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Research Article

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The Study of Nanoparticle Size and Coating Influence on the Protein Corona Thermodynamic

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ABSTRACT

Nanoparticle-protein interaction is one of the important aspects of xenobiotic materials in biological environments. Larger nanoparticles can effect on proteins structure and caused more decrements in their hydrophilicity. All proteins have certain binding affinity to nanoparticles called, Vroman effect. This effect is coupled with free Gibbs energy alteration in energy profile of free proteins and their adsorbed on nanoparticles. However, there are no evidences that show how and why hard corona is a protein fingerprint. This study showed that, larger proteins having higher molecular weight and less helix structures attitude to larger nanoparticles. According to statistical analysis, unlike previous studies, it found that the size of nanoparticles is more important than their hydrophobicity. From a thermodynamic point of view, larger nanoparticles have more contact positions than the smaller; so whole energy transmitted from larger nanoparticles is higher. This result indicated that the energy transmitted from smaller nanoparticles to protein, could be transferred to aqueous environment more than larger ones. In addition it demonstrated that, energy parameter could be used as reference parameter for explaining protein attitude to nanoparticle. Whereas nanoparticles can play a role as seeds for protein fibrillation, it is important to consider that how they selected for a specific experiment. For example, in targeted drug delivery systems, nanoparticles act as good carriers. So according to nanoparticles characteristics and protein features in target cells, tissues and organisms nanoparticle-protein interactions could be predicted and then experimentally tested with respect to decrements in probability errors.

Keywords*: Thermodynamic interaction, Corona, Free Gibbs energy, Protein fibrillation*

INTRODUCTION

Special characters of nanoparticles (NPs) can affect most organisms and biological systems. One of the most important NP's characters is their surface-to-volume ratio[1-3]. It is well known that biological fluids that have proteins, interaction with NPs-proteins is necessary. It is proposed when the NPs enter into a cell or biological fluid, they are surrounded with proteins[4]. Proteins girdle the NPs and build an environment so called corona[5-8]. One well cited assertion in a literature about corona is that it is constructed according to different affinity of proteins toward NPs[4, 6, 9-11]. It is constructed from an outer and an inner layer namely soft and hard, respectively[10,12].

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While the outer layer has more exchange rates (**Eq. 1**) and lesser affinity to NP; the inner layer has more affinity and less exchange[2,5,12]. It is established that protein corona is an unstable and reversible mechanism[13]. In some references this equation illustrated as **Equ. 2**. As a consequence, the hard corona considered as a fingerprint of a NP in a biological fluid[6,11].

Equation 1: $$Exchange\ rate = \frac{Dissociation\quad Rate}{Association\quad Rate}$$

Equation 2: $$P + NP \underset{K_{off}}{\overset{K_{on}}{\leftrightarrows}} P - NP \quad K_d = \frac{[P].[NP]}{[P-NP]} = \frac{K_{off}}{K_{on}}$$

Where the K_d is dissociation constant, [P], [NP] and [P-NP] are the equilibrium concentration of free protein free NP and the protein-NP complex, respectively. K_{off} and K_{on} are the dissociation and association rate constants, respectively. It should be noticed that in some references the word adsorption is used instead of affinity[14-17]. However the proteins placed in the hard and/or soft corona may be different in several factors such as hydrophobicity, kinetics[6], charge, secondary and tertiary structures and etc.; there are other factors can affect the interactions of NP-proteins. These interactions belong to NPs characteristics such as hydrophobicity, particle type and even inequality of NP surface or shape[18-20]. Among those, hydrophobicity has the most influence on the interaction of NP-protein[10]. The proteins hydrophobicity lays on the presence of hydrophobic amino acids in their structure. Hydrophobicity is also related to the NP affinity to hydrophobic regions of proteins which depend on their covered chain derivatives[21, 22]. In this report it had been shown that, activity of α-chymotrypsin (ChT) almost blocked via negative Au-NP[19]. While the use of long chain surfactant such as C_{11} alkan thiol, alcohol and C_6 alkan, considering the surfactant nature, the activity of ChT was restored[15]. This research mentioned that however thiol containing surfactant made more reactivation; the C_6-chain alkan had slighter reactivation activity. Probably it resulted in the hydrophobic effect of surfactants. Although the thiol-gold interaction encourages the hydrophobicity of NPs, the C_6-chain alkan exposed less hydrophobic surface area[15]. NPs hydrophobicity can manipulate by change of the coating area that covered NPs. Coatings based on different surface charge can classify into three categories: positives, neutrals and negatives. On the other hand, hydrophobicity may relate to the other NPs surface characters. So chemical features can be considered as criterion for the surface of NPs[22]. Nevertheless, investigators use zeta potential in the case of large-scale screening of NPs and proteins interaction[12,23,24]. It means that the NPs charge variations can affect the proteins structure and its affinity to NPs. Furthermore, it leads to assign the corona formation, which may then change the zeta potential[10]. Therefore, the variations on zeta potential is an indicator of the proteins binding to NPs. Goy-lopez *et al* has been shown that NPs having high hydrophobicity need more albumins to saturate their surface[7]; so the increment in NPs hydrophobicity will increase the stoichiometry. They also claimed that, whatever NPs hydrophilicity is, the binding time of albumin will increase and the exchange rate decreased. Therefore, it seems the hydrophilicity increment (with manipulated NPs coating) will lead to thicker hard corona formation. In other word, NPs coat can decrease the NPs agglomeration, non-specific conjugation and NP-toxicity[9,19,25]. Moreover, according to Lynch *et al*. following the human adult hemoglobin (Hb) bonded to NPs, its secondary structure changed and the percentage of α-helix has decreased[12]. In addition, proteins were having more hydrophobic amino acids rather than those having more hydrophilic amino acids, on their native structure, got stronger change following the adsorption on NPs[2].

There are several metallic, metal oxide, semiconductor and silica cores such as Au, Ag, CdSe, Fe_2O_3 and single wall nanotubes (SWNTs) used to construction of NPs[26-28]. It is obvious that each core has its own features that can affect the corona. One of the most important features is Surface Plasmon Resonance (SPR)[2]. SPR generally is the collection of conductive electrons oscillation attributable to the resonant excitation by the incident photons[29].

