Delivered to Perfection at Nanoscale. A Coupled Multiscale Simulation/Experimental Investigation of Protein Release from Nanochannel Delivery Systems

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ABSTRACT

Transport and surface interactions of proteins in nanopore membranes play a key role in many processes of biomedical importance. Although the use of porous materials provides a large surface-to-volume ratio, the efficiency of the operations is often determined by transport behavior, and this is complicated by the fact that transport paths (i.e., the pores) are frequently of molecular dimensions. Under these conditions, wall effects become significant, with the mobility of molecules being affected by hydrodynamic interactions between protein molecules and the wall. Modeling of transport in pores is normally carried out at the continuum level, making use of such parameters as hindrance coefficients; these in turn are typically estimated using continuum methods applied at the level of individual diffusing particles.

In this work we coupled experimental evidences (e.g., contact angle estimation, z-potential measurements, and atomic force microscope imaging) to multiscale computer simulations for the analysis of the adsorption/diffusion properties of three different proteins (hen egg-white lysozyme, bovine serum albumin, and interferon) through a microfabricated silicon membrane, having pores of nanometric size in only one dimension. Our joint efforts allowed us a) to elucidate the specific mechanisms of interaction between the biopolymers and the silicon surface, and b) to use molecular

simulations to calculate molecular parameters to be subsequently included in an appropriate mathematical equation of diffusion.

In so doing, the global computational ansatz proposed constitutes an "ab initio" recipe, for which no experimental data are needed to predict the protein release, and can be tailored in principle to match any different protein and any different surface, thus filling gap between the nano and the macroscale.

1. Introduction

Considerable advances have been made in the field of drug delivery technology over the last three decades, resulting in many breakthroughs in clinical medicine.[1] However, important classes of drugs have yet to benefit from these technological successes. One of the major requirements for implantable drug delivery devices (DDDs) is controlled release of therapeutic agents, especially biological molecules, as a continuous delivery over an extended period of time. The goal here is to achieve a continuous drug release profile consistent with zero-order kinetics, where the concentration of the drug in the bloodstream remains constant through the entire delivery period. Injected drugs have first-order kinetics, with initial high concentration in blood (above the therapeutic range), followed by an exponential fall. Toxicity occurs at the drug concentration peak, whilst efficacy diminishes as the drug concentration decreases below this range. Therefore, the therapeutic advantages of continuous release of the drug by implantable delivery devices are evident: minimized adverse reactions by reducing the peak levels, predictable and extended duration of action, reduced inconvenience of frequent dosing, and thereby, improved patient compliance.

Different technologies have been developed to achieve this goal. Silicon microfabrication technology, however, can permit the creation of DDSs that possess a combination of structural, mechanical, and electronical features that may surmount some of the challenges posed by all other available systems.[1,2] Easy of use, reproducibility, tightly controlled dimensions, and ability to manufacture in high volumes are other advantages. In this framework, nanochannel delivery systems (or nDSs) were recently envisioned for the delivery of therapeutic molecules.[3] The fundamental embodiment of the first device employed high precision nanoengineered channels to yield the long-term zero-order release.[4] However, a detailed understanding of the nanoscale molecular phenomena with overarching transport and related effects in

microfluidic devices, leading to strong deviations from Fick law, is far from being entirely understood.

In an attempt to concur to fill this gap of knowledge, we concentrated our efforts in the development of a novel multiscale simulation tool for integrated nanosystem design, analysis and optimization based on a three-tiered modeling approach consisting of (i) molecular models, (ii) atomistic molecular dynamics simulations, and (iii) dynamical models of protein transport at the continuum scale.[5,6] The generalized three-level modeling paradigm under development integrates nanoscale effects of arbitrary biosystems accurately, efficiently, and seamlessly using continuum models carrying molecular information. The first step in the paradigm is to perform molecular simulations to elucidate the essential molecular behavior. Next, selected molecular parameters are derived from these calculations. Finally, the information from the atomistic models is coupled to the continuum model. The validity of this approach is demonstrated in this work through a proof-of-concept study of the non-Fickian release of three proteins (lysozyme, bovine serum albumin, and interferon-α 2b) through microfabricated silicon membranes, consisting of arrays of parallel rectangular channels. The channels have a width of 45 μm, and their length is 4 μm. The microfabrication process allows to precisely tuning the channel height in the nanometer range. Accordingly, these membranes have pores of nanometric size in only one dimension, thus differing from the majority of the cases analyzed in literature, where a cylindrical pore configuration is usually considered, leading to non-Fickian diffusion phenomena, like single file diffusion, different from the one investigated in the present work.

