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Màster oficial en

Modelització Computacional Atomística i Multiescala en Física, Química i Bioquímica

Treball Final de Màster

Modelització de processos enzimàtics de reacció-difusió en medis crowded mitjançant simulacions de Dinàmica Browniana.

Modelización de procesos enzimáticos de reacción-difusión en medios crowded mediante simulaciones de Dinámica Browniana.

Modelling enzymatic reaction-diffusion processes in crowded media by Brownian Dynamic simulations.

Martí López Berbel

September 2017

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Sóc de les persones que pensa que la ciència té una gran bellesa. Un científic al seu laboratori no es tan sols un tècnic: és també un nen davant de fenòmens naturals que l'impressionen com un conte de fades.

Marie Curie

Agrair al grup *BioPhysicalChemistry* l'oportunitat d'haver entrat a formar part del món de la investigació i la recerca científica i ajudar-me en el propi creixement personal com a investigador, agrair també el tracte gaudit durant aquest any per part dels membres del grup;

Als companys del màster amb qui he compartit molts moments i esforços i que han fet que aquest any sigui únic i ple de moments inoblidables;

Als companys de despatx amb qui he compartit algunes tardes ben llargues;

A la família per recolzar-me en tot moment;

I per acabar, al Sergio per guiar-me durant tot el procés i ser el recolzament necessari per a tirar endavant en tot moment.

Títol: **Modelització de processos enzimàtics de reacció-difusió en medis crowded mitjançant simulacions de Dinàmica Browniana.**

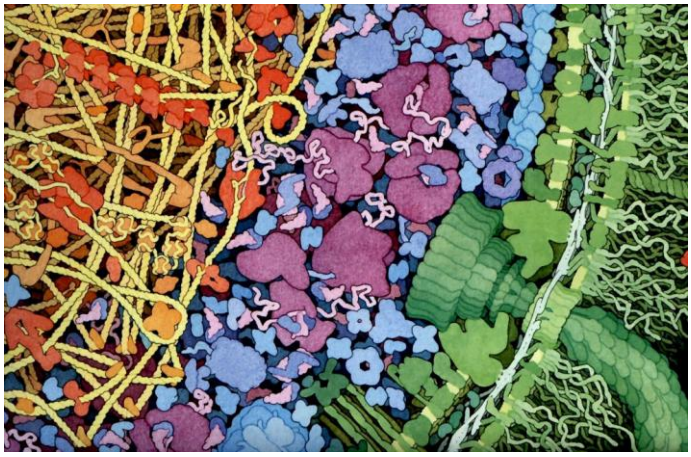
Estudiant: Martí López Berbel

Data: Setembre 2017

Director/s: Dr. Sergio Madurga Díez
Departament de Ciència dels Materials i Química Física

Les reaccions enzimàtiques han sigut àmpliament estudiades però habitualment sota condicions ideals. Per entendre més profundament com aquestes reaccions esdevenen en medis realistes tals com un medi cel·lular, s'han d'estudiar en medis "*crowded*". Experimentalment, encara no tenim les eines adequades per monitoritzar aquestes reaccions però amb eines computacionals, els medis "*crowded*" es poden generar amb facilitat. A més a més, la reacció es pot seguir sense dificultats. Per realitzar aquestes simulacions es descriu un sistema "*off-lattice*" de reacció-difusió amb Dinàmica Browniana. Aquest codi és suficient per a realitzar simulacions amb constants cinètiques ràpides ($k_1=10^9$ M^{-1} s^{-1}) però no quan aquestes són realistes. Aquest treball se centra en resoldre aquest inconvenient mitjançant un seguit de simulacions curtes i la seva conseqüent extrapolació per obtenir els perfils de concentracions de totes les espècies que hi estan involucrades. No es mostrarà cap perfil de reacció amb constants realistes però el mètode per aconseguir-les es testeja reconstruint els perfils d'una reacció amb constants cinètiques accelerades.

Les dades simulades s'ajusten al model de Michaelis-Menten amb el mètode Levenberg-Marquardt. Per saber fins on seria acceptable admetre les extrapolacions com a vàlides s'empra una extensió del mètode Levenberg-Marquardt. Aquesta es basa en un procediment Monte Carlo per generar grups de dades extres. Les noves dades es comparen estadísticament per a obtenir la màxima extrapolació possible sense afectar significativament els resultats. S'ha considerat que amb 100 extra grups de dades era suficient. Els resultats mostren com es possible d'obtenir els perfils de reaccions enzimàtiques reduint, com a mínim, el temps de simulació dos ordres de magnituds amb menys de dos simulacions. Aquest mètode també permet accelerar el temps de simulació de sistemes ràpids atorgant la possibilitat d'utilitzar el temps estalviat per a simular més repliques i aconseguir una estadística més acurada.