In NPs, SPR is a criterion to show the oscillation of electrons on the cores. Resonance is related to the interfacing of two parts such as NPs and reaction milieu and it can be vary based on different NPs and/or reaction milieu. Actually, when a coat for a NP was choosing, the goal is change the effects of core on NPs surrounded environment. Another factor, that affects NP-protein interaction intensively, is the size of NPs and even proteins[30-32].

Molecular weight (MW) of proteins formed from two parts, the numbers of amino acids (size of proteins) and the amino acids side chain. It has been reported that proteins with less flexibility, will have less tendency to small NPs[2,33]. Small NPs have more surface curvature rather than large NPs[2,7,34]. Whatever NP-curvature be increased, numbers of its surface atoms will increase; so chemical properties will increase too[2]. In other words, small NPs have more surface-to-volume ratio rather than large NPs. Additionally; it had been shown that the increment in NP-size, the proteins adsorption on NPs or corona thickness will increase[7]. With the increment in NP size, the numbers of proteins binding position will increase. If n_i be the appropriate contiguity positions on NPs for protein *i*, the numbers of binding positions on NP calculated by **Equ. 2** [6]:

Equation 3: $$n_i = 4\pi\ (rNP + ri)/\pi ri$$

Which r_{NP} is NP-radius and r_i is protein radius. In some studies, investigators reported that NPs (in case of C_{60} fullerenes) does not influence on adsorbed conformational protein[35]. But NPs size influence on chemical ligands positioned around the NPs. Furthermore, the interaction of chemical ligands to each other could affect proteins adsorption[36]. It has been reported that proteins adsorbed on NPs caused decrement in α-helix[12,37] and the protein hydrophilicity had been decreased. Proteins such as enzymes have different behaviors when they adsorbed on NPs[29,38,39]. There are many reports that showed enzyme activity had decreased after they had been adsorbed on NPs. When proteins adsorbed on NPs, from their active sites, cause structural perturbation (direct contact or alter structure such that effect on activity) which cause the enzyme activity prevent or at least it decrease[9]. In spite of this, if it doesn't have any perturbation in active site, enzyme activity will be protected[9]. It has been proposed there are at least four approaches for NP-protein binding involved : 1) electrostatic adsorption, 2) covalent interaction to the NP ligand, 3) protein cofactor attachment on NP and 4) direct protein adsorption, based on an amino acid, on the NP core[9]. Lynch *et al.* reported that, electrostatic adsorption is the most important one amongst them[12]. Moreover,Aubin-Tom *et al.* proposed that direct attachment of proteins on NPs and the attachment of proteins based on protein cofactor on NPs caused proprietary attachment[9]. One of the important aspects of nanomaterial study is protein fibrillation[40,41]. In one side, it is believed that the NPs that are presents in brain cells can act as a core for amyloid proteins and catalyze formation of protein fibrillation[20,35,42]. But there is no complete quantitative data and logic models can explain this phenomenon. On the other side, there are some evidences shows that, NPs and biocompatible phospholipid nanomiseles prevent aggregation of proteins in Alzheimer's disease[20,27]. Also, hydrated C_{60} fullerenes prevent beta amyloid fibrillation[12]. In fact, the knowledge about potential protein fibrillation is far from comprehensive reason. Furthermore, there are parameters which should be considered, such as biochemical properties of those proteins placed in hard and soft corona individually, interaction between proteins and reaction milieu molecules before and after adsorption on NPs and finally protein-protein interaction before and after contact to NPs and etc. Previous study showed that NP-coatings have more effect on corona construction and composition than NP-size[2]. These investigations usually more focused on NP properties[19,22,30,43,44]. Also, they mainly considered on corona composition and their change following the adsorbed[12,13]. In this study, firstly statistically analyze the NP-size which is more important than coting in corona composition. Thereafter the protein adsorbed on NPs and their structural and thermodynamic properties were studied. This study will help to design special NPs and apply them in biological media to reduce perils. In one side, it had shown that hydrophilic coating construct lighter corona than hydrophobic ones[45].

On the other side, small NPs could cross out through membranes and barriers, such as blood brain barrier[46-48]. So, the design of NPs according to target cells will help to better management and utilize them into the biological environments. Investigations showed that larger NPs could better change the protein structures and caused their fibrillation. But there are no comprehensive reasons for this phenomenon. This study tries to explain mentioned phenomenausing the thermodynamic and structural properties of proteins.

MATERIALS AND METHODS

Dataset generation

Interaction of NP-Protein was extracted from publication by Lundqvist *et al*. entitled "Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts"[10]. All proteins were extracted from the protein database of NCBI (http://www.ncbi.nlm.nih.gov/protein/). The signal peptides of proteins had identified from UNIPROT database (www.uniprot.org) and removed.

Protein characterization

Protein sequences were characterized with the basis of molecular weight, isoelectric point,GRAVY, aliphatic index and instability index using EXPASY database tools(http://web.expasy.org/protparam/). Average flexibility of proteins had been calculated based on other tools of EXPASY database (http://web.expasy.org/protscale/). Amino acid characterization including aromatic and aliphatic amino acids, charged and non-charged amino acids, polarity and size of amino acids was carried out EMBOSS (http://www.bi.up.ac.za/cgi-bin/emboss.pl?_action=input&_app=pepstats). According to Rose *et al*. Tyr was placed in hydrophilic amino acids[49]. Amino acid composition of proteins was calculated based on CLC Main Workbench V. 6.6.2.2. Secondary structure of all proteins was computed using Geneious V 6.0.6.Enthalpy of proteins had been calculated based on spdb viwer V.4.1.0. At first, structure of those proteins that experimentally exist in PDB database (http://www.rcsb.org/pdb/home/home.do) had been extracted. But these structures did not exist for all proteins. Therefore, structures of other proteins had predicted based on Phyre (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

It should be noticed that, some proteins had not good prediction and removed. At last, all structures imported to program. For increment of energy minimization accuracy, it had taken H-bonds construction.

Protein pairwise alignment for those proteins adsorbed on each size of NPs had been taken through NCBI database tools and blastp, separately (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq).

