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Molecular modeling of enzyme attachment on AFM probes

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ABSTRACT

The immobilization of enzymes on atomic force microscope tip (AFM tip) surface is a crucial step in the development of nanobiosensors to be used in detection process. In this work, an atomistic modeling of the attachment of the acetyl coenzyme A carboxylase (ACC enzyme) on a functionalized AFM tip surface is proposed. Using electrostatic considerations, suitable enzyme–surface orientations with the active sites of the ACC enzyme available for interactions with bulk molecules were found. A 50 ns molecular dynamics trajectory in aqueous solution was obtained and surface contact area, hydrogen bonding and protein stability were analyzed. The enzyme–surface model proposed here with minor adjustment can be applied to study antigen–antibody interactions as well as enzyme immobilization on silica for chromatography applications.

1. Introduction

The design of functionalized atomic force microscope tips (AFM tips) to build biosensors with high definition and accuracy requires the quantification of the interaction energetics between organic and inorganic compounds, such as protein–surface and protein–ligand complexes. The complexity of the adsorption process of enzymes onto inorganic surfaces can be better understood by a combination of theoretical and experimental studies. Therefore, the achievements presented here are useful to build biosensors capable to detect inhibitors (e.g. herbicides) by using enzymes as a probe.

In a previous paper of our research group [1] we have been developing prototypes of nanobiosensors to detect herbicides used in agriculture. In addition, our obtained results from combining theoretical and experimental data to investigate the interaction of two pesticides (diclofop and atrazine) with the active sites of the acetyl-coenzyme A carboxylase (ACC enzyme) were in a good agreement [2–4]. In the present paper, the earlier studies were reassumed and the aim is the development of a molecular model and a computational protocol to study enzyme–surface interaction to build biosensors. These investigations are part of a general research project aimed to investigate natural products capable of interacting with important enzymes of plants and animals to be efficiently used in insect plague control [5].

AFM experiments have been successfully employed to measure intra-molecular unfolding forces of individual proteins [6,7] and intermolecular forces between various ligands and receptor pairs [8–14]. Compared with other biophysical approaches for direct measurements of intermolecular forces, such as optical tweezers, magnetic torsion devices, and bio-membrane force probes (BFP), AFM has the unique advantage of possessing the high spatial resolution needed to detect molecular interaction. Additionally, recent progress in AFM studies has made it possible to directly quantify the range and magnitude of the interactions forces between proteins and other molecules [15]. Nevertheless, the AFM technique is very useful for quantifying single molecule interaction, but it does not reveal neither molecular mechanisms behind the binding of ligands nor conformational changes in biomolecules in atomic time scale [1]. The goal of quantifying intermolecular forces, binding energies as well as complex stabilization of protein–protein, protein–ligand and protein–surface systems can be obtained by combining AFM results with theoretical studies. Generally, the specificity of an AFM tip is obtained by using enzymes as molecular probe. Therefore, the combination of computational methods such as quantum mechanics (QM) and molecular dynamics (MD) calculations with

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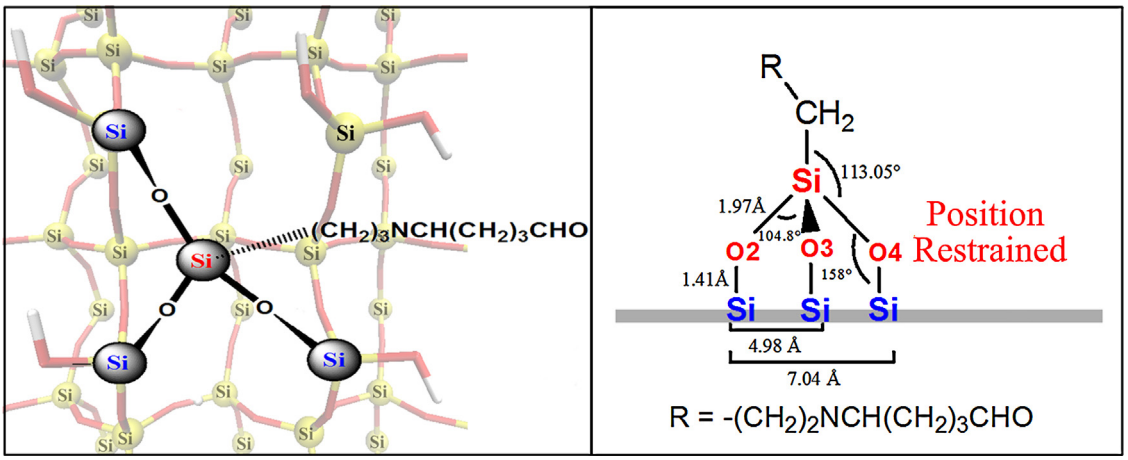

Fig. 1. Connection of the linker SSA with atoms from the AFM tip surface. The silicon and oxygen atoms (Si-O-Si) were position restrained. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

experimental analysis will be useful to make optimal estimative of enzyme immobilization to propose AFM tips with enhanced sensibility. In this paper, we report the use of computer simulation methodologies (MD and QM) to investigate proper conditions to immobilize an enzyme as to build functionalized AFM tips.