Il·lustració 1 Model de l'interior "crowded" d'una cèl·lula

Título: **Modelización de procesos enzimáticos de reacción-difusión en medios crowded mediante simulaciones de Dinámica Browniana.**

Estudiante: Martí López Berbel
Fecha: Septiembre 2017
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Las reacciones enzimáticas han sido ampliamente estudiadas, pero habitualmente bajo condiciones ideales. Per entender más profundamente como estas reacciones transcurren en medios realistas tales como un medio celular, hay que estudiar las en medios "*crowded*". Experimentalmente, aun no hay las herramientas adecuadas per monitorizar estas reacciones, pero con herramientas computacionales, los medios "*crowded*" se pueden generar con facilidad. Además, las reacciones se pueden seguir sin dificultades. Para realizar estas simulaciones se describe un sistema "*off-lattice*" de reacción-difusión con Dinámica Browniana. Este código es suficiente para realizar simulaciones con constantes cinéticas rápidas ($k_1=10^9$ M^{-1} s^{-1}) pero no cuando estas son realistas. Este trabajo se centra en resolver este inconveniente mediante un seguido de simulaciones cortas i la su consecuente extrapolación pera obtener los perfiles de concentraciones de todas les especies que están involucradas. No se mostrará ningún perfil de reacción con constantes realistas pero el método para conseguir las se testea reconstruyendo los perfiles de una reacción con constantes cinéticas aceleradas.

Los datos simulados se ajustan al modelo de Michaelis-Menten con el método Levenberg-Marquardt. Para saber hasta dónde sería aceptable admitir las extrapolaciones como válidas se usa una extensión del método Levenberg-Marquardt. Esta se basa en un procedimiento Monte Carlo per generar grupos de datos extras. Los nuevos datos se comparan estadísticamente para obtener la máxima extrapolación posible sin afectar significativamente los resultados. Se ha considerado que con 100 extra grupos de datos era suficiente. Los resultados muestran cómo es posible de obtener los perfiles de reacciones enzimáticas reduciendo, como mínimo, el tiempo de simulación dos órdenes de magnitud con menos de dos simulaciones. Este método también permite acelerar el tiempo de simulación de sistemas rápidos otorgando la posibilidad de utilizar el tiempo ahorrado para simular más replicas y conseguir una estadística más acerada.

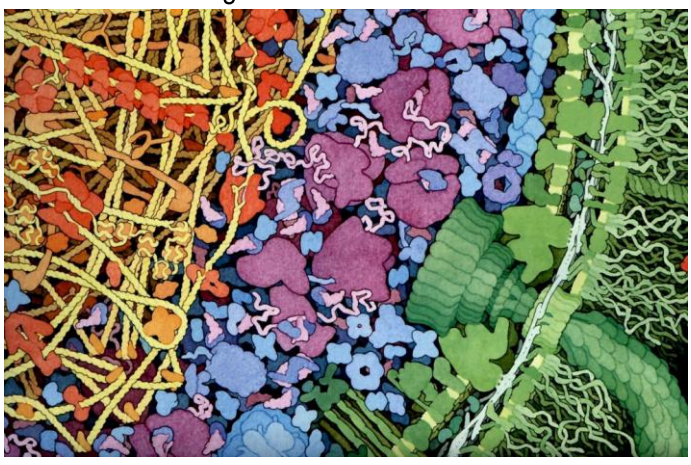

Figura 1 Representación del interior "crowded" de una célula.

Title: **Modeling enzymatic reaction-diffusion processes in crowded media by Brownian Dynamic simulations.**

Student: Martí López Berbel

Date: September 2017

Supervisor/s: Dr. Sergio Madurga Díez
Department of Materials Science and Physical Chemistry

The enzymatic reactions have been deeply studied but usually in an ideal environment conditions. To understand more deeply how such reactions evolves in more realistic media as cell-like environments those reactions have to be studied in crowded media. Experimentally, we still do not have the adequate tools to monitor it but with computational tools crowded environments can be easily generated. Furthermore, the reaction can be tracked without difficulties. To perform the crowded simulations the system is described by an off-lattice reaction-diffusion system with Brownian Dynamics. That code is good enough to perform simulations with fast kinetic constants ($k_1=10^9$ M^{-1} s^{-1}) but not when they are realistic. This work is focused to solve this setback through a sum of small simulations and the complementary extrapolation to get concentrations profiles of all the species involved on such reactions. Any realistic reaction profile is get but the method is tested rebuilding a fast kinetics system.

