 nm, GM252 Tandem monochromator (ABI Analytical Kratos Division, Spectral Energy, USA) via a Predispersion Prism (LHA 165/2), and PMA-11 (Hamamatsu, Japan) for detection, has been done. Nanosilica (VP OX 10, degussa, Germany) and nanodiamond (KDM, Kay Diamond Products, USA) with diameter 100 nm as substrates, and lysozyme (molecular weight 14.370 kg/mol, 4.7×10^4 unit/mg solid, 5×10^4 unit/mg−95% protein, Sigma Chemicals, USA) as adsorbate have been chosen for the experiment. Nanodiamond was carboxylated and contained carboxyl group approximately lower than 10% on the surface [21, 22]. The Langmuir and BET surface areas [12] of nanodiamond and nanosilica have been obtained by use of adsorptive dose rate, and static volumetric measurement technique (Gemini V series, Micromeritics, Norcross, USA). BET surface areas of nanodiamond and Nanosilica are 55 and 15, their Langmuir are 80 and 20 m^2/g, respectively.

For the investigation of reactions, both nanodiamond and nanosilica dissolved in PPBS with concentration 0.25 μg/μL (2.5 mg nanoparticle dissolved in 10 mL PPBS) were applied as substrates; lysozyme molecule was used as adsorbate. 7 mmol/L PPBS was used to prepare lysozyme solutions of different concentrations 0−1000 nmol/L, and suspension solutions of both nanoparticles. First of all, 2 mL lysozyme solution of different concentrations from 0 to 1000 nmol/L was taken and put into the quartz cuvette of 10 mm×10 mm×30 mm for the measurement. The fluorescence spectra in 200−650 nm were recorded, in which the illumination light of Xe, water, and lysozyme peaked at 285, 314, and 345 nm, respectively. The cubic data (Figs. S1, S3, S5, and S7 in Supplementary material) show the obtained fluorescence of lysozyme of 0−1000 nmol/L. It can be simulated with formula "Allometric1" in Origin 6.0,

$$y = cx \tag{1}$$

where y, c, and x represent intensity of fluorescence, differential of this straight line, and concentration of lysozyme, respectively. The differential is necessary to obtain the respective coverage [11, 12, 15−17, 19, 20, 23, 24]. The recording range of spectrum can be reduced into 250−500 nm, in order to save time.

Each of 2 mL lysozyme solution of different concentrations was treated with 20 μL nanoparticle suspension solution, which contained 5 μg nanoparticles (either nanodiamond or nanosilica). They were well mixed for 1 h, and then centrifuged (1.3×10^5 r/min, MIKRO 20, Hettich Zentrifugen, D-78532 Tuttlingen, Germany).

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The upper layer of approximately 1.8 mL after centrifugation was taken out for the fluorescence measurement. The coverage as well as quantity of adsorbed lysozyme molecules by nanoparticles from the fluorescence differences between lysozyme solutions treated and untreated with nanoparticle can be obtained (in Supplementary material). This procedure was repeated for systems of lysozyme solutions of 10, 20, 30, 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 500, 750, and 1000 nmol/L with one of the nanoparticles, either nanodiamond or nanosilica, and at pH either 7.0, 9.0, or 13.0. As soon as all coverages within the concentration range 0−1000 nmol/L at one pH value were obtained, a simulation procedure named "LangmuirEXT1" given in Origin 6.0 was applied:

$$Y = \frac{abX}{1 + bX} \tag{2}$$

where Y is the curve of coverages, X is the "rest" concentration of lysozyme after centrifugation, and a is fixed as 1 in order to match the Langmuir isotherm equation [11, 19], then b can be simulated and obtained as the adsorption constant or adsorption reaction constant for this nanoparticle at the respective pH value [11, 19, 20].