The target enzyme used in this investigation was the ACC which has crucial roles in fatty acid metabolism of humans and most other living organisms. ACC is also an attractive target for drug discovery against a variety of human diseases, including diabetes, obesity, cancer, and microbial infections. In addition, ACC from plants has been a target of herbicides used commercially for more than 20 years. Haloxyfop, diclofop (FOPs), sethoxydim and butroxydim (DIMs) are herbicides capable to occupy the carboxytransferase (CT) domain of the ACC and hamper its enzymatic function.

AFM tips are made of silicon (Si) or silicon nitride (Si_3N_4) and, in order to attach enzymes acting as a probe, this surface must be modified. Among the existing methodologies to chemical modification of AFM tip surface, two techniques have been used mostly in the last few years. The first uses tips covered with gold [16] and thiol entities which are properly bonded to biomolecules. The second uses the oxidation of the AFM tip surface due to its exposure to air or water, as a result a large amount of reactive Si—OH groups are formed reacting strongly with silanes, alcohols and also polymers such as polyethylene glycol (PEG) [17,18]. In the present study, a surface spacer agent (SSA) obtained by combining 3-(aminopropyl) triethoxysilane (APTES) and glutaraldehyde [19,20] was used to cover the AFM tip surface. For biosensor purposes, the glutaraldehyde reacts with APTES establishing a bridge for attachment of biomolecules. Glutaraldehyde is a common cross-linking agent due its reaction with residues such as lysine to form stable adducts [20,21]. In order to ensure that the cross-linker molecules can efficient adsorb an enzyme, the interaction forces between AFM tips surface and enzyme must be greater than those between inhibitors and proteins; otherwise, the enzyme might be pulled off from the AFM tip. The linker provides stable adsorption conditions and flexibility for biomolecules; as a result receptor–ligand interactions capabilities are preserved. The geometric dimensions of the system were chosen as to provide the appropriated computer simulation conditions discussed below.

2. Methodology

In this work an atomistic model was proposed to study the interaction of the ACC enzyme with an AFM tip surface using molecular dynamics methodology. In the next sections this atomistic model and the procedures used to determine the force field (FF) parameters are presented.

2.1. System definition

According to the experimental data reported by Etchegaray et al. [19], Bhushan et al. [20], and Deda et al. [22], AFM tip probes can be modified using APTES as a linker. When AFM tips are exposure to environment conditions an oxidation occurs naturally; as a result, the AFM surface is hydroxylated forming —SiOH groups at the top of the surface allowing the interaction with APTES molecules. Further, this surface is treated with glutaraldehyde forming an APTES–glutaraldehyde complex which is capable of reacting with amino acids groups such as lysine and arginine. As can be seen in Figs. 1 and 2, the linker is bounded to the SiOH surface considering

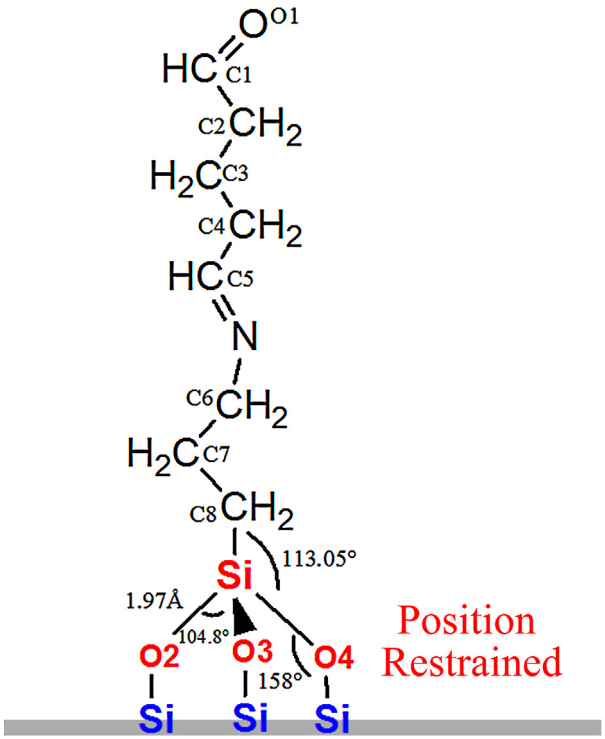

Fig. 2. Final structure of the SSA linker. The atoms labels were used in the molecular geometry scanning and electrostatic potential calculations.