Data analysis

Related calculation of protein datasets was performed using Microsoft Excel. Then the average of all proteins in different class (each size *vs* coating proposed as a class) for each parameter was calculated.

Net charge of a protein was the algebraic sum of all charged amino acids presented in each protein[50]. It had been computed based on:

Equation 4:

$$\sum_{i=1}^{20} \alpha i \, fi$$

Where, the 20 referred as amino acids illustrated by *i*. The αi for positively charged amino acids was 1 and for negatively charged amino acids was -1 and for other amino acids was 0. The *fi* was the frequency of occurrence of amino acids *i*. All proteins related parameters were expressed as mean values which were calculated based on summation of each parameter for each size and coating divided into number of proteins in each class.

Analysis of Variance and multiple comparison of those treatments performed based on two and three levels factorial of sizeand coating, respectively. In addition the experiments was designed imbalanced completely random using Statistica V. 10.

RESULTS

It had shown that NPs size decrease, with no attention to their coat, caused to change in the secondary structure of proteins and lead to impressive decrease in helix blocks (**Table 1**). Most parts of helix and turn blocks included polar amino acids, most part of strand blocks constructed from non-polar amino acids[51]. It seems that with increments in NPs size, protein hydrophilicity had decreased. According to Kyte*et al*., hydrophobic proteins have more GRAVY index than the hydrophilic ones[52-54]. The GRAVY score is the average hydropathy score for the whole amino acids involved in protein[52,55,56]. Our data showed if NPs size increased, the protein hydrophobicity would increase. Thus, according to secondary structure data and GRAVY scores, with increasing NPs size, protein hydrophobicity have increased. It had been reported that proteins with lesser MW had more affinity to smaller NPs[57], while low MW ones have more flexibility. Whereas flexibility have invers relation with thermostability[58], it seems that the thermo stability of native proteins attached to small NPs was lesser than the thermostability of native proteins adsorbed on larger NPs. The thermostability of native proteins means the thermostability of proteins before their attachment to NPs. Although the thermostability of proteins after their sorption on NPs had not direct relation with its natives, it had reported that the thermostability of proteins adsorbed on NPs had been increased[59]. It seems after proteins adsorbed on NPs, their flexibility had decreased, and the thermostability of them had increased. Aliphatic index is a criterion that shows the thermostability measurement of a protein and calculated from Expasy dataset[60]. According to our investigations this index was higher for smaller NPs, except in the case of neutral coat. It seems that based on small amino acids and aliphatic index (thermostability index), flexibility of those proteins that attached to larger NPs must be higher than those attached to smaller NPs. But it should be noticed that in addition to flexibility, there are more factors which influence the thermostability of proteins such as “Cys+Ser”, “Tyr+Thr+Asn”, helix blocks and solubility[60]. In one side, it had shown that helix structures were more in adsorbed proteins on smaller NPs, on the other side literature asserted that the entropy of helix structures is more than strands[51]. Therefore, this index revealed that the flexibility of proteins adsorbed on larger NPs should be more. According to Mahmoudi*et al*., proteins that adsorbed on specified NPs, with respect to their size, had rather narrow pH range[2]. Our study suggested that isoelectric point of proteins that adsorbed on smaller NPs was lower than proteins *pI* that adsorbed on larger NPs (**Table 1**). Interestingly, proteins adsorbed on both small and large NPs demonstrate more *pI* than each of them.

It is well known that the presence of certain dipeptides in N-terminal of several proteins is significantly different in the unstable proteins compared to stable ones[61,62]. Based on the impact of these dipeptides, other index had calculated namely instability index[60,63]. According to this index, instable proteins will have more than 40 values, while stable proteins will have less than 40 values. Our results showed that, instability index of proteins adsorbed on larger NPs, werehigher than the smaller NPs. Moreover; effect of size on the adsorbed proteins instability index was significant. Therefore proteins adsorbed on smaller NPs physiologically were more stable than those adsorbed on larger NPs.

Table 1 showed that the Ala in adsorbed proteins on smaller NPs was higher than larger NPs. Furthermore, Cys, Asp and Pro percentage were higher in the case of proteins adsorbed on larger NPs than smaller ones. Mean scores of other amino acids percentage, in each size, was almost equal (**Table1**). In addition, the presence of some amino acids such as charged Pro and Gly as gatekeeper regions in beside of aggregation-prone regions, naturally preserve the protein aggregation[64-66]. Our data showed that charged amino acids were almost equal for small and large NPs. While Pro and Glyattached to larger NPs were more than smaller NPs, (table 1); the presence of Cys in proteins increase the probability of protein aggregation too[67]. It had been found that the amount of Cys in proteins adsorbed on larger NPs were more than the smaller one. Additionally, previously mentioned that as positions contactof proteins on larger NPs were bigger than the smaller NPs, they had more surface area than the smaller NPs. So it seems protein perturbation on larger NPs occurred more than the smaller NPs.

NP’s coating is one of the important factors affecting on protein adsorption of charged amino acids. Neutral coating had lower effect on proteins adsorption on NPs than charged amino acids. Nevertheless, the percentage of Pro and Gly for neutral coating was more than charged coating.