The measurement was very difficult, time-consuming, and must be repeated many times, *e.g.* 10 times for concentrations 0−1000 nmol/L at each pH value, until reasonable data and curve were obtained. Simulation of coverage is very "experience"-dependent. The simulation of coverage according to the formula mentioned above, depends nearly only upon, whether the data point at lower concentrations, *e.g.* ≤50 nmol/L, cause the simulated curve perpendicularly increasing or not [11, 12]. The data at higher concentrations, *e.g.* ≥200 nmol/L, do not nearly influence the simulation results. The steeper the coverage curve in the region of lower concentrations increases, the stronger the adsorption between lysozyme and nanoparticle is; the larger the adsorption constant of the nanoparticle-lysozyme system is (Table I). The coverage of system ND-lysozyme at pH=13.0 (Fig.S8(a)) seems the most effective. On the contrary, the coverage of system NS-lysozyme at pH=7.0 (Fig.S2(b)) is clearly the most ineffective, and the adsorption constant is the smallest (Table I).

Although data points, at 180, 385, and 1040 nmol/L in Fig.S2(a); 319 nmol/L in Fig.S4(a); 96, 334, 697, and 1218 nmol/L in Fig.S4(b); 67, 118, and 198 nmol/L in Fig.S6(a); 134, 197, 288, and 1002 nmol/L in Fig.S6(b); 173, 227, 277, 450, 782, and 1262 nmol/L in Fig.S8(a); 228, 301, 449, 734, and 1215 nmol/L in Fig.S8(b), are located either slightly or enormously far from the simulated curve, their effects can be neglected; their deviations are purposeless for the simulation results. The coverages, at 1040 nmol/L in Fig.S2(a); 1218 nmol/L in Fig.S4(b); 1002 nmol/L in Fig.S6(b); 450, 782, and 1262 nmol/L in Fig.S8a; 301, 449, 734, and 1215 nmol/L in Fig.S8(b), are even nearly zero, because the measured fluorescences of lysozyme molecules before adsorption with nanoparticles mixed were either identical or even smaller than those after adsorption with nanoparticles mixed. These abnormal data which appear at concentrations larger than 200 nmol/L, even 500 nmol/L, cannot have any influence upon the simulation results as well as adsorption constants.

III. RESULTS AND DISCUSSION

Adsorption reaction constants at pH values 7.0, 9.0, 11.0, and 13.0 for both systems ND-lysozyme and NS-lysozyme have been obtained and shown in Table I. Quotient of constants of system ND-lysozyme over those of NS-lysozyme is ≈4 [24] (Fig.1 and Table I). It seems there are factors or similar factors, which influence the adsorptivities or adsorption strengths at different pH values (pH=7, 9, 10, and 13) for both systems: ND-lysozyme and NS-lysozyme. The maximal adsorption reaction constants for both nanoparticles appear at around 11−13. Lysozyme molecules behave as neutral at pH=11.4 (or pI=11.4) [25, 26] during the interaction with the nanoparticles. They should come more easily close to the surfaces of the nanoparticles and be adsorbed. The lysozyme molecule adsorbed on the surface of nanodiamond around pI as well as pH=11 or 13 in this experiment may better keep its helicity as well as its activity. The system ND-lysozyme shows its adsorption capability stronger than NS-lysozyme.

The linear regions of coverages at pH=9 (Fig.S4 and Table II), 11 (Fig.S6 and Table II), and 13 (Fig.S8 and Table II) are similar and can roughly be chosen between 10−30 nmol/L of system ND-lysozyme and 10−50 nmol/L for system NS-lysozyme. Detailed estimation procedures of the related values given in Tables II and III for systems ND-lysozyme at pH=11.0 and NS-lysozyme at pH=13.0 are shown in the Supplementary material.