The simulated data is fitted to the Michaelis-Menten kinetics with the Levenberg-Marquardt method. To ensure until where the extrapolation of the simulated data should be accepted an extension of the Levenberg-Marquardt method is used. It is based in Monte Carlo procedure to generate extra simulated data. This extra data is compared statistically to obtain the degree of maximum extrapolation without affecting significantly the final results. It was considered that 100 extra sets are enough to ensure it. The results show that is possible to get the concentration profiles of enzymatic reactions reducing the simulation time at least two magnitude orders and no more than two simulations are need it. This method gives also the opportunity to have good approximations for systems with fast kinetic constants given the possibility to spend the saved time simulating more replicas to get more accurate results.

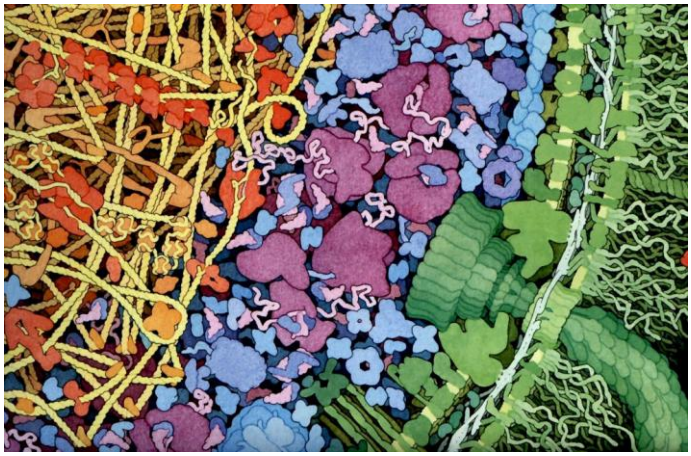

Figure 1 Visual model of the inside of a crowded cell.

Descripció justificada en català/castellà de la contribució de l'estudiant a cadascuna de les etapes del treball desenvolupat, si s'han fet servir resultats previs del grup de recerca on s'hagi desenvolupat, si hi ha hagut alguna col·laboració per assolir algun dels resultats, etc.

Aquest treball es pot considerar una continuació, des d'una certa distància, del treball final de Màster de la Mireia Vía Nadal, realitzat fa un any al mateix grup que ha supervisat el meu treball final de màster. El programa necessari per a dur a terme las simulacions del processos enzimàtics (RK3D) es producte del seu treball. Tot i així, el programa s'ha hagut d'adaptar per a satisfer els nous requisits demanats. En concret s'ha actualitzat des de la versió RK1_3D.11.8 a la versió final RK1_3D.12.3. Aquesta actualització ha implicat de l'adaptació de la subrutina "*inicialitzacio_sistema*" i petites correccions per encaixar les modificacions realitzades.

El següent pas ha estat la creació d'un codi (ERFAC) per aconseguir l'objectiu principal del treball, aquest consta d'un seguit de codis en fortran, octave i bash que permetent generar perfils de reacció similars als obtinguts en base a una simulació complerta mitjançant simulacions més curtes i extrapolacions.

L'objectiu final, però, era aconseguir utilitzar el mètode provat per aconseguir un perfil de reacció amb constants més realistes impossible de realitzar amb la versió RK3D inicial sense el mòdul ERFAC.

FORMATTED REPORT

Modeling enzymatic reaction-diffusion processes in crowded media by Brownian Dynamics simulations

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Advisor: Sergio Madurga Díez.

Abstract: Enzymatic reactions have been deeply studied but usually in an ideal environment conditions. To understand more deeply how such reactions evolves in more realistic media as cell-like environments those reactions have to be studied in crowded media. Experimentally, we still do not have the adequate tools to monitor it but with computational tools crowded environments can be easily generated. Furthermore, the reaction can be tracked without difficulties. To perform the crowded simulations the system is described by an off-lattice reaction-diffusion system with Brownian Dynamics. That code is good enough to perform simulations with fast kinetic constants (k_1=10^9 $M^{-1}s^{-1}$) but not when they are realistic. This work is focused to solve this setback through a sum of small simulations and the complementary extrapolation to get concentrations profiles of all the species involved on such reactions. Any realistic reaction profile is get but the method is tested rebuilding a fast kinetics system. The simulated data is fitted to the Michaelis-Menten kinetics with the Levenberg-Marquardt method. To ensure until where the extrapolation of the simulated data should be accepted an extension of the Levenberg-Marquardt method is used. It is based in Monte Carlo procedure to generate extra simulated data. This extra data is compared statistically to obtain the degree of maximum extrapolation without affecting significantly the final results. It was considered that 100 extra sets are enough to ensure it. The results show that is possible to get the concentration profiles of enzymatic reactions reducing the simulation time at least two magnitude orders and no more than two simulations are need it. This method gives also the opportunity to have good approximations for systems with fast kinetic constants given the possibility to spend the saved time simulating more replicas to get more accurate results.