Besides, this percentage in negative coating was more noticeable than positive coating. Also, the percentage of Cys in adsorbed proteins on neutral coating NPs was higher than charged amino acids. Alike Pro and Gly, negative coating NPs had more percentage of Cys than positive coating NPs.
It seems with respect to these factors; neutral, negative and positive coating will have most effect on protein fibrillation, respectively. Finally, these data increase our knowledge that larger NPs with neutral coating had more influence on protein fibrillation and smaller NPs with positive coating had lesser influence on fibrillation.
Lynch *et al*., have drawn a great deal with attention on binding of Hb (Hemoglobin) on Cds QDs, which alters the conformation of Hb and decreased the helix structure of proteins[12]. What we found suggested that those proteins that attached into smaller NPs had more helix structure in comparison with larger NPs. While, proteins adsorbed on positive coating had more differentiation in secondary structures; other coating had lesser turn structures. It seems that as proteins adsorbed on smaller NPs had lower connection, accessible residues to reaction milieu probably more than adsorbed proteins on larger NPs. It is because contact positions on larger NPs were more than smaller ones. Helix structures have more accessible residues and hydrophilic proteins have more helix structures than hydrophobic proteins. So, hydrophilic proteins have more accessible residues than those hydrophobic. Our data revealed that, proteins attached to charged coating had higherMW than those attached to neutral coating. In addition, attached proteins to charged coating NPs had higher MW than proteins attached to negative charged coating. According Goy-lopez*et al*. and Mahmoudi *et al*.relevant studies, the numbers of proteins that adsorbed on larger NPs were more than numbers of proteins that adsorbed on smaller NPs[2,7]. However data mentioned that NP coating had very much influences on numbers of proteins adsorbed on NPs; it has been observed that positive coating had most adsorbed proteins on smaller NPs. Moreover, larger NPs with negative coating had adsorbed proteins more than other coatings. In addition, those proteins that adsorbed on both 50 and 100 nanometer had most numbers on neutral coating (**Table1**). According to Deiana*et al*., hydrophobicity of proteins having less than 200 residues was more than those with 200 residues[68.] On the other hand, it had been reported that hydrophobic amino acids have more entropy than hydrophilic or polar amino acids[32,68]. Furthermore, the strand structures have more entropy and hydrophobic amino acids than helix ones[51]. Our study demonstrates that helix blocks percentage, which showed percentage of helix structures, and hydrophobic percentage of proteins adsorbed on smaller NPs were more than larger NPs. It should be noticed that proteins with more hydrophobic residues, will have better spatial conformation.In some references, these amino acids called "order-promoting region"[41,63,69].
It seems larger NPs tend to positive charge proteins, while smaller NPs attitude to negative net charge proteins. Therefore those proteins attached to larger NPs had more basic amino acids than proteins attached to smaller NPs.
According to statistical analysis, enthalpy, weight, instability index, alanine percentage, asparagine percentage, Pro percentage, acidic amino acids percentage and net charge had a significant relationship only with size alterationof NPs (**Table 2**). Also, helix structures, *pI* and the percentage of Cysteine had a significance relation with size and the interaction of size and coating. In addition, aliphatic index, Leu and Ser percentage, the percentage of small, moderate, large and aliphatic amino acids percentage, had a significant relation with only interaction of size and coating of NPs. While, there are no remarkable relation between the coating of NPs and protein characteristics (**Table 2**); Duncan test showed levels in each factor had statistical significant differences (supplementary data).
Lundqvist *et al*. claimed that "the coronas around two different sized neutral polystyrene particles are very similar, with≈80% homology between the two coronas, suggesting that the molecular (e.g., hydrophobic) properties are more important than size for that case"[10]. They suggested that whereas those adsorbed proteins on small and large NPs (in case of neutral coating) were almost identical, the NP coating had more influence on protein adsorption than its size. But for study of NPs size and its influence on protein adsorption, proteins that only adsorption small or large NPs must be considered. As a result, all proteins that adsorbed only on smaller NPs had aligned with those adsorbed on larger NPs. This alignment had been taken based on pairwise alignment (not multiple alignments). Thereafter the identity of each two proteins, having similar coating, had been determined. According to pairwise alignment results, those proteins adsorbed on smaller and larger NPs had no significant identity (or no homology, this word applied when two proteins or nucleic acids descendent from common ancestor) (**Table 3A,B,C**).

Table 1: mean scores of all proteins adsorbed on each nanoparticle with special size and coating

	100			100&50			50		
	Amine	**Plain**	**Carboxyl**	**Amine**	**Plain**	**Carboxyl**	**Amine**	**Plain**	**Carboxyl**
Average flexibility	0.604	0.593	0.596	0.603	0.604	0.609	0.591	0.597	0.591
Enthalpy	-2322	-15428.5	-18400	-10012	-18874	-12907	-10999	-2803	-10775
Coil(block)	0.297	0.318	0.293	0.301	0.292	0.289	0.299	0.289	0.292
Helix(block)	0.119	0.207	0.165	0.198	0.161	0.18	0.197	0.218	0.225
Strand(block)	0.224	0.186	0.217	0.231	0.22	0.227	0.209	0.179	0.205
Turn(block)	0.358	0.287	0.324	0.268	0.326	0.302	0.294	0.312	0.276
Weight	74.83	37.476	78.059	93.954	71.162	73.266	52.181	12.706	30.52
Total residues	679.875	333.8	716.562	847	637	669	492.461	114.333	292
Weight(side)	37.727	19.638	39.836	48.103	35.945	37.252	26.761	7.328	16.357
Isoelectric point	7.717	5.956	6.345	6.712	6.747	6.946	5.753	5.136	6.395
Aliphatic index	66.81	83.618	80.781	74.64	72.145	70.303	81.139	71.606	75.27
Instability index	46.083	42.736	43.073	45.404	47.675	47.357	41.547	39.613	37.395
GRAVY	-0.457	-0.363	-0.339	-0.594	-0.482	-0.618	-0.37	-0.445	-0.44
Alanine (A)	0.044	0.068	0.056	0.062	0.052	0.054	0.067	0.073	0.077
Cysteine (C)	0.038	0.013	0.025	0.017	0.03	0.025	0.014	0.019	0.015
Aspartic Acid (D)	0.043	0.044	0.049	0.044	0.045	0.045	0.044	0.062	0.042
Glutamic Acid (E)	0.057	0.078	0.062	0.081	0.069	0.078	0.071	0.069	0.079
Phenylalanine (F)	0.037	0.041	0.037	0.033	0.034	0.032	0.037	0.026	0.036
Glycine (G)	0.059	0.054	0.056	0.04	0.053	0.048	0.052	0.036	0.047
Histidine (H)	0.025	0.018	0.02	0.018	0.021	0.019	0.018	0.014	0.014
Isoleucine (I)	0.031	0.033	0.04	0.026	0.03	0.027	0.031	0.034	0.025
Lysine (K)	0.068	0.055	0.057	0.071	0.066	0.073	0.057	0.05	0.063
Leucine (L)	0.068	0.1	0.091	0.09	0.08	0.079	0.095	0.049	0.076
Methionine (M)	0.012	0.016	0.015	0.015	0.012	0.012	0.015	0.011	0.015
Asparagine (N)	0.042	0.032	0.042	0.034	0.043	0.037	0.037	0.025	0.019
Proline (P)	0.079	0.054	0.058	0.047	0.068	0.058	0.051	0.041	0.037
Glutamine (Q)	0.036	0.05	0.044	0.054	0.045	0.05	0.05	0.023	0.047
Arginine (R)	0.034	0.049	0.038	0.044	0.037	0.04	0.034	0.036	0.044
Serine (S)	0.097	0.066	0.078	0.088	0.086	0.085	0.072	0.078	0.072
Threonine (T)	0.08	0.059	0.068	0.064	0.072	0.066	0.063	0.071	0.059
Valine (V)	0.077	0.069	0.067	0.06	0.074	0.066	0.063	0.07	0.066
Tryptophan (W)	0.016	0.015	0.012	0.013	0.014	0.013	0.012	0.014	0.015
Tyrosine (Y)	0.034	0.023	0.028	0.027	0.033	0.032	0.025	0.03	0.026
Small	0.57	0.487	0.526	0.486	0.54	0.509	0.508	0.569	0.496
Large	0.429	0.512	0.473	0.513	0.459	0.49	0.491	0.43	0.503
Hydrophobic	0.391	0.436	0.423	0.383	0.394	0.378	0.425	0.4	0.424
Very polar	0.368	0.387	0.371	0.407	0.387	0.409	0.378	0.367	0.377
Moderately polar	0.24	0.176	0.205	0.208	0.218	0.212	0.196	0.232	0.198
Charged	0.233	0.261	0.24	0.283	0.246	0.277	0.247	0.277	0.276
Non charged	0.766	0.738	0.759	0.716	0.753	0.722	0.752	0.722	0.723
Basic	0.13	0.131	0.123	0.145	0.128	0.143	0.12	0.121	0.138
Acidic	0.102	0.13	0.117	0.137	0.117	0.133	0.127	0.156	0.138
Net charge	0.027	0.0005	0.005	0.008	0.011	0.01	-0.006	-0.034	-0.0001
Aliphatic	0.179	0.214	0.21	0.19	0.19	0.183	0.206	0.182	0.189
Aromatic	0.115	0.104	0.103	0.097	0.106	0.102	0.102	0.103	0.106