At the beginning of the linear region (Table II), 10 nmol/L for system ND-lysozyme at pH=11.0, every adsorbed lysozyme molecule possesses approximately 23 nm^2 (Eq.S(4)) on the surface of ND. There are approximately 1.1 mg/m^2 (Eq.S(6)) lysozyme molecules adsorbed on the surface of ND; approximately 60 mg (Eq.S(7)) lysozyme molecules can be adsorbed by using one gram ND. At end of this linear region of 30 nmol/L for this system, every adsorbed lysozyme molecule possesses approximately 8 nm^2 (Eq.S(9)) on the surface of ND (Table II). There are approximately 3.2 mg/m^2 (Eq.S(11)) lysozyme molecules adsorbed on the surface of ND; approximately 170 mg (Eq.S(12)) lysozyme molecules can be adsorbed by every gram of ND. The adsorbed lysozyme molecules can still well keep their helicities and activities. At adsorption threshold concentration of 290 nmol/L (Tables III and V) for this system ND-lysozyme at pH=11.0, every adsorbed

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TABLE II Available surfaces during the adsorption reactions and adsorbed quantities of lysozyme molecules on unit surface of ND and NS at pH=7.0, 9.0, 11.0, and 13.0.

Parameter	ND-lysozyme				NS-lysozyme			
pH	7.0	9.0	11.0	13.0	7.0	9.0	11.0	13.0
BET surface/(m^2/g)	55	55	55	55	15	15	15	15
Nanoparticle treated/μg	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Linear region/(nmol/L)	10−50	10−30	10−30	10−30	10−20	10−50	10−50	10−50
Total volume/mL	2.02	2.02	2.02	2.02	2.02	2.02	2.02	2.02
Molecules involved/10^{13}	1.2−6.1	1.2−3.6	1.2−3.6	1.2−3.6	1.2−2.4	1.2−6.1	1.2−6.1	1.2−6.1
Available surface/(nm^2)	5−23	8−23	8−23	8−23	3−6	1.2−6	1.2−6	1.2−6
Adsorbed lysozyme/(mg/m^2)[a]	1.1−5.3	1.1−3.2	1.1−3.2	1.1−3.2	3.9−7.7	3.9−19	3.9−19	3.9−19
Adsorbed lysozyme by nanoparticle/(mg/g)[a]	60−290	60−170	60−170	60−170	60−115	60−280	60−280	60−290

[a] At both ends of the linear region.

TABLE III Available surfaces during the adsorption reactions and adsorbed quantities of lysozyme molecules on unit surface of nanodiamond and nanosilica at pH=7.0, 9.0, 11.0, and 13.0 at the respective threshold concentrations.

Parameter	ND-lysozyme				NS-lysozyme			
pH	7.0	9.0	11.0	13.0	7.0	9.0	11.0	13.0
BET surface/(m^2/g)	55	55	55	55	15	15	15	15
Nanoparticle treated/μg	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Threshold/(nmol/L)	140	200	290	140	130	190	150	100
Molecules involved/10^{13}	17.0	24.3	35.3	17.0	15.8	23.1	18.3	12.2
Available surface/(nm^2)	1.6	1.1	0.8	1.6	0.5	0.3	0.4	0.6
Adsorbed lysozyme/(mg/m^2)[a]	14.8	21.1	31.2	14.8	50.3	73.5	58.1	38.7
Adsorbed lysozyme by nanoparticle/(mg/g)[a]	810	1160	1680	810	750	1100	870	580

[a] At threshold concentration.

lysozyme molecule can possess approximately 0.8 nm^2 (Eq.S(14) and Table III) free space on the surface of ND. There were approximately 31 mg/m^2 (Eq.S(16)) lysozyme molecules adsorbed on the surface of ND. Approximately 1680 mg (Eq.S(17)) lysozyme molecules as the most packed can be adsorbed by using one gram ND.