2. Materials and methods

The available X-ray structures of the hen egg-white lysozyme (LYS) (Walsh et al., 1998) and recombinant human interferon-α 2b (INF) (Radhakrishnan et al., 1996) (Protein Data Bank (PDB) entry codes 4LZT[7] and 1RH2[8], chain A, respectively) were used as starting structures. The 3D model structure of the bovine serum albumin (BSA) was built from the human serum albumin (HAS, available in the PBD as code 1AO6, chain A[9]) by a combination of homology-based techniques.[10] The quality of the model was assessed by using different the programs PROCHECK and WHATIF. Ramachandran plot statistics indicated that 97% of the main-chain dihedral angles were

found in the most favorable region, thus confirming the excellent quality of the 3D model of bovine serum albumin obtained. This optimized 3D model was then used as the entry point for molecular dynamics (MD) simulations.

The silicon surface was prepared from scratch. We first built a single plane of silicon using appropriate templates from the simulation package *Materials Studio* (v. 3.2, Accelrys Inc., San Diego, CA, USA), and saturated with hydrogen atoms the dangling bonds at the edges. After full optimization in vacuum, we replicated this plane to obtain a final surface of 84 Å ×65 Å. A unit cell with periodic boundary conditions in three dimensions was then constructed for each system, containing the silicon slab, the protein, and free mobile explicit water molecules of the TIP3P type.

We then carried out atomistic molecular dynamics (MD) simulations according to the following schema: (a) direct energy minimizations of each protein close to the silicon surface, and (b) MD runs with explicit solvation over 10 ns of time using the Amber 7.0 molecular platform.[11] The first procedure should correspond, in principle, to the initial adsorption stage, but it should also yield the preferred conformations if the adsorption process is either under kinetic control, or at large surface coverage. Conversely, the second procedure should yield the best overall conformation on a clean surface with the largest interaction energy under thermodynamic control. Starting from the work of Peskir,[12] we then developed a mathematical model of biomolecular diffusion through nanochannels to provide a reasonable interpretation of the observed non-Fickian phenomenon. Importantly, the model depends on two molecular parameters which are obtained from the MD simulations described above. The model was implemented in the Matlab-Simulink™ software environment to run virtual experiments by means of pc-based simulations at the continuum scale. The experimental measurements of the contact angle between the proteins and the silicon surface were obtained by dipping a silicon wafer into hydrofluoric acid to remove the native oxide layer. The wafer was then immersed in the corresponding protein solution (5mg/mL in PBS) at room temperature for two days. The wafer was finally rinsed and dried under nitrogen current. A micropipette was used to place a solution drop of 5 μL onto the surface, and the contact angle was calculated by fitting the shape of sessile drop. The reported values are averaged over three repeated experiments. Pure Millipore water was used for comparison. The AFM imaging of adsorbed proteins was obtained with a Veeco Nanoscope instrument (Digital Instruments, Santa Barbara, CA), using an NP-S20 tip in

the tapping mode. The applied force was always minimized, so as not to deform the soft protein layer.

3. Results and discussion

The average contact angles of water on the protein-covered silicon surfaces were in the range between 30 and 50°, whilst that obtained in the case of clean Si surface was 73°. These values, in line with similar literature evidences for other proteins absorbed onto hydrophobic surfaces, clearly indicate that the proteins have adsorbed onto the Si layer, rendering it more hydrophilic.

The AFM image, topography, and cross sectional profile along the line in topography were obtained simultaneously, and are shown in Figure 1 for BSA as an example. Several, important information can be deduced from these AFM evidences. Indeed, Figure 1 shows that the coverage of the silicon surface by BSA is extensive, although not complete as observed, for instance, in the case of LYS. Interestingly, the height of the adsorbed protein lies around 7 nm, with some peaks around 12-13 nm, as can be seen on the profile in Figure 1. These dimensions are slightly higher than the average dimensions of the protein (~ 4×4×14 nm), indicating that BSA undergoes a slight conformational change in the direction normal to the surface, as expected for 'soft' proteins. Further, the relative frequencies of appearance of the smaller peaks with respect to the higher ones seem to support a side-on prevalent configuration of adsorbed BSA molecules. An opposite behavior was observed for LYS, a 'hard' protein.