Table 2: the summery of particle size and coating and their interaction show by 2×3 factorial ANOVA. "*" means p<0.05, "" means p<0.01 and "ns" means non-significant.**

	Size	Coating	Size & coating
Average flexibility	ns	ns	ns
Enthalpy	**	ns	ns
Coil (blocks)	ns	ns	ns
Helix (blocks)	**	ns	*
Strand (blocks)	ns	ns	ns
Turn (blocks)	ns	ns	ns
Weight	**	ns	ns
Isoelectric point	**	ns	*
Aliphatic index	ns	ns	**
Instability index	*	ns	ns
GRAVY	ns	ns	ns
Alanine(Ala)	**	ns	ns
Cysteine(Cys)	*	ns	*
Aspartic acid(Asp)	ns	ns	ns
Glutamic acid(Glu)	ns	ns	ns
Phenylalanine(Phe)	ns	ns	ns
Glycine(Gly)	ns	ns	ns
Histidine(His)	ns	ns	ns
Isoleucine(Iso)	ns	ns	ns
Lysine(Lys)	ns	ns	ns
Leucine(Leu)	ns	ns	*
Methionine(Met)	ns	ns	ns
Asparagine(Asn)	*	ns	ns
Proline(Pro)	**	ns	ns
Glutamine(Gln)	ns	ns	ns
Arginine(Arg)	ns	ns	ns
Serine(Ser)	ns	ns	*
Threonine(Thr)	ns	ns	ns
Valine(Val)	ns	ns	ns
Tryptophan(Try)	ns	ns	ns
Tyrosine(Tyr)	ns	ns	ns
Small amino acids	ns	ns	**
Large amino acids	ns	ns	**
Hydrophobic amino acids	ns	ns	ns
Very polar amino acids	ns	ns	ns
Moderately polar amino acids	ns	ns	**
Charged amino acids	ns	ns	ns
Non-charged amino acids	ns	ns	ns
Basic amino acids	ns	ns	ns
Acidic amino acids	**	ns	ns
Net charge	**	ns	ns
Aliphatic amino acids	ns	ns	*
Aromatic amino acids	ns	ns	ns

Table 3: (A) the pairwise alignment of proteins adsorbed on each size of nanoparticle separately and their identity percentage for neutral coating

100	**P01877.3**		**P02649.1**		**P01008.1**		**P01009.3**		**P02743.2**		**P04114.2**	
50	**query coverage**	**identity**	**query coverage**	**identity**	**query coverage**	**identity**	**query coverage**	**identity**	**query coverage**	**identity**	**query coverage**	**identity**
P01591.4	29	23	-	-	18	39	-	-	-	-	55	38
P02656.1	-	-	75	30	46	22	-	-	-	-	97	45
P02766.1	7	44	-	-	48	20	14	39	-	-	23	30

Table 3: (B) the pairwise alignment of proteins adsorbed on each size of nanoparticle separately and their identity percentage for positive coating

100	**P01857.1**		**P01859.2**		**P01861.1**		**P01871.3**		**P02749.3**		**P00747.2**		**P12259.4**		**P03951.1**	
50	**query coverage**	**identity**	**query coverage**	**identity**	**query coverage**	**identity**	**query coverage**	**identity**	**query coverage**	**identity**	**query coverage**	**identity**	**query coverage**	**identity**	**query coverage**	**identity**
P01876.2	100	32	10	32	100	32	100	35	7	46	14	29	4	23	19	23
P06727.3	4	43	6	71	4	43	3	35	9	36	9	54	0	38	13	21
P02652.1	8	33	8	32	13	32	8	44	7	38	5	47	1	32	-	-
P02656.1	-	-	-	-	-	-	2	60	-	-	1	33	-	-	-	-
P05090.1	27	26	19	27	34	21	4	32	15	50	-	-	2	86	4	38
Q13790.2	-	-	-	-	2	57	-	-	4	31	3	83	1	24	2	33
O14791.5	11	31	11	24	9	36	-	-	5	67	10	29	4	32	4	23
P01009.3	6	100	7	83	7	83	-	-	31	18	2	33	2	45	8	22
P01011.2	6	63	-	-	-	-	10	24	19	20	1	47	1	27	5	57
P02766.1	-	-	-	-	-	-	2	30	8	44	-	-	8	28	1	100
P02751.4	14	30	37	29	26	34	23	52	53	31	6	32	16	25	2	44
P07225.1	3	38	-	-	-	-	10	29	17	50	8	35	2	38	12	36
P08697.3	17	26	16	46	18	40	9	47	11	30	4	31	4	36	4	25