For system NS-lysozyme at pH=13.0 (Table II), at beginning of the linear region 10 nmol/L, every adsorbed lysozyme molecule possesses approximately 6 nm^2 (Eq.S(20)) free space on the surface of NS. There are approximately 4 mg/m^2 (Eq.S(22)) lysozyme molecules adsorbed on the surface of NS, and 60 mg (Eq.S(23)) lysozyme molecules can be adsorbed by every gram of NS. At end of this linear region, 50 nmol/L for this system, every adsorbed lysozyme molecule possesses approximately 1.2 nm^2 (Eq.S(25)) free space on the surface of NS (Table II). There are approximately 19 mg/m^2 (Eq.S(27)) lysozyme molecules adsorbed on the surface of NS, and 290 mg (Eq.S(28)) lysozyme molecules can be adsorbed with every gram of NS, where the adsorbed lysozyme molecules can still well keep their helicities and activities. There are approximately 39 mg/m^2 (Eq.S(32)) lysozyme molecules adsorbed on the surface of NS; approximately 580 mg (Eq.S(33)) lysozyme molecules as the most closely accumulated can be adsorbed by using one gram NS, and every adsorbed lysozyme molecule can obtain 0.6 nm^2 (Eq.S(30) and Table III) free space on the surface of NS, at the threshold concentration 100 nmol/L and at pH=13.0 (Tables III and V).

The respectively involved molecules can be deduced as 1.2−3.6×10^{13} and 1.2−6.1×10^{13} for cases with ND and NS, respectively. The available surfaces which each adsorbed molecule possesses can be approximated as 8−23 and 1−6 nm^2 for both systems respectively. They can be averaged as 15 and 4 nm^2, respectively. Both values correspond well to the data 10 and 2 nm^2 reported in Refs.[11, 19], where they have been estimated from smaller linear regions, 20−30 and 40−50 nmol/L for ND-lysozyme and NS-lysozyme systems, respectively. The values 15 and 4 nm^2 obtained from three different pH values (pH=9, 11, and 13), instead of 10 and 2 nm^2 from only one pH value (pH=11), are certainly more significant, in the sense that the interaction circumstances at these three pH values are rather similar to those at pI (=11.4). At adsorption threshold concentrations, every adsorbed lysozyme molecule ob-

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TABLE IV The (rest) lysozyme concentrations where 80% and 90% of the nanoparticle surfaces at different pH values have been covered by ND and NS.

Coverage	Nanoparticle	(Rest) lysozyme concentration at different pH value/(nmol/L)			
		pH=7.0	pH=9.0	pH=11.0	pH=13.0
80%	ND	100	25	25	20
90%	ND	210	65	55	45
80%	NS	400	110	90	70
90%	NS	>700	280	200	160

tains approximately 1.3 and 0.5 nm^2 on the surfaces of ND and NS, respectively. There is nearly no difference, whether either all the four pH=7, 9, 11, and 13, or only three pH=9, 11, and 13 are considered.

Because of the stronger charge-charge interactions within the protein molecule, among the molecules, and between the molecule and nanoparticle surface, it is difficult to choose the linear regions of coverages (Figs. S2, S4, S6, and S8) for both adsorption systems−ND-lysozyme (Figs. S1a, S3a, S5a, and S7a) and NS-lysozyme (Figs. S1b, S3b, S5b, and S7b), especially for case at pH=7. The adsorbed lysozyme molecules $1.2\times10^{13}-6.1\times10^{13}$ and $1.2\times10^{13}-2.4\times10^{13}$ (Table II), available nanoareas 5−23 and 3−6 nm^2 (Table II) for every adsorbed lysozyme molecule on the respective surfaces of ND and nanosilica at pH=7.0 are therefore rather insignificant.

In general, not only charge-charge (charges on the surface of nanoparticle, charge of lysozyme, charges within lysozyme, and charge of water) interaction, but also conformational or orientational collocation of the adsorbed molecule is the considerable factors to influence the adsorption reaction. Roughness of the surface of nanoparticle may also be one of the decisive factors besides charge on the surface, Zeta potential [27], pH value of the surrounding, and modification of the surface, *e.g.* carboxylation [21, 22] in order to increase the adsorption capability.

Alternatively, in order to differentiate the adsorption capabilities and conformations of the adsorbed lysozyme molecules in both systems at four different pH values, the obtained coverages (Figs. S2, S4, S6, and S8) can be summarized with 80% and 90% as listed in Table IV. It is understandable, that (i) from Fig.S6a, 80% and 90% coverages of the ND surface by lysozyme molecules can be interpolated roughly at 25 and 55 nmol/L at pH=11.0; and (ii) from Fig.S8b, 80% and 90% coverages of the NS surface by lysozyme molecules can be interpolated roughly at 70 and 160 nmol/L at pH=13.0.