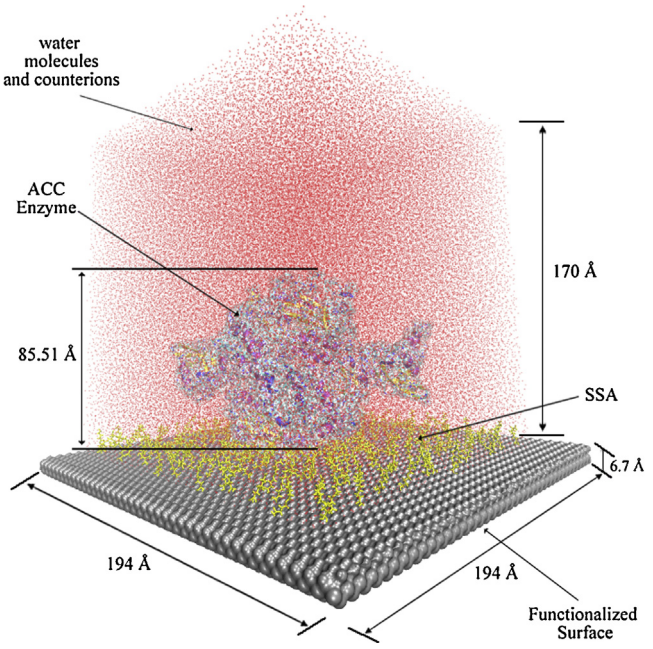

Fig. 3. Description of the simulated system using molecular dynamics methodology. System dimensions are shown for each component of the system.

the silicon atom of the surface. This model was called surface-spacer agent (SSA). The interaction of this surface model with biomolecules can be straightforwardly obtained using FF parameters allowing a computational insight in the physical–chemical process occurring on AFM tips. In the next section the FF parameters were calculated and this atomistic model is discussed.

2.2. Description of the surface model

The silanol (SiOH) surface representing the oxidized AFM tip was modeled using a SiO_2 network with hydrogen atoms bonded to the oxygen of the top of the surface. The initial SiO_2 geometry was obtained using standard procedures available in Inorganic Builder Plugin implemented in VMD program [23]. Schematic representations of the linker surface spacer agent (SSA) model on the surface are shown in Figs. 1 and 2. The surface geometry was optimized and positions obtained for some silicon atoms were used to distribute196 linker molecules on a squared surface with dimensions 194 Å × 194 Å as can be seen in Fig. 3. The silicon atoms bounded to the linkers were constrained to their original surface position during the simulations in order to represent solid state like strong interactions. Table 1 summarizes the number of atoms of the simulated system.

The remaining atoms on the SiOH surface network were not explicitly included in the simulation but their influence were considered using a potential function facility available in the NAMD program [24], as discussed by Aksimentiev et al. [25]. This atom less representation reduces the degrees of freedom of the system decreasing the computational demands of the simulations.

Table 1
Number of particles (N) of each system component.

Component	N
SSA model	6076
ACC enzyme	23,150
Tip3p water	143,198
Na^+ contra-ions	30
Total	172,454

Table 2
Bond distances and bond angles (r^0_μ Å , θ^0_μ) and force constants (k^s_μ, k^b_μ) for the SSA model reported in Fig. 2.

Bond (r)	k^s_μ (kcal mol^{-1} Å^2)	r^0_μ (Å^2)
C8—Si	306.432	1.5080
Si—O	Fixed	1.9780
O—Si	Fixed	1.4105
Angle (θ)	**k^b_μ (kcal mol^{-1} rad^{-2})**	**θ^0_μ (°)**
C8—Si—O	418.400	113.050
C7—C8—Si	488.273	112.700
Si—O—Si	Fixed	158.070

2.3. Surface-spacer agent (SSA) parameterization

To perform molecular dynamics simulations using a classical FF approach to be implemented in OPLS-AA FF protocol [26], the total energy is divided into two terms, the E^{intra} and E^{inter}. The E^{intra} interaction energy term is defined as:

$$E^{intra} = E^{strech} + E^{bend} + E^{Rots} + E^{Ftors} + E^{nb} \quad (1)$$

The first three terms have a harmonic expression:

$$E^{strech} = \frac{1}{2}\sum_{\mu}^{N_S} K^s_\mu (r_\mu - r^0_\mu)^2; \quad E^{bend} = \frac{1}{2}\sum_{\mu}^{N_b} K^b_\mu (\theta_\mu - \theta^0_\mu)^2$$

$$E^{Rtors} = \frac{1}{2}\sum_{\mu}^{N_{Rt}} K^t_\mu (\phi_\mu - \phi^0_\mu)^2 \quad (2)$$

where k^s_μ, k^b_μ, k^t_μ and r^0_μ, θ^0_μ, ϕ^0_μ are the force constants and equilibrium values for bond stretching, angle bending, and rigid torsional dihedral angle, respectively. The E^{Ftors} term is the potential contribution for flexible proper dihedral angle Ψ and can be represented using a five terms Ryckaert–Bellemans (RB) function [27].

$$E^{Ftors} = \sum_{n=0}^{S} C_n(\cos(\Psi))^n \quad (3)$$

The OPLS-AA FF was selected to be used in this work because the molecular structure of the ACC enzyme in our previous paper [1] was built according to this FF protocol and most of the potential parameters needed were available in this force field. The missing ones were obtained using geometries and energies results calculated with the ORCA quantum chemistry program [28] at the Hartree–Fock level using 6-31g* basis set. The same procedure used for creation of the potential energy curves were obtained according to Kirschner et al. [29], constrained optimizations were performed by specifying an internal coordinate to be frozen (as can be seen as a fixed terms in Tables 2 and 3) while all other degrees of freedom were allowed to relax fully. The results were analyzed using

Table 3
Dihedral angles ϕ^0_μ and force constants k^t_μ for the SSA model* reported in Fig. 2.