Table 3: (C) the pairwise alignment of proteins adsorbed on each size of nanoparticle separately and their identity percentage for negative coating

50	P02766.1		P03951.1		P01008.1		P02652.1		P02656.1		P02649.1	
100	query coverage	identity	query coverage	identity	query coverage	identity	query coverage	identity	query coverage	identity	query coverage	identity
P01859.2	-	-	5	35	6	31	32	33	-	-	4	21
P01876.2	31	28	17	23	-	-	-	-	-	-	-	-
P01591.4	-	-	10	43	5	39	-	-	-	-	-	-
P01871.3	7	30	1	63	-	-	51	44	12	60	20	33
P04114.2	23	30	-	-	15	63	65	58	97	45	84	19
Q13790.2	10	46	1	33	3	29	30	24	20	38	-	-
O14791.5	-	-	-	-	-	-	75	29	27	27	24	56
P00751.2	9	50	43	25	7	70	18	40	16	57	-	-
P05156.2	35	42	59	33	29	23	18	67	15	40	-	-
P05155.2	16	29	5	32	87	26	32	37	32	27	5	25
P01009.3	14	39	7	22	87	30	12	50	-	-	5	38
P01011.2	4	83	4	29	87	35	48	26	34	38	32	21
P00747.2	-	-	54	37	4	39	42	47	-	-	17	25
P02743.2	-	-	4	36	11	50	24	45	-	-	-	-
P07225.1	14	39	10	36	12	30	62	24	-	-	11	42
P08697.3	-	-	4	25	91	30	18	50	37	27	67	25

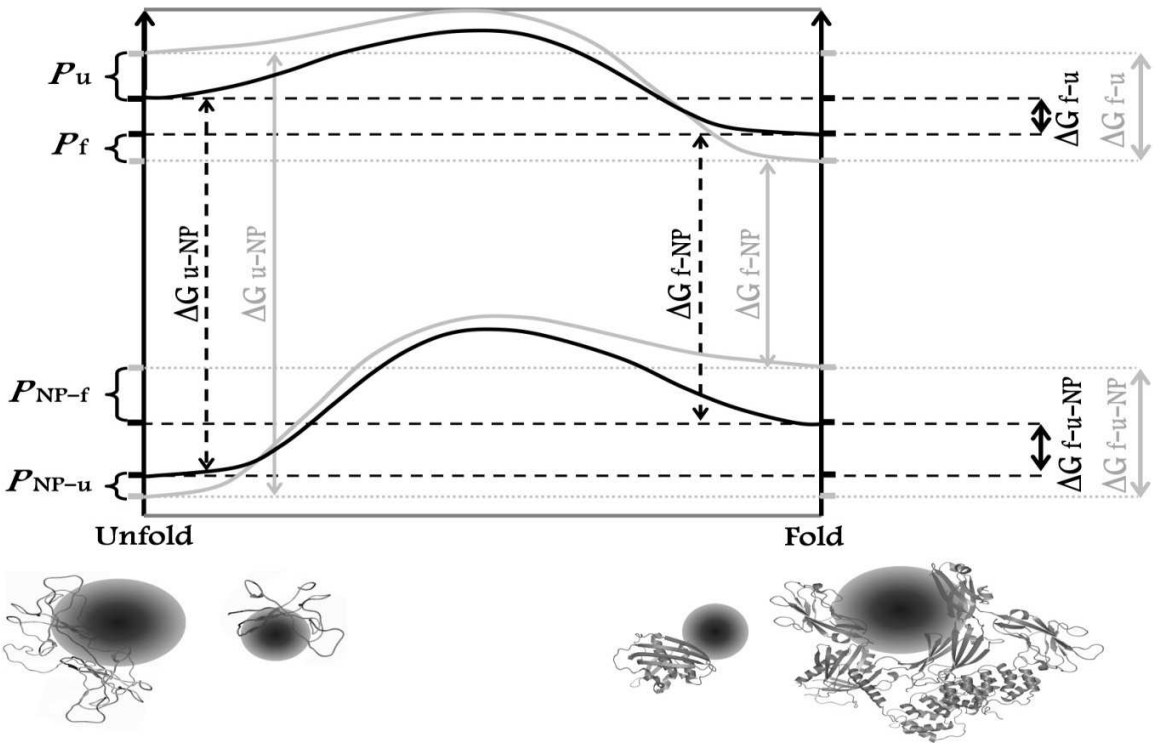

Fig.1: Comparison of the small (**Gray**) and Large (**Black**) proteins Gibbs energy, (GE), before and after their interaction with nanoparticles. P_u: Unfold proteins GE, P_f: Fold proteins GE, P_{NP-f} : Folded proteins attached to NPs GE, P_{NP-u}: Unfolded proteins attached to NPs GE, $\Delta G_{\text{f-u}}$: Folding free change energy, $\Delta G_{\text{u-NP}}$: Unfolded protein-NP absorption free change energy, $\Delta G_{\text{f-NP}}$: Folded protein-NP absorption free change energy, $\Delta G_{\text{u-f-NP}}$ Folding free change energy adsorbed on NP.