I. INTRODUCTION

Enzymatic reactions have a particular interest in the biochemical field. Understand better how they are performed can give us light about one of the most important biological reaction mechanism. They are involved in almost all biological organisms regulating many vital functions.

Enzymatic reactions have been studied for many years. The complexity of the real systems with those reactions, as inside the cells, has made that important approaches has been made in order to face the problem. The cell environment is crowded with a huge number of macromolecules, these but, are present in a tiny concentration. When a reaction is occurring inside a cell the media will be non homogeneously mixed and crowded, and due to that facts, the reaction agents (will not have a behaviour as an ideal model). We can say then, that a reaction produced in a cell-like media will involve a considerably amount of non interaction particles and the diffusion will play an important role in the kinetics. To recreate the cell-like environment experimentally and study the enzymatic reaction-diffusion processes it has not been possible to due to technical limitations that, currently, does not permit to perform it. Due to all this inconveniences all the research focused on the enzymatic reaction-diffusion processes have supposed an ideal homogeneous and dilute system. That is far from the real systems and to get deep into that issue an have a more realistic overview the computational sources can help as to simulate the behaviour of the reaction agents in a cell-like environment.

The model used to simulate the enzymatic reactions is the **Michaelis-Menten kinetics mechanism**, (as the model is well-known, we will not explain it deeply):

$$\begin{cases} \frac{dS}{dt} = -k_1 \cdot S(t) \cdot (E_0\text{-}C(t)) + k_{m1} \cdot C(t) \\ \frac{dC}{dt} = k_1 \cdot S(t) \cdot (E_0\text{-}C(t)) - k_{m1} \cdot C(t) - k_2 \cdot C(t) \end{cases} \quad (1)$$

The original model of Michaelis-Menten kinetics can be reduced to those final differential equations Eq. (1) and the concentration of the enzyme and the concentration of the product can be obtained from them.

Computational tools give us an advantage to study such systems but, as in the experimental case, there are many technical limitations. To simulate a system big enough to be representative with real values of concentration of the reaction agents and kinetic constants the computational power required is extremely huge so to get the final profile curve of concentrations an extrapolation will be need it.

II. COMPUTATIONAL METHOD

The code used **(RK3D)** [1] to perform the simulations has been done for Mireia Vía as her Master's thesis in the

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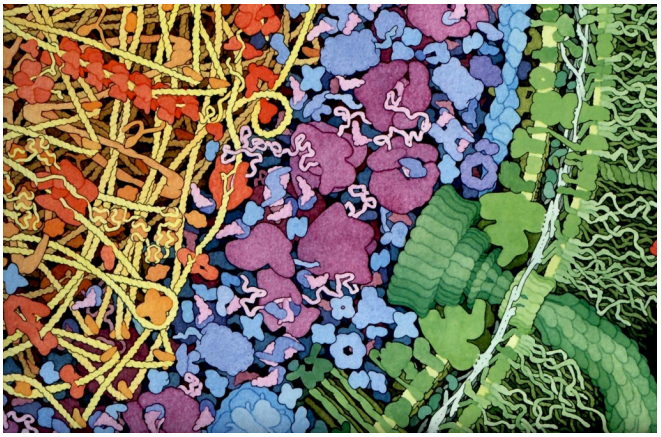

FIG. 1: Visual model of the inside of a crowded cell.

group of *BioPhysicalChemistry*. Then the exact mechanism of the program will not be extensively explained. However, a few relevant details will be discussed.

The system follows the Brownian Dynamics motion (BD) to simulate the diffusion of the the particles. Thinking in the cellular environment, an small confined space with many species that usually some reactant ones has a population not many orders of magnitude larger than the others, the stochastic methods become more appropriated to describe it. Between all the stochastic simulation algorithms, we need one who takes into consideration the effect of diffusion upon reactivity and the effect of macromolecular crowding onto diffusion itself. The BD accomplishes those requirements and is the most used stochastic algorithm to simulate such biochemical networks.