Dihedral (ϕ)	k^t_μ (kcal mol^{-1})	ϕ^0_μ
O2—Si—O3—Si	Fixed	37.84
C7—C8—Si—O	Fixed	178.600
H8—C8—Si—O	−0.327	66.290
C8—Si—O—Si	Fixed	154.077
C6—C7—C8—Si	0.051	179.820
C3—C4—C5—N	0.200	117.330
H7—C7—C6—N	0.200	61.920
C5—N—C6—H6	0.102	118.180
H5—C5—N—C6	0.344	0.620

the GRACE program [30] and parameters obtained are presented in Tables 2 and 3 as well.

All the Lennard–Jones parameters needed for the non-bonded interactions were taken from the OPLSAA force-field. The charges needed to calculate the Coulombic interactions were obtained using the RESP method [31] implemented in the NWChem program [32].

2.4. Molecular dynamics simulations

In Section 2.2 a surface model suitable to represent a functionalized AFM tip surface in molecular dynamics simulations was presented. The dimeric model of the ACC enzyme proposed by Franca et al. [1] containing 1655 residues (23,185 atoms) was initially placed 5 Å above this surface using a steered molecular

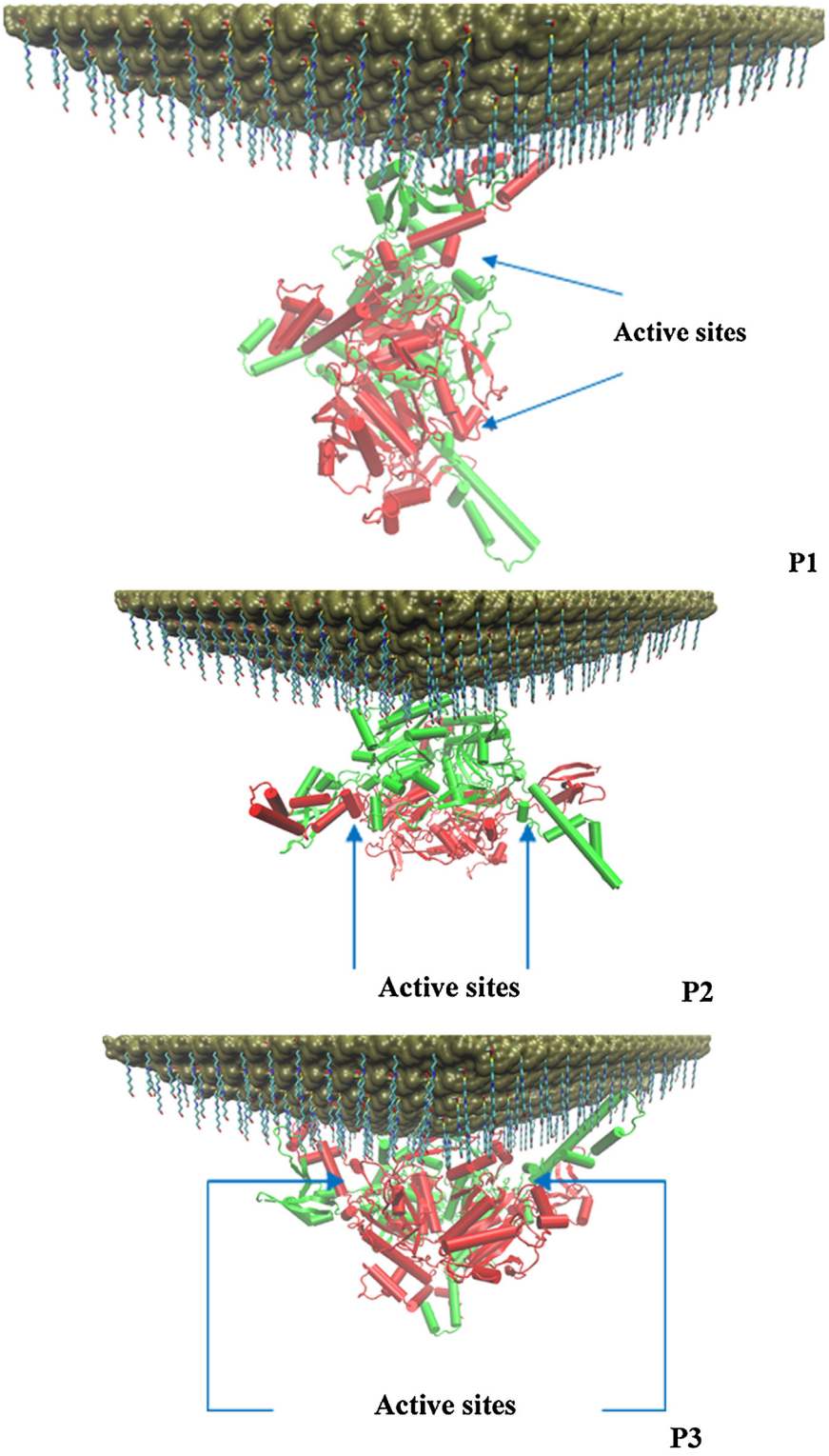

Fig. 4. Schematic representation of enzyme attachment on the SSA surface with some of the possible active sites orientations. ACC enzyme is represented in cartoon view using VMD program [23].

dynamics (SMD) [33,34] protocol. The enzyme was pushed toward the surface with a constant velocity of 0.001 Å/fs during 50 ps until the first residue reaches 5 Å from the surface. After this, the steering force was removed and a free equilibration was run for 1 ns. The SSA-ACC enzyme set was immersed in a box containing 143,198 tip3p water molecules [35] and 30 Na^+ contra-ions were added. In Fig. 3 we present a schematic picture of the system under study: (i) a silanol surface covered by a surface spacer agent (SSA) layer forming a functionalized AFM surface; (ii) an enzyme adsorbed onto the SSA and the system was solvated.