DISCUTION

Our results showed that the hydrophilicity of proteins attached to larger NPs was more than of those attached to the smaller. So the interaction of the first group with aqueous medium was more than another group. It seems for this reason proteins got more structural variations, facing the larger NPs rather than the smaller ones. Literature which might shed light on this is limited and contradicting. Little is known that the larger NPs in comparison with the smaller, have better SPR (Surface Plasmon resonance) because with increase of size, the SPR had red-shift[70,71]. So at a similar surface area, larger NPs transmitted lesser energy to each contact position of their interacted proteins. On the other hand, the more surface area of individual larger NP in comparison with the smaller NP caused the more contact positions of it[7,9]. Overalls of SPR and contact position study can hypothesize that larger NP transmitted more energy to their attached proteins. Few reports exist on NPs mixed with plasma proteins, the first visiting one is human serum albumin, (HSA) comparing to fibrinogen and high density lipoprotein, (HDL)[6]. They assume that it is because of the higher abundance of HAS in plasma than other proteins. On the other hand the equilibrium constant of Fibrinogen and HDL was higher than HSA. This mechanism so-called Vroman effect[37,72-75]. So after a while NPs preferred to attach to Fibrinogen and HDL. We want to emphasize that facing the NPs and proteins is random, and this feature resulted in NPs "preferred" to be adsorbed on select proteins following the higher equilibrium constant development. Our data revealed that, flexibility of those proteins adsorbed on large and small NPs was almost equal as well as the entropy of whole proteins. Statistical analysis proved that there is no significant difference between average flexibility of adsorbed proteins on large/small NPs (**Table 2**). It is not clear why proteins adsorbed on larger NPs were heavier than those adsorbed on smaller's; hence their enthalpy is higher too. According to Gibbs energy equation, because of the entropy of mentioned proteins collection was almost equal, the only factor could affect Gibbs free energy was enthalpy.

Equation 5: $$\Delta G = \Delta H - T\Delta S$$

Which ΔG is the protein-folding free energy change that is equal to amount of transmitted energy from a NP to a protein. ΔH is total connection energy of each protein and T is temperature, (K, it had been proposed that this parameter was constant for all proteins). ΔS is the entropy of each protein.

Therefore, it seems change free energy of proteins that adsorbed on larger NPs was more than those adsorbed on smaller NPs. Since free Gibbs energy of a protein laid on the differences between folded and unfolded structure of a protein, its decrement means that the protein can tolerate and take more environmental energy while its folded structure getting lesser folding variations. Consideration research mentioned that the proteins adsorption on larger NPs with more contact positions was more than smaller NPs[7]. The related report revealed that the SPR of smaller NPs are weaker than larger NPs as well as their surface contact position which are lesser[71]. As a consequence, the energy transmission from a contact position of smellers is more. On the other hand larger NPs having more contact positions and transmitted more total energy. For lightening, it seems after the first proteins and NPs visiting, larger NPs preferred to attach to proteins having more negative free Gibbs energy. In contrast; proteins adsorbed on smaller NPs have lesser free Gibbs energy (**Fig. 1**).

Equation 6 : $$\Delta G_{\text{u-NP}}=\Delta G_{\text{f-u}}+\Delta G_{\text{f-NP}}+\Delta G_{\text{u-f-NP}}$$

Where$\Delta G_{\text{f-u}}$ is the protein folding free change energy, $\Delta G_{\text{u-NP}}$ and $\Delta G_{\text{f-NP}}$ the unfolded protein-NP absorption and the folded protein-NP absorption free energy change respectively. Finally, $\Delta G_{\text{u-f-NP}}$ is the protein folding free energy change adsorbed on NP.

It had proposed that, the helix structures of smaller proteins were more than larger ones. Also, MW of proteins had considered at least 5 KDa.

At the constant temperature, native structures of larger proteins have more free Gibbs energy than smaller proteins; because they have more enthalpy and entropy (**Equ. 7.1**). It had been reported that free Gibbs energy of folded proteins adsorbed on NPs had been more than free Gibbs energy of native folded proteins[13].

From thermo dynamical point of view, folded protein-NP free Gibbs energy of a smaller NP was more than larger one. This index refers to first collision of a folded protein and a NP. As previously mentioned, amount of transmitted energy from a local position of smaller NP to a protein, was more than larger one. So, smaller NPs at first collision prefer to bind to proteins, because of transferring more energy to them, than larger NPs (**Equ. 7.2**). Thereafter, contact positions of proteins on larger NPs and whole transmitted energy to proteins will increase. So according to equation (**Equ. 7.3**) the free Gibbs energy of larger proteins on larger NPs should be more than smaller proteins on smaller NPs. Folded proteins adsorbed on larger NPs prefer to preserve their structures and on the other side, larger NPs transfer more energy to proteins. Consequently, larger NPs prefer to attach to larger proteins as they can tolerate more transmitted energy. When larger NPs attach to unfolded larger proteins, they may construct more connection with them than smaller NPs attached to unfolded smaller proteins. So it seems that the enthalpy and entropy of unfolded larger proteins on larger NPs was more than unfolded smaller proteins on smaller NPs. Therefore, the free Gibbs energy of unfolded larger proteins on larger NPs should be more (equation 7.4).

Equation 7 (**Fig 1**):

7.1: $|\Delta G^{L}_{f\text{-}u}| > |\Delta G^{S}_{f\text{-}u}|$

7.2: $|\Delta G^{S}_{f\text{-}NP}| > |\Delta G^{L}_{f\text{-}NP}|$

7.3: $|\Delta G^{L}_{f\text{-}u\text{-}NP}| > |\Delta G^{S}_{f\text{-}u\text{-}NP}|$

7.4: $|\Delta G^{L}_{u\text{-}NP}| > |\Delta G^{S}_{u\text{-}NP}|$

Where the superscripts L and S show larger and smaller proteinsrespectively. So there is a question that, why proteins adsorbed on smaller NPs have less structure perturbation? Some reasons can hypothesize. First, whole transmitted energy from smaller NPs to a protein is less than larger NPs. Second, it seems, smaller NPs tolerate perturbation because adsorbed proteins have more helix structures and, can transfer the energy to the milieu easily. In another words, the decrements of proteins hydrophilic regions resulted in the decrements of the interaction between adsorbed proteins and aqueous environment. As a result proteins can easily change to get the best structure in that situation. Moreover, helix structures have more hydrophilic regions as other proteins secondary structures. So proteins having more helix structures will have more connection with aqueous environment. It seems proteins having more helix structures and less contact positions will have more interaction with aqueous environment. Consequently, these data guide our knowledge that proteins adsorbed on smaller NPs supposed to be able to transfer more energy from smaller NPs to aqueous environment. Logically, the energy transferred from smaller NPs to aqueous environment was more than larger NPs. So it seems that the enthalpy of surrounded environment of smaller NPs should be more than larger NPs. The transmitted energy to aqueous environment manifested by thermal energy, so the term “enthalpy” can use to explain this. Ningthoujam *et al*. reported that the excess enthalpy of SnO_2 NPs had been increased by the decrease of NPs size[76].