We then determined the energy of attraction between a protein chain adsorbed onto the silicon surface and another, free biopolymer molecule. For the sake of simplicity, we considered that the free protein molecule would sit on top of the bound one upon adsorption. Each simulation, again carried out by keeping the silicon surface atoms static, was repeated six times. The distance of the free protein from the bound one was then changed, and the simulation repeated until 19 different protein/protein distances were evaluated. The resulting interaction energies were then averaged, and the corresponding standard deviations were recorded. Figure 2 shows a plot of the interaction energies on the free LYS as a function of position from the LYS adsorbed onto the surface, as an example. As we can see, the interaction energy follows the standard particle dispersion interaction pattern: a very repulsive potential at close distances, followed by an attractive region, and ending with forces approaching zero at

long distances. In details, the energy becomes negligible at distances greater than 5-6 Å, is at a minimum at approximately 2.5 Å, and become increasingly repulsive as the free polypeptide approaches the bound protein. The average calculated value at the minimum is equal to E_{min} = - 93.93 kJ/mol.
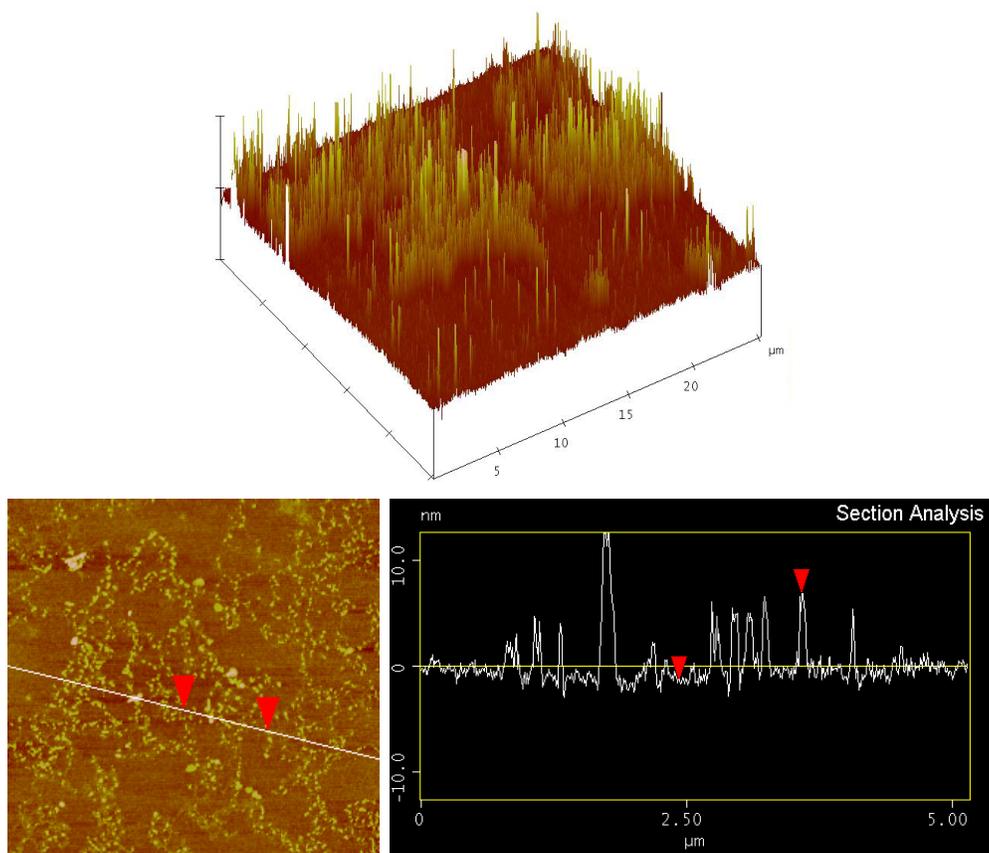