Molecular dynamics simulations were performed with the NAMD 2.7 program [24] using standard periodic boundary conditions and the following protocol: (i) after volume adjustment at 1.0 atm by a Langevin piston [36] the calculations were performed in the NVT ensemble at 310 K and 1.0 atm during 1 ns, and temperature was controlled using a Langevin thermostat; (ii) a cut-off distance of 16 Å was used and long-range corrections were considered using the Ewald sum formalism [37]. With the cut-off value used, numerical stability in the trajectory generation using the NAMD code was achieved. Raut et al. [38] also reported a similar procedure in their investigation of peptide–surface interaction; (iii) after (i) a NPT equilibration step with 20 ns was run; (iv) using the average volume obtained in step (iii) a NVT ensemble calculation was performed to obtain a new 30 ns simulation trajectory.

The results were analyzed using VMD [23] and GRACE [30] programs.

3. Results

Some of the force field parameters needed to run molecular dynamics was calculated as follows:

3.1. SSA force field parameters

The molecular dynamics calculations were performed using the OPLS-AA parameters. Some SSA model parameters such as bond distances, bond angles, dihedrals and the corresponding force constants needed in Eq. (2) were calculated and are presented in Tables 2 and 3. Geometric mean combining rules were used to obtain cross interactions potential parameters. As discussed before these calculations were performed using the ORCA quantum chemistry software at the HF/6-31g*level, as mentioned in Section 2.3. The standard deviation between calculated energy curves and the ones obtained using the parameters below was 0.05 kcal mol^{-1}.

3.2. Enzyme orientation on the surface

To a further understanding of the ACC enzyme orientation on the SSA modeled surface a combination of experimental and theoretical analysis is needed. The final structure achieved by the enzyme in the adsorption process depends basically on its initial orientation on the SSA surface. Experimentally, it is possible to increase the probability of a determined enzyme orientation by controlling environment conditions, such as temperature, pH and reaction time [2,3,39]. From electrostatic considerations the protonated $-NH_3$ groups of an enzyme interacts strongly with aldehyde groups (—COH). Therefore, the availability of the positively charged sites on enzyme surface can be used to control its interaction with the SSA through aldehyde groups [19]. In a previous study, upon solving the nonlinear Poisson–Boltzmann equation using a finite-difference procedure, Franca et al. [1] reported the electrostatic charge distribution of the ACC enzyme. Therefore, using these considerations, some initial orientations of the enzyme upon the SSA can be chosen to set initial geometric conditions for molecular dynamics simulation. As already discussed by those authors, the enzyme orientation should be chosen keeping the active site exposed to interact with substrates molecules from the bulk.

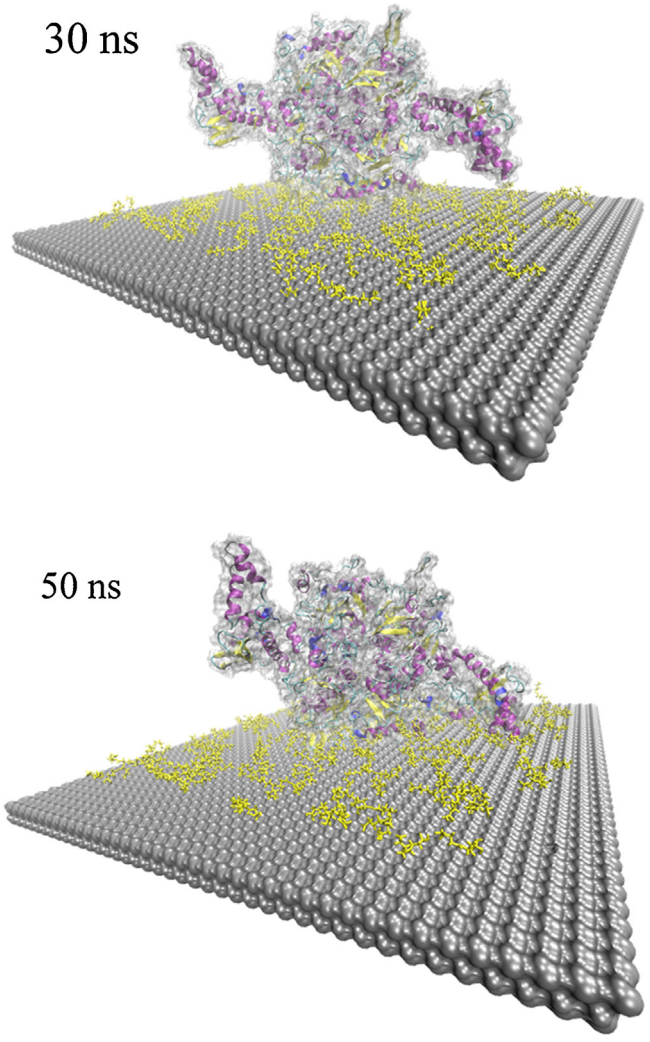

Fig. 5. Structural mobility of the ACC enzyme after 30 and 50 ns of MD simulation. Water molecules were not represented.