The Langevin equation is implemented. The deterministic force corresponds to the non specific interaction potentials:

$$V(x,y,z) = \begin{cases} \frac{1}{2}k_{pair}(d_{ij} - r_{ij})^2 & d_{ij} < r_{ij} \\ 0 & d_{ij} > r_{ij} \end{cases} \quad (2)$$

The potential is a quadratic, harmonic potential by pairs where $d_{ij} = \left[(x_i - x_j)^2 + (y_i - y_j)^2 + (z_i - z_j)^2\right]^{1/2}$ is the pairwise distance between two particles and $r_{ij} = (r_i + r_j)$ is the sum of the radius. The k_{pair} is the inter-particle repulsion force constant. In the RK3D it is set to 10 KJ mol^{-1} nm^{-2}. [2]

The stochastic term of the Langevin equation Eq. (2) needs a random Gaussian distribution number to be calculated. As the program has a random uniform distribution generator the Box-Muller transformation [3] permits us to get the set of variables normally distributed. The polar form of the Box-Muller transformation is widely implemented in stochastic modelling due to its velocity and robuster. The algorithm can be found in the numerical recipes. [4]

In order to get the profiles of reaction with realistic constants a main code in bash called *'Enzymatic Reactions: Adjustment and Extrapolation Code'* (**ERAEC**) has been performed. The code uses a sort of fortran and octave codes to analyze statistically the simulation outputs and get finally the profiles.

To be able to get a realistic reaction profile this method is based on simulating a short time of the reaction, and from that fit the obtained data upon the Michaelis-Menten kinetics and extrapolate the adjustment to get a bit longer simulation data. After, the reaction is simulated again but starting forward, using the final concentrations of the extrapolation as the initial ones for the next simulation. The data is again fitted to Michaelis-Menten kinetics and extrapolated to move forward on the reaction profile. Repeating such process we will get the reaction profile of a system with extremely slow kinetic parameters just with few short simulations instead of spending huge amount of sources and time to complete one full simulation. The theoretical background and the sub-codes are explained deeply in the following sections.

A. RK3D adjustments

The main idea behind the method to get those realistic reaction profiles is the possibility to get parts of that profiles extrapolating small simulations. In order to use the RK3D few adaptations have been done.

To validate this step, the method is tested with the well described system with accelerated constants (k_1=10^9 M^{-1}s^{-1}). Using the ODE from the complete simulated set of data the profile curve is constructed. The objective then is to rebuild the ODE using the purposed method. In order to demonstrate the utility of the method a simulation of 10^4 ns of the first system, it will be referred as fast system since now, will be re-simulated with 50 ns simulation reducing the magnitude order of time per three.

The previous RK3D simulated systems that starts with only substrate and enzyme in it. To be able to initialize the program when a reaction is already started and modifying briefly the program to avoid non direct mismatches, the initializing function, *inicialitzacio_sistema*, has been adapted. Initial quantities of complex and product particles have to be introduced. In the first step they will be set to 0 but at the rest of them, these amounts have to be upload according to the extrapolations. The amounts of initial enzyme and substrate even they will change along the process they will remain constant in the input file.

As the program was not initially designed to be initialized with a reaction in progress, the variables containing the initial amounts of substrate and enzyme have to be included in other functions or subroutines. Due to that the real initial amount of those reaction agents are set using basic arithmetic with the initial amounts of product and complex and the amount of enzyme and substrate at the beginning of the reaction.

$$[\mathrm{E}] = [\mathrm{E\_initial}] - [\mathrm{C}]$$
$$[\mathrm{S}] = [\mathrm{S\_initial}] - [\mathrm{C}] - [\mathrm{P}] \quad (3)$$

To complete the adjust, all the complex particles must have a pair associated to permit their decay into E plus S or E plus P. For all C particles then, another one is added without coordinates and set as type 0 (fake particle) to be reintroduced in the system as S in case of decay.

The last correction is to link by pairs, as the original RK3D does, every C particle with a fake particle to set all the initial particles as they are when the program is already on progress to be sure the new system will be properly recognized by the code.

B. Data adjustment

As is explained at the beginning of the section the main part of the method is the fitting. As better and more reliable is the adjust, more fast is the whole method. The process will be explained deeply in the current section.

The simulated data is fitted to the Michaelis-Menten kinetics Eq. (1) using the octave function lsode from the odepkg package to solve the differential equations Eq. (1) and the leasqr function from the optim package to fit the differential equation solution. [5] Leasqr function is based in the Levenberg-Marquardt nonlinear regression method.