Figure 1. AFM image (top), topography (bottom left) and cross sectional profile along the line in topography (bottom, left) of the silicon surface after adsorption of BSA from solution.
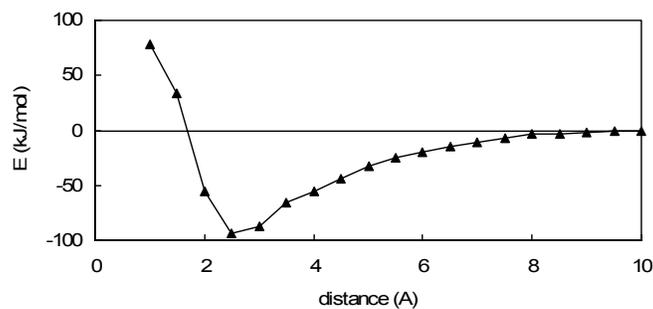

Figure 2. Free/bound LYS interaction energy as a function of position from the LYS molecule bound to the silicon surface.

To proceed to the third level of our multiscale procedure, we can start by recalling that classical diffusion theory is based on the Einstein's relation, which expresses the diffusion coefficient of spherical Brownian particles in a solution as:

$$D_{AB} = \frac{kT}{6\pi r\eta} \tag{1}$$

where k is the Boltzmann constant, T the temperature, r the radius of the solute particles, and η the viscosity coefficient of the liquid. In order to derive a more general model, one needs to take into account the fact that the mixture does not satisfy the hypothesis of ideal gas law. When the state of Brownian particles in the Einstein argument deviates from the ideal gas law, it assumes the form of the van der Waals equation:

$$\left(p + \frac{a}{\gamma^2}\right)(\gamma - b) = RT \tag{2}$$

where p is the pressure, R is the universal gas constant, and $\gamma = V/n$ is the molar volume, V being the total volume and n the number of moles. It is well known that the constants a and b have a physical interpretation: The term $a/\gamma 2$ represents the additional positive pressure caused by the presence of other solute particles, as a consequence of the long-range attractive forces; the constant b, instead, represents the volume occupied by the gas molecules, so that the term $(\gamma - b)$ represents the effective "free volume". We have then exploited the work of Peskir,[12] who derived from equation (2) the generalized expression for the diffusion coefficient (compare with equation (1)):

$$\tilde{D}_{AB} = \frac{kT}{6\pi r\eta}\left[\frac{1}{(1 - b_0 v)^2} - \frac{2a_0}{kT}v\right] \tag{3}$$

where $v = v(t,x)$ is the average number of Brownian particles at position x at time t, $a_0 = a/N_0^2$, $b_0 = b/N_0$, and N_0 is Avogadro's number. Note that the generalized coefficient $\tilde{D}_{AB}$ recovers the Einstein coefficient D_{AB} when $a = b = 0$. Expression (3) yields the following relation:

$$\frac{\partial v}{\partial t} = \left(\frac{kT}{6\pi r\eta}\frac{2b_0}{(1 - b_0 v)^3} - \frac{2a_0}{6\pi r\eta}\right)\left(\frac{\partial v}{\partial x}\right)^2 + \left(\frac{kT}{6\pi r\eta}\frac{1}{(1 - b_0 v)^2} - \frac{2a_0}{6\pi r\eta}v\right)\frac{\partial^2 v}{\partial x^2} \tag{4}$$

which is a generalized diffusion law. A finite-element model has then been built upon relation (4).

In order to predict the release behavior of each protein from the nanochannel membrane, we simply equated the parameter b to the protein molar volume. The parameter E_0 was calculated, according to its definition as the potential energy of interaction exerted on the free biopolymer molecule by the bound one at minimum. For example, in the case of LYS, since the calculated van der Waals molecular volume $V_{vdW,LYS} = 16651$ Å^3, the parameter b is equal to:

$$b = V_{vdW,LYS} \times N_0 = 0.0100 \text{ m}^3 \tag{5}$$

The value of the interaction parameter a is then calculated as:

$$a = -\frac{E_0 V N_A^2}{2 N_m} = 6.95 \times 10^3 \text{ Pa m}^6 \tag{6}$$

in which the number N_m of interacting molecule is 2 (i.e., the bound and free LYS molecules), and the total volume is set equal to the volume of the simulation cell: $V = 4.91 \times 10^{-25}$ m^3. The value of E_0 is obtained from the minimum of the potential energy of interaction curve between the free and bound LYS molecules, as explained above: $E_{min} = -93.93$ kJ/mol, as $E_0 = E_{min}/N_0 = -1.56 \times 10^{-19}$ J/molecule.