Other values shown in Table IV can be obtained according to the similar steps. The higher "rest" lysozyme concentration reflects the smaller number of lysozyme molecules adsorbed on the nanoparticle surface. Rest lysozyme concentration is contributed by the lysozyme molecules, which are still free in the solution and not adsorbed on the surface of nanoparticle after centrifugation. Considering the same diameter (100 nm) and different sizes of surfaces of both nanopaticles used, it can easily be predicted, that more, even most lysozyme molecules are possibly adsorbed into the deep wells or pores so that their conformations are well collocated [23, 25, 26, 29−34, 39−43] within the rougher surfaces, and every molecule can occupy greater surface in the system of ND-lysozyme. Surface of NS is rather flat, and the adsorbed lysozyme molecules in this system lie nearly flat on the surface [23, 25, 26, 29−34, 39−43]. The contact surfaces are not big or interactions are not so strong for the case of ND-lysozyme. The adsorbed molecules have looser spaces at the concentrations lower than threshold concentration [12]. If the concentration is low enough, *e.g.* lower than 50 nmol/L, the adsorbed molecules may lie nearly flat on the surface, especially for the case of NS [12, 26]. If the concentrations are higher and around threshold, the adsorbed molecules are crowded, even squeezed together [25, 26, 39−45]. The higher the concentration is, *e.g.* keeps increasing to around 50−200 nmol/L (Table III), the smaller the space is, which every adsorbed molecule can obtain. The molecules will be squeezed and like standing.

The threshold concentration, at which the lysozyme molecules are adsorbed and saturated on the surface of the nanoparticle, can be obtained at position, where the fluorescence data (Figs. S1, S3, S5, and S7) intersect the x axis of concentration of lysozyme. In the region of higher concentrations of lysozyme, these blue fluorescence data keep running in parallel to those in back as well as red. This phenomon signifies the saturated coverage on the nanosurface. All the respective threshold concentrations [11, 12, 15−20, 23, 24, 28−34, 39−43] on either ND or NS surface and at different pH values can be obtained and shown in Table V.

The NS-lysozyme system shows the weakest adsorption at pH=7 and the ND-lysozyme system at pH=13 shows the strongest (Table IV). The interaction between the surface and molecule is relaxed as soon as the pH value is reduced. It is obviously that the adsorption between ND and lysozyme molecule is stronger than that between NS and lysozyme molecule (Fig.1).

Suspension solutions of both nanoparticles for the adsorption experiment with lysozyme at the four pH=7.0, 9.0, 11.0, and 13.0, have been prepared in 0.25 μg/μL.

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TABLE V Saturation thresholds of lysozyme on the surfaces of ND and NS with suspension solutions 0.25 μg/μL and 2.5 μg/μL.

pH	Nanoparticle	Concentration/(nmol/L)	
		0.25 μg/μL	2.5 μg/μL [12]
7.0	ND	140	–
	NS	130	–
9.0	ND	200	–
	NS	190	–
11.0	ND	290	190
	NS	150	175
13.0	ND	140	–
	NS	100	–