In Fig. 4, three favorable adsorption positions of the ACC enzyme on SSA surface are presented. The optimal enzyme orientation requires a large surface contact area to ensure strong adsorption on but living the active sites unblocked to allow interactions. From the plugin Volarea [40] implemented in VMD program, the surface contact area values calculated for the three analyzed areas were: A(P1) ≅ 2623.47 $Å^2$, A(P2) ≅ 5000.25 $Å^2$ and A(P3) ≅ 9825.23 $Å^2$, therefore increasing from P1 to P3 showing that P3 is expect to have an optimal adsorption energy. Nevertheless in the P3 conditions the ACC active sites are not exposed to the bulk and the interaction with a given substrate is blocked. As the surface contact area of P2 is greater than P1 and its adsorption conditions are more favorable, therefore this structure was chosen to proceed with molecular dynamics analysis.

Using position 2, the setup of initial coordinate was performed placing the ACC enzyme 5 Å above the SSA. The MD simulation was performed using Steered Molecular Dynamics [34] protocol. In the equilibration process, the ACC enzyme was enforced toward to the surface using facilities implemented in the NAMD configuration file [24]. In this procedure, a constant acceleration of 0.001 Å ps^{-2} (0.13896 pN) was used until the first amino acid had reached 5 Å distance of the surface. Then, a 20 ns NPT simulation was carried out to equilibrate the system. After this equilibration procedure a 30 ns

Table 4
Analysis of the hydrogen bond during 30 ns of simulation runs. H-bonds average detection was resumed for each percentage of the trajectory.

%	25	50	75	100
Enzyme	13	17	23	22
Arginine	6	7.5	6.5	6
Lysine	6	7	7	7.5

of simulation trajectory was performed for further analysis. The final orientation and energy equilibration curve behaviors during the simulation process are shown in Figs. 5 and 6, respectively. One observes that after 20 ns the total energy is almost constant in agreement with an NVT ensemble calculation.

Using the 30 ns last simulation trajectory some parameters were analyzed to obtain an insight in the dynamical behavior of the system. Hydrogen bonding analysis between SSA and the ACC enzyme were obtained monitoring the interactions of ARG–$NH_3^+$and LYS–NH_3^+ enzymatic groups with the aldehyde group from SSA linker as function of time and the results obtained are shown in Table 4. The hydrogen bonding populations were calculated using geometric rules implemented in VMD program [23] but using structural considerations proposed by Torshin et al. [41].

In Table 4 the H-bonds analysis shows an average of 18H-bonds between ACC enzyme and SSA in the last 30 ns of trajectory, as

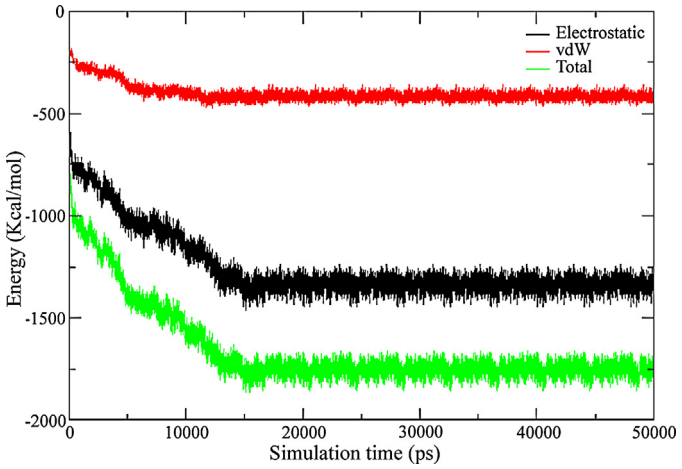

Fig. 6. Electrostatic, van der Waals and total energies involved during 50 ns of MD simulation. After 15 ns the system energies stabilized with average of electrostatic energy = −1306.46 kcal mol^{-1}, van der Waals = −417.682 kcal mol^{-1} and total energy = −1724.15 kcal mol^{-1}.

expected, the main contributions were from arginine and lysine side groups due to its positive charge.