Even the function already has a default values for all the inputs required apart from the data to adjust, our code include all of them to give to the user the capability to approach the fitting for any individual case as the importance that this step has for the whole process. The independent variable and the dependent variable have chosen to be the time and the complex concentration respectively. Additionally, the dependent variable can be changed to any other one to test if the adjustment fits better.

This is also the problematic step of the method, depending on the initial data, the system cannot be able to do the adjust as the convergence of lsode function has a failure. The main reasons usually are a bad Jacobian, a wrong integration method or a non adequate tolerance. However, if the inputs are chosen carefully the performance of the function must be good enough.

Once the fitting is done, the extrapolation can be as long as wanted but longer simulations can be too heavy and will generate a considerably amount of data that will be wasted.

From the fitted ordinary differential equation obtained from the simulated data, new profiles are generated. To get those data a Monte Carlo calculation of confidence intervals is used. This is an extension of the Levenberg-Marquardt method [6] that permits to generate a new set of data as the simulated one. The set of concentration values has to be a Gaussian-distributed variable μ and variance σ^2. The Monte Carlo simulation method for non linear regressions:

$$\mathrm{x} = \mathrm{z}\sigma + \mu \quad (4)$$

where

$$\mathrm{z} = (-2\ln(p_1))^{1/2}\cos 2\pi(p_2) \quad (5)$$

z is a normally distributed variable with mean=0 and variance=1 and p_1 and p_2 are uniformly distributed random numbers between 0 and 1.

Michaelis-Menten has different reaction agents, and consequently, different reaction profiles for each. The chosen one to perform this research was the *Complex* concentration. In Eq. (4) the x become the complex concentration. μ is the complex concentration from the adjusted differential equation and $\sigma = \chi$. The adjust program use the fitted data obtained during the adjustment to calculate reduced χ by:

$$\chi = \frac{\sqrt{\sum_i (\mu_i - x_i)^2}}{\mathrm{dof}} \quad (6)$$

where *dof* means the degrees of freedom of the system, in our case will be the number of points evaluated less the independent variables. With the reduced χ we avoid the dependence between the number of points evaluated and χ value in order to do robust the method.

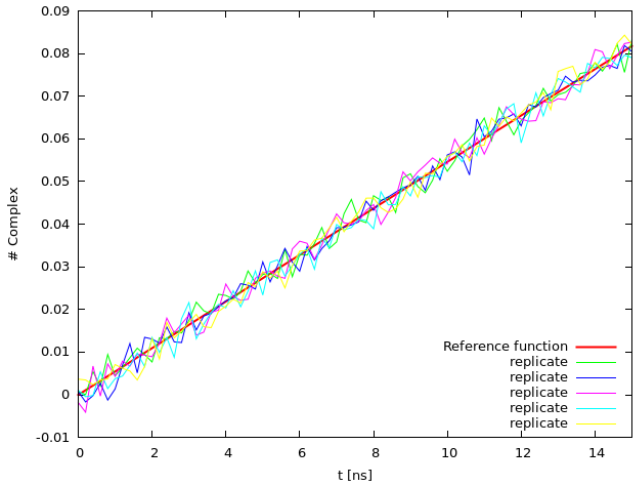

FIG. 2: Example of the data generated by the extension of the Levenberg-Marquardt method. In red is plotted the base function from the new simulated data comes from.

Following the process, a sort of 100 sets of Monte Carlo simulations is generated and adjusted with and adaptation of the adjustment octave code. An ODE is calculated for each set of data and the σ of the sort of profile curves is calculated each 25 time steps. The cut off to

accept the extrapolation is the reduced χ from the fast system.

Once using the extended Levenberg-Marquardt method an extrapolation of data non-simulated can be accepted, the RK3D is run again using those final data as the initial conditions. The new simulated data is treated using the same method but now this data is added to the previous simulated one (non including the data accepted by extending the simulation). Due to that, the new extrapolation could be more longer as more simulated data is used and successively each new step will accelerate the process.

As, in each step, the whole profile curves are readjusted, the final results will not show the simulation steps.

III. RESULTS

All the comparisons are done it varying k_1 because the other two constants (k_{-1}, k_2) depend strongly from it.[7]

The Fig.(3). shows the extrapolations have done it in each step for the $\mu \pm 3\sigma$ acceptance case. The simulations were 50 ns long and at third step, the new extrapolation was already worst that the second one. The first simulation was done between 0-50 ns and the extrapolation could be done until 260 ns. From there another 50 ns simulation was launch and the new extrapolation could be done until 530 ns. Here appears the main problem of the procedure. The next extrapolation only could be done until 420 ns.