Ten time higher suspension solutions 2.5 μg/μL of both nanoparticles at pH=11.0 have been used [12], in order to determine the threshold concentrations of adsorption of lysozyme, at which the surfaces of the nanoparticles can be saturated as well as maximal covered. However, this effort seems unnecessary or overflowed. The threshold concentrations with suspension solution 0.25 μg/μL of nanoparticles at pH=11.0 are 290 and 150 nmol/L; those with 2.5 μg/μL are 190 and 175 nmol/L for ND and NS, respectively (Table V). Although the measurement was quite difficult, the evaluation was very time-consuming, and there are certainly large fluctuations of the values, the threshold concentrations for both nanoparticles at pH=11.0, which is near pI (=11.4), are higher than those at pH=7.0, 9.0, and 13.0, because the lysozyme molecules behave like neutral facing the nanoparticles, the repulsion seems the smallest. The adsorbed quantity of lysozyme is the largest and the threshold concentration is located higher. 290 nmol/L at pH=11.0 and 190 nmol/L at pH=9.0 are for cases of ND and NS, respectively. For the case of NS: there are 21×10^{13} lysozyme molecules involved and adsorbed on its surface at the threshold concentration 175 nmol/L by using suspension solution 2.5 μg/μL [12]; the threshold concentration becomes 150 nmol/L by using 0.25 μg/μL. The fewer nanoparticles are used, the threshold concentration for saturation coverage is understandably direct-proportionally lower. However, it is on the contrary for the case of ND. There are 23×10^{13} lysozyme molecules [12] involved on the surface of ND at the threshold concentration 190 nmol/L by using the suspension solution 2.5 μg/μL [12]. The threshold concentration increases to 290 nmol/L by using lower suspension solution 0.25 μg/μL, where approximately 2.4×10^{13} (averaged value of 1.2×10^{13} and 3.6×10^{13}), only one tenth of lysozyme molecules involved (Table III). The fewer nanoparticles (0.25 μg/μL) are taken, the higher concentration of lysozyme is necessary for the preparation of saturated surface of ND. The threshold concentration at 290 nmol/L for ND with suspension solution 0.25 μg/μL is higher than 190 nmol/L with 2.5 μg/μL, which is difficult to be explained.

Comparing the results of adsorption reaction constants of ND-lysozyme 1.6×10^8 and NS-lysozyme 4.5×10^7 at pH=11.0 (pI=11.4) (Table I) with ND-BSA 3.7×10^7 and NS-BSA 1.2×10^8 at pH=4.7 (pI=4.70) [39−43], the adsorption of lysozyme on the surface of ND is stronger than that of NS; on the contrary, BSA can be more strongly adsorbed on the surface of NS, at the respective pI value of 4.70. Certain assumptions can be made regarding to the factors which influence the adsorptions. It is possible that the surface of ND is rougher than that of NS. Lysozyme molecules are easierly adsorbed into the wells, low-lying places, or pores on the surface of ND and enlarge the collocation surfaces between lysozyme and ND. There is no rough or uneven surface for NS to increase the collocation of the interaction surfaces. However, it is difficult for the case of BSA molecules to be adsorbed into the pores on the surface of ND because of its larger dimensions with $4\times4\times14=224$ nm^3, which is more than 5 times of lysozyme with $3\times3\times4.5=40.5$ m^3 [11, 25, 26]. Surface of nanosilica is rather smooth, whatever doesn't make much difference for the adsorption of two proteins. The larger volume of BSA and rougher surface of ND may be the reasons, that BSA molecules which cannot be well adsorbed into pores, don't obtain enough contact or interaction surfaces for the adsorptivity or adsorption strength. The smooth surface of NS doesn't influence the adsorptivity or adsorption strength of lysozyme (4.5×10^7) and BSA (1.2×10^8) much.

There were 2.91×10^{-4} mg lysozyme molecules adsorbed on the surface 7.5×10^{-5} m^2 (Eq.S(19)) of NS in the suspension solution of 0.25 μg/μL at pH=7.0 and 10 nmol/L (Fig.S1(a), and Table II). It can be approximated that 3.9 mg/m^2 (Eq.S(22)) lysozyme molecules are adsorbed. Furthermore, it can be very roughly interpolated from Figs. S2(b), S4(b), S6(b), S8(b), and Table II, that 10 mg/m^2 as well as 150 mg/g lysozyme molecules are adsorbed within the linear regions of the coverages, maintaining their helicities and activities; 55 mg/m^2 or 580−1100 mg/g or in average as the tightest accumulated form at threshold concentrations and all four pH values for system NS-lysozyme. The distinguished values to take at different pH values and threshold concentrations should be insignificant. 10 mg/m^2 within the linear regions is just very roughly estimated. According to results in Fig.1(c) of Dordick *et al.* [26], there may be 2.5 mg/m^2 for 3000 lysozyme molecules adsorbed on the surface of NS with diameter 100 nm at 10 nmol/L and pH=6.9. The saturated adsorption appears approximately with 6500 molecules and 5.0 mg/m^2. Our results (10 mg/m^2) within the linear region by fluorescence spectroscopy and results of Dordick *et al.* (5 mg/m^2) by CD spectroscopy seems to fall in the same order of values. However, 5.0 mg/m^2 mentioned above is located at around 2.1×10^{-5} mol/L. Threshold from our measurement is much lower located