The release profile obtained by our model is shown in Figure 3 for interferon-α 2b, together with the experimental data, and the profile corresponding to a Fickian release. From Figure 3 it is evident how the diffusion profile estimated by Fick law is completely different from the experimental one. Further, in the same figure it is also reported the diffusion profile of the model obtained by selecting the parameters a and b which better fit, according to a mean square error policy, the experimental data. It is worth noting that the release curve obtained from the model using the a and b parameters obtained from our molecular modeling approach works even better than the model derived from the data fitting for a period covering the first 20-25 days (actually our model fits almost perfectly the experimental data for the first 20 days). Moreover, the last part of the experimental curve is not completely reliable, due to the offset caused by the experimental errors which cumulate day after day; this could explain the discrepancies between the proposed model and the data after day 25.
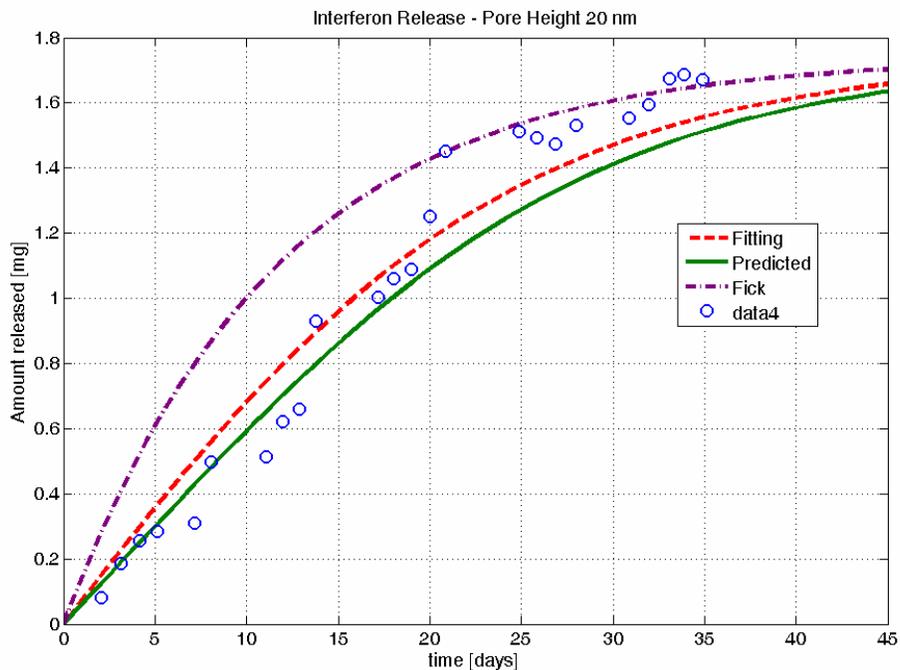
Figure 3. INF mass flux through a 20 nm pore height membrane: experimental data (○), Fick's law prediction (−·−), model based simulation with parameters derived by fitting (—), and model based simulation with parameters derived by molecular dynamics simulation (continuous line).

Conclusions

In conclusion, in this work we have formulated a multiscale simulation approach to the biomacromolecule diffusion in nanochannels, based on computer simulations spanning from the atomic world to the continuum dimension. Long regarded as a purely mathematical subject, molecular multiscale modeling can now be considered as most relevant for its physical significance. Molecular engineering is indeed entering a new era, characterized by an unprecedented control over chemical reaction, as well as product molecular architecture, conformations and morphology. Molecular multiscale modeling thus provides a sort of unifying set of principles to understand and interpret the behavior of seemingly different systems on common grounds. In other terms, it provides a common language that enables practitioners of molecular engineering to approach problems in areas as diverse as, for instance, chemical sensing, microfluidics, genetics, and last but not least, nanomechanical microdevices such as that experimentally employed in this study. Therefore the main contribution of this paper is

that of establishing a sort of *ab-initio* methodology which allows predicting the kinetics of biomolecular diffusion through membrane nanochannels without requiring, in principle, expensive laboratory experiments.

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