Another important parameter to be monitored was the enzyme integrity as a function of time, in order to prevent enzyme

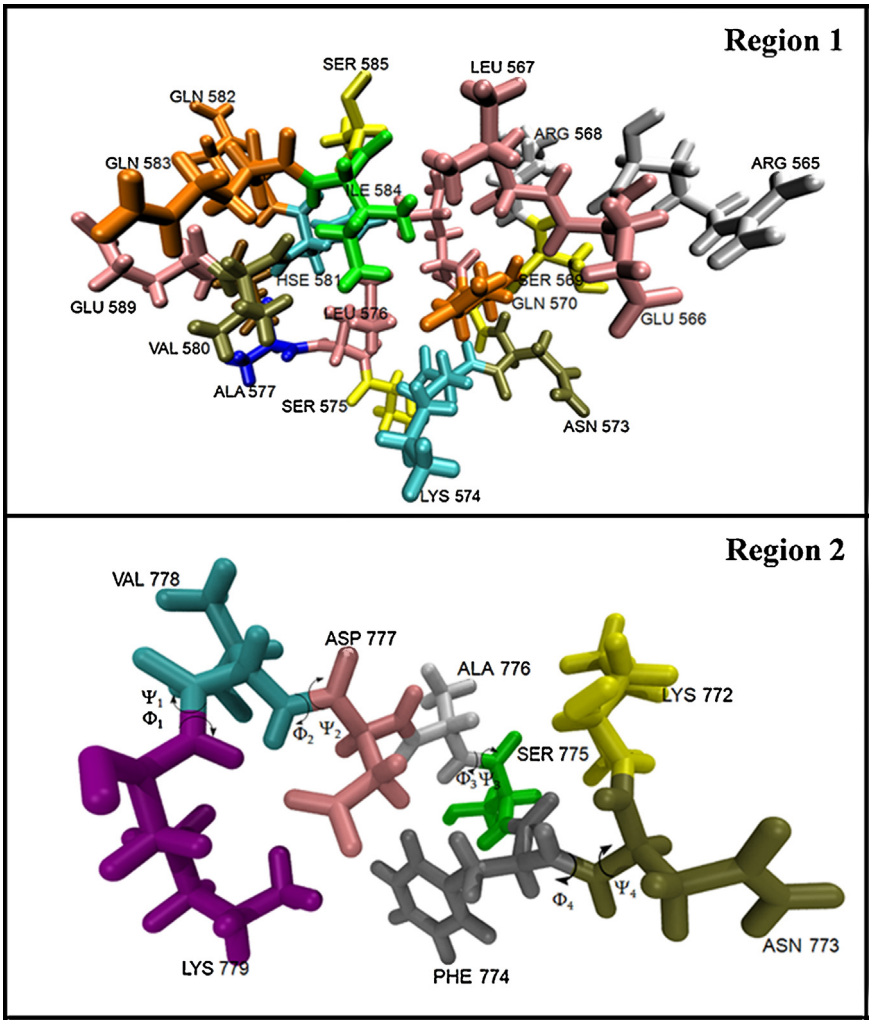

Fig. 7. Monitored dihedral angles (ψ and Φ) as a function of time for region 2.

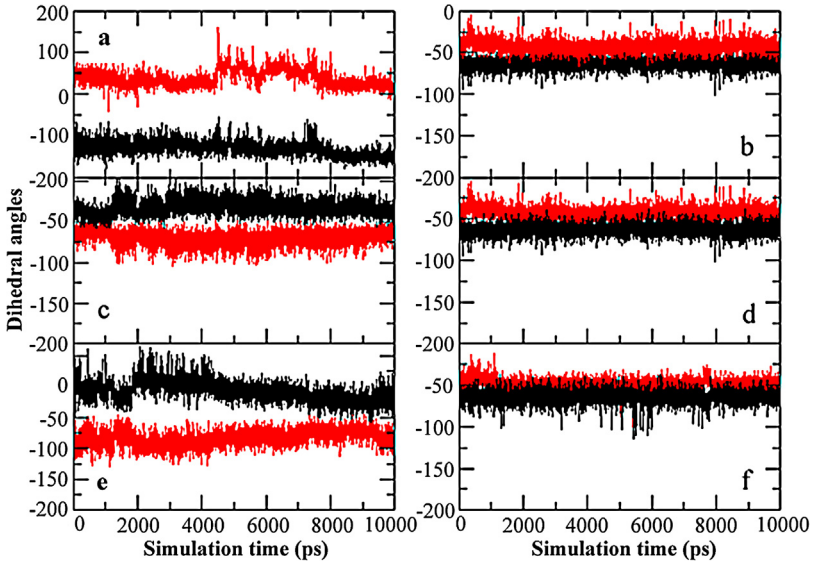

Fig. 8. Selected ϕ (in red) and Ψ (in black) dihedral angles of the region 2: (a, c and e) represent 10 ns of the trajectory after equilibration, and (b, d and f) the last 10 ns of the trajectory. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