The rebuild of that reaction profile has been performed using the semiautomatic ERFAC code. One important limitation of the actual version of the RK3D is that is not possible to start it with a non integer amount of particles. This seems to be obvious as a BD simulation code cannot simulate half particles or similar. The problem is that it is required usually, if the extrapolation will finish when the amount of the reaction agents are not integer. At Fig.(3) is shown how sharply is this effect.

In the Table I is shown how change the accepted extrapolation depending on the initial simulation. In particular, how different behaves when the initial simulation is 50 ns longer (from 100 to 150 ns). Looking at $\mu \pm 2\sigma$ case for the 0-150 ns simulation it is already accepting all the extrapolation but only 400 ns for the 0-100 ns simulation. Does can give us an idea that how many magnitude orders the simulation time can be reduced if the code is adapted to start statistically with real initial amount of particles.

	k_1 (nm^3 ns^{-1})	$\mu \pm \sigma$	$\mu \pm 2\sigma$	$\mu \pm 3\sigma$	$\mu \pm 4\sigma$
0-100 ns	0.151	165 ns	400 ns	840 ns	10000 ns
0-150 ns	0.155	325 ns	10000 ns	10000 ns	10000 ns

TABLE I: Extrapolation accepted depending on precision.

As this is not developed yet at ERFAC, to follow the

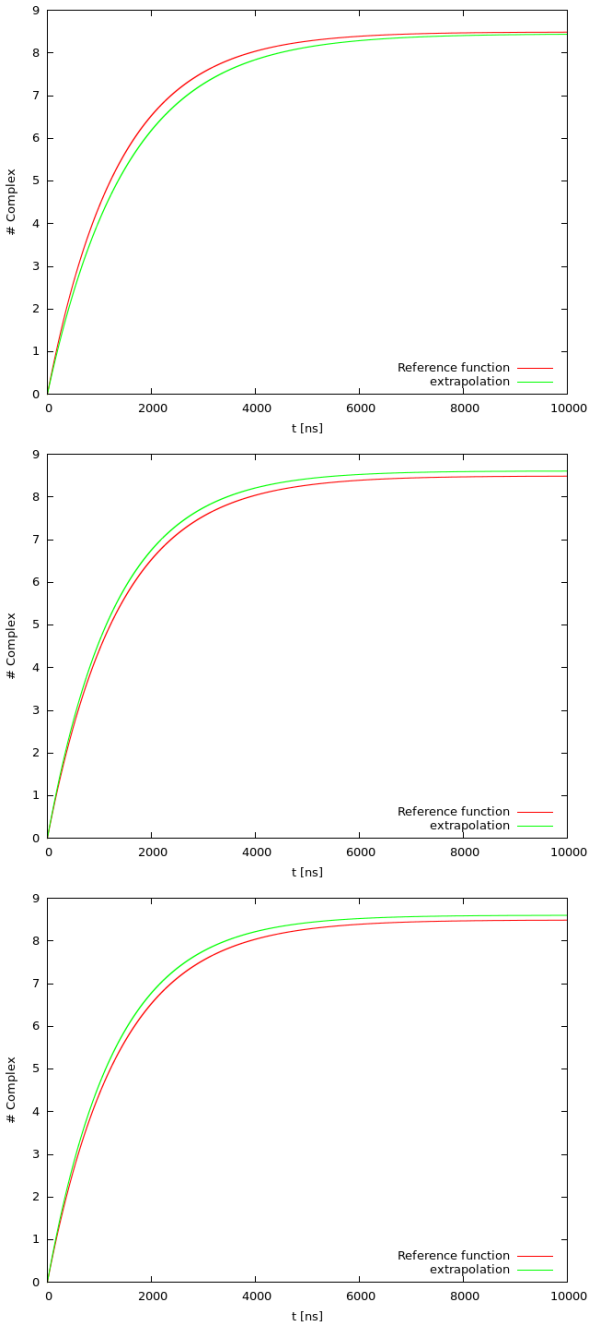

FIG. 3: Representation of the extrapolations from the three first steps. The k_1 are respectively 0.142, 0.169 and 0.171 (nm^3 ns^{-1}). The k_1 of the complete simulation is 0.159nm^3 ns^{-1}. The third step is the visualization of the lack of the program, when the concentration of the product at the profile reaction grows enough that if its set to 0 again for the next step, the following fitting become worst.

rebuild of the reaction profile the extrapolation has to reach, at least, the presence of the first product particle. This moment, but, is further on the reaction. As it shown on Table I adding few simulation time the fitting becomes better fast so, to get an extrapolation that reaches the presence of the first product particle to launch the next extrapolation but does not reach the whole reaction profile does not appear easily. Due to this issue the magnitude order or the time it has been reduced only by two.