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at 1.3×10^{-7} mol/L, where concentrations lower than μmol/L may not be resolvable using CD. Besides, there are 50 mg/m^2 as well as 750 mg/g (Table III) lysozyme molecules adsorbed by NS. Our value (50 mg/m^2) at threshold is 10 times larger than that (5.0 mg/m^2) obtained by Dordick *et al.* The linear region of our measurement is located at around 10−50 nmol/L, where the experimental concentrations shown by Dordick *et al.* are located nearly zero and not interpolable. The fluorescence method may be more competent than CD.

Regarding to ND-lysozyme system, approximately 2.0 mg/m^2 as well as 130 mg/g (Table II) within the linear regions of coverages at four pH values are obtained. 20 mg/m^2 as well as 810−1680 mg/g (1100 mg/g as averaged) at the respective threshold concentrations and four pH values have been obtained (Table III).

The fluorescence method is very useful for the concentration detection down to nmol/L, and CD might be still as usual for detection of μmol/L concentration. There is nearly 1000 times measuring sensitivity difference. Our values (Table II) obtained within the linear regions should be reliable. However, the respective values (Table III) estimated at threshold concentrations should still be kept questionable, before the helicities or activities of the adsorbed lysozyme molecules by the respective nanoparticles at different pH values are available and comparable with those of the adsorbed molecules within the linear regions of coverages, *i.e.* the adsorbed molecules keep nearly 100% of their helicities or activities, also at threshold concentrations and four pH values.

IV. CONCLUSION

Because the lysozyme molecule shows charge neutral at approximately pH=10.4, the lysozyme adsorbed on the surface of both nanoparticles should be the most packed, could keep its smallest volume or space, *i.e.* the optimal conformation as well as activity, especially if the surface is rough like that of ND, where lysozyme molecules can be adsorbed and well collocated into the pores with larger contact as well as interaction surfaces. The data at pH=13 confirm this argument (Table IV). The threshold concentrations for adsorptions with both nanoparticles are higher at pH around 11. The adsorption capability can be the best and the strongest at pH=13, where the adsorbed lysozyme molecule may still keep its original conformation as well as helicity and show the highest activity. The preparation for optimal adsorption reaction at pH=13 is preferable. Having the same diameter as NS, ND has rougher surface. Lysozyme molecules are small enough and can be adsorbed more compacter into the pores on the surface of ND. The adsorption strength and adsorptivity are therefore higher and the adsorption reaction constant is larger.

ND and NS can adsorb lysozyme molecules with roughly 2 and 10 mg/m^2, 130 and 150 mg/g, respectively, within their linear regions of coverage (Table III). The averaged adsorption capabilities are approximated as 20 and 55 mg/m^2, 1100 and 800 mg/g for the respective systems, at the threshold concentrations and four pH values (Table III).

The significant preparation concentrations of saturated lysozyme molecules with nanodiamond and nanosilica as carriers are located roughly at 150−250 nmol/L at four pH of 7, 9, 11, and 13 [12].

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Supplementary material: Total 16 figures of Fig.S1−Fig.S8 are given, so readers can have easy and clear comparison. Detailed estimation procedures of the related values given in Tables II and III for systems ND-lysozyme at pH=11.0 and NS-lysozyme at pH=13.0 are also exemplified.

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