According to **Table 2** interaction effects of size and coating had significant relation with proteins thermostability. The relation shows using aliphatic index[51,77,78]. This index is calculated based on aliphatic amino acids, such as Ala, Leu, Ile and Val[79]. So the interaction effect of size and coating had statistical significant difference with thepercentage of aliphatic amino acids too. The aliphatic index has almost no significant difference between small and large NPs. As a result, it seems the transmitted energy that turned to excess enthalpy in surrounded aqueous environment of NPs would not have meaningful effect on proteins structural variations.

As previously mentioned, the stability of proteins adsorbed on smaller NPs was naturally more than larger NPs. Consequently, the presence of dipeptides on N-terminal regions of proteins that adsorbed on larger NPs may have synergistic effect on their instability.

It is doubtful whether the dipeptides nature affects the protein adsorption and/or aggregation on larger NP or their location.

Not only the percentage of small amino acids in proteins attached to larger NPs had been more than smaller NPs, but also, the flexibility of proteins is affected by their numbers and side chains length. In addition, attachment of proteins to NPs was mainly affected by amino acid numbers or MW of proteins rather than the side chain flexibility. Generally larger proteins are likely to bind NPs stronger than smaller ones[13]. Statistical analysis showed that the size of NPs have significant effect on proteins MW that adsorbed on large and small NPs. It means that size alteration of NPs has influence on the selection of NPs by proteins and attachments to them. From **Table 2** can prove that the interaction of protein-NPs is affected not only by the percentage of protein's small amino acids; but also the size of NPs and the interactionof size and coating. On the other hand, it found that mentioned interaction have been much more affected by the size of NPs than the contaminant of size and coating. The publication demonstrated that the numbers of proteins in smaller NPs hard corona were more than those of larger NPs[10], while there are some others that mentioned *vice versa*[6,7]: the increase in size of NPs caused better protein adsorption, which is logical based on **Equ. 3**.

Previous studies showed that proteins adsorbed on different size of NPs had different *pI* [2]. Despite of this our statistical analysis *pI* of proteins that adsorbed on different NPs demonstrate that the attachment have meaningful relation with NPs size and interaction of size and coating. It seems larger NPs prefer to attach to proteins having more basic amino acids. It is because, in this situation the numbers of contact positions will increase and NPs are able to transfer more energy to attached proteins. Also, as Duncan test showed, larger NPs with positive coating had statistically significant difference with interaction effect of each level of other factors (supplementary data).

The presence of some amino acids such as Ala, Cys, Asp and Pro respecting to adsorbed proteins size is statistically significant. Additionally, Cys, Leu and Ser had significant relation with interaction of size and coating of NPs. Ala plays a role in substrate recognition; especially the interaction with non-reactive atoms such as carbon[80]. But it is unclear that why Alaexisting in proteins that adsorbed on smaller NPs, yet? As it mentioned previously, total energy that transmitted to adsorbed proteins on larger NPs was more than smaller NPs; so it seems the presence of Cys, and its resulted disulfide bonds in proteins that adsorbed on larger NPs, could increase their tolerance to transmitted energy. Leu is one of the amino acids that prefer to place in helix structures[80]. Whereas helix structure had meaningful relation with interaction effect of size and coating of NPs. It seems statistical significant of this interaction is logical. One well-cited assertion in the literature is that the helix role in this statistical significance may lays on Leu. Furthermore, adsorption of proteins on NPs is a kind of physiochemical interaction, so it can be assumed with the increase of gate keepers (involved charged amino acids, Gly and Pro) and/or the increase of NPs surface area, the probability of protein structure disruption will increase. In another words, naturally presence of gate keepers in native structure of proteins helps to prevent structural damage and proteins aggregation[41,64-66,81,82]. But contacting NPs with such proteins, caused loose of these regions and leaded to protein aggregation.

Unlike Lindqvist*et al.* that claimed protein adsorption is more affected by coating not size, our data revealed that; identity of proteins that adsorbed on NPs with different size but similar coating was not enough. So it can be claimed that, the coating had more effect on protein adsorption than size. It had observed that, the density of coating[36], functional groups and derivatives length could effect on protein adsorption[15]. It can be concluded that if NPs indifferent size with same coating characteristics were used, the density of coating would be different on each NPs size. Therefore, size has indirect effect on protein adsorption by manipulate coating density.From thermodynamic point of view, larger NPs have more contact positions, so they can transfer more energy to proteins. Therefore they prefer to attach to larger proteins, because larger proteins can tolerate more transmitted energy and transfer it to environment. Against previous studies that showed coat have more effect on protein types placed in corona[10], it can concluded that the identity of proteins placed in different coating were not significant. So, NP-coating had not remarkable effect on corona composition. The results showed that the adsorbed smaller unfolded proteins on smaller NPs are more thermodynamically stable than other proteins in any situations.

NPs could act as seeds in protein fibrillation. In addition the illustrated and confirmed that NP-size have more effect on protein fibrillation based on protein structural features.

CONCLUSION

it may some reasons for more proteins aggregation attached to larger NPs. Briefly, 1) the presence of more gate keepers and more disruption of them, 2) the presence of more free Cys, 3) the presence of more contact positions on larger NPs, 4) transfer of more energy to proteins, 5) low helix structures on adsorbed proteins, and 6) the presence of certain dipeptides that naturally caused to instability of adsorbed proteins (more instability index) which could influence on more alteration in structure of proteins adsorbed on larger NPs and caused their easier aggregation.

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