One way to solve it is using statistics. To setback that issue is not necessary to modify the RK3D code itself. If the RK3D is run several times starting at the same reaction time, the initial amount of enzyme, substrate, complex and product of each simulation must be set different to fit the average of each concentration at the end of the previous extrapolation. For instance, if the product present a final concentration of 0.25 particles, the RK3D can be run 100 times, 25 times with the initial amount of 1 product particles and the 75 remaining with initial amount of 0 product particles.

The procedure is illustrated at Eq. (**??**)

It is suggested to write that update to the code in order to get the final objective of generate the reaction profile of an enzymatic reaction with realistic kinetic constants.

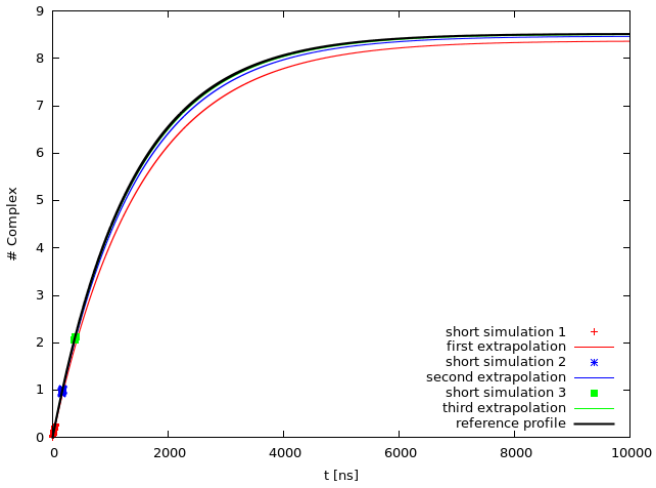

FIG. 4: The procedure is exemplified. In red the first step, in blue the second one and in green, the third one.

IV. CONCLUSIONS

The method used to rebuild the profiles of enzymatic reactions has been correctly implemented and the results agree with the objective.

- The method purposed permits to get the reaction profiles faster than the BD complete simulations with the previous RK3D code, spending less computational sources and time. That properties make ERFAC a seed code to get reaction profiles with realistic constants.
- To improve the procedure two steps should modified. The fitting with the leasqr and lsode functions present often failures if the function parameters are not accurately introduced. The other one is how the simulation are initialized after the first step. The extrapolations are strongly affected by the approximations with the initial amounts of reaction agents.
- The ERFAC code can be accelerated if it is redesigned to be all in octave language.

V. APPENDIX

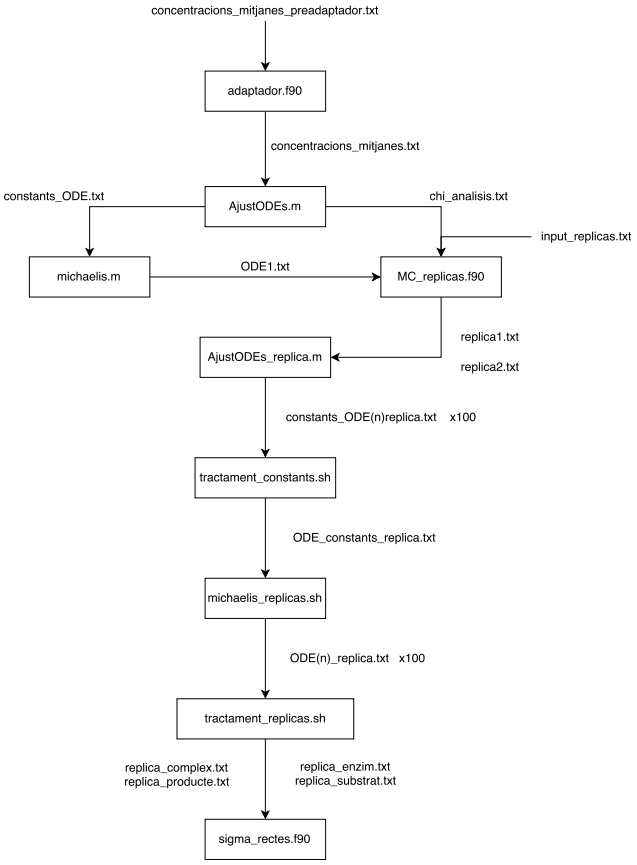

FIG. 5: Diagram of the ERFAC code.

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