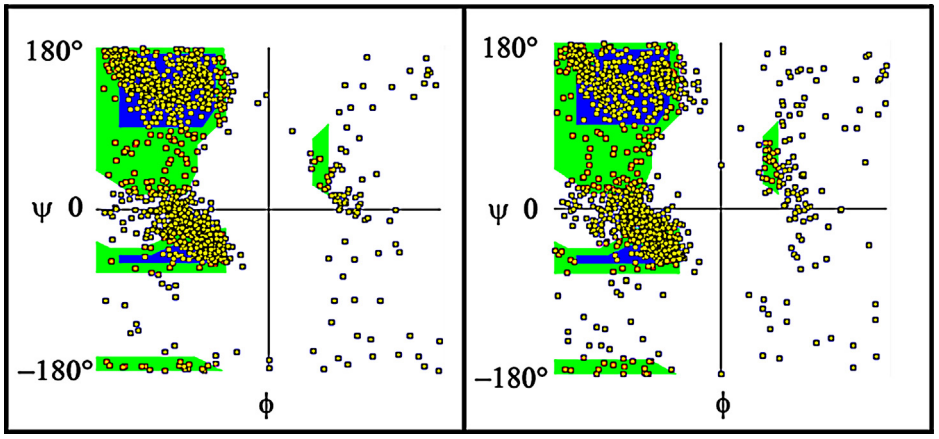

Fig. 9. Ramachandran plotting of the ACC amino acids after 50 ns. Most of the amino acids were not affected by the presence of the SSA surface.

denaturation. To perform this analysis, some selected amino acids were divided into two regions according to their interactivity with the SSA (Fig. 7). Region 1 is defined as the one with initial interaction with the SSA and region 2 is the one achieving relevant interaction after 20 ns. Therefore, the internal flexibility of the regions 1 and 2 was monitored following the dihedral angles values ϕ and Ψ. One should expect different structural behavior in these regions as a consequence of enzyme–SSA interaction. Nevertheless the dihedral angles distributions as a function of time are almost the same for both regions. Fig. 8 represents time progression of the selected dihedral angles from the region 2 in the last 20 ns of the trajectory. In the first part (Fig. 8a, c and e) dihedral undergo some changes due the few H-bonds exhibited. In contrast, the last part, the number of H-bonds was increased (Fig. 8b, d and f), as a consequence, almost stable values were observed.

One observes better adsorption conditions as a function of time in agreement with the increasing of the surface contact area. An average energy/contact area of $-88.9546\,\text{kcal mol}^{-1}\,\text{A}^{-2}$ for region 1 and $-102.802\,\text{kcal mol}^{-1}\,\text{A}^{-2}$ for region 2 were obtained, to be compared with $-52.4201\,\text{kcal mol}^{-1}\,\text{A}^{-2}$ and $-89.2358\,\text{kcal mol}^{-1}\,\text{A}^{-2}$ respectively in the beginning of the simulation (Fig. 7). The increase of surface contact area is clear but the ACC enzyme active sites are still available to interact with molecules from the bulk.

The initial and final enzyme structures were compared using the Ramachandran plotting presented in Fig. 9. Results obtained from Ramachandran plotting shown no eligible differences between the ϕ and Ψ dihedral angles from the initial and final structure after 50 ns of MD trajectory. To access the dynamic stability of the immobilized ACC enzyme dimeric form an overall deviation from the starting structure was computed and a RMSD value of 0.22 Å was found. Therefore, the ACC enzyme was not denatured by the interaction with the model surface. Nevertheless, one must be aware that, according to Gunsteren et al. [42], larger trajectory could be necessary to fully investigate the stability and unfolding of some enzymatic systems. Regarding the ACC enzyme dimeric system presented here, such investigation is under way and will be reported in the future.

4. Conclusions

This paper reports an atomistic model for a functionalized surface of the AFM tip. This surface is covered by a cross

linker molecules to the attachment of enzymes for nanobiosensor purposes. The atomistic model was called SSA, this term stands for surface-space agent. The OPLS-AA force field was used but some parameters needed in the atomistic model were calculated at HF/6-31g level. Molecular dynamic simulations were employed to simulate the functionalized AFM tip interacting with ACC enzyme, modeled by Franca et al. [1] in aqueous solution. According to the electrostatic potential analysis, three possible enzymatic orientations of the ACC on the AFM tip were proposed. The final molecular dynamics calculations revealed that after 50 ns time step of simulation, the achieved enzyme geometry preserved the active sites of ACC enzyme to interact with molecules from the bulk.

From electrostatic potential analysis the enzyme–AFM tip interactions were assumed to be of major importance in two peptide regions. Hydrogen bonds were monitored as a function of time and an average of 18H-bonds was found. Most of the H-bonds were attribute to arginine and lysine side groups with the aldehyde group of SSA. It was observed that an enhancement of surface contact area between enzyme–SSA and the actives sites availability to bulk molecules was preserved. The possibility of enzyme denaturation due to interactions with SSA was investigated monitoring some dihedral angles from contact area. Ramachandran plotting showed that after 50 ns of MD simulation no significant enzyme structure modifications were detected.

The results obtained show that model proposed in this paper leads to a further understanding of the enzyme–surface interactions and immobilization process on AFM tips from an atomistic point of view. It is also worth to note that with minor modifications the model can be straightforwardly used to study enzyme immobilization on silica for chromatography applications [43,44]. Therefore, the interactions of a given substrate with an enzyme active site can be studied considering all the important features needed for the atomistic modeling in different detection process, which is helpful in the development of new ligands screening methodologies, as already discussed by Comer et al. [45].

Finally, the quantum chemical calculations were performed in Theoretical Chemistry Laboratory at Federal University of São Carlos whereas the molecular dynamics calculations were carried out using the Texas Learning & Computation Center (TLC2) super computer cluster facilities at the University of Houston.

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