# ENMET: Endothelial Cell Metabolism Mathematical Model

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## Abstract

*Endothelial cells have central role in controlling blood vessels homeostasis, secreting vasodilator (Nitric Oxide, NO) and vasoconstrictor (Endothelin-1, ET-1) molecules, in response to various shear-stress levels due to blood flow. ENMET (Endothelial cell METabolism) is mathematical model that mimics completely the principal metabolic pathways in endothelial cell, connecting mechanical-stimuli responses (Et-1 and NO production) to main biochemical reactions. It is based on a set of non-linear differential equations, implemented in Simulink®, based on the cellular energetic state.*

*The validation phase is based on experiments performed in a dynamic culture chamber where cell culture is exposed to a laminar flow that generates a well controlled shear stress.*

## 1. Introduction

Mathematical and computational modelling of biological systems [1] provides a functional frame for a systematically reading together data coming from biological, physiological, and clinical research.

These models could provide a system-level understanding and patho-physiology simulation and could predict how, when and in whom diseases will develop. In-silico modelling of biological processes is very low cost and has no ethic problems, because no animal experiments are needed, and user-friendly interfaces can permit also to users with little experience to have good predictions on phenomena.

From engineering point of view, the metabolic reactions that take place inside a cell or a tissue can be considered in terms of constitutive and state equations, boundary and initial conditions. The main role in these systems is played by enzymes, which are proteins that function catalysing specific biochemical reactions that convert reactants into products. Many attempts have been tried in literature to simulate these molecular processes, in particular several software packages for a quantitative simulation of biochemical metabolic pathways, based on numerical integration of rate equations, have been developed [2,9].

However, all these models/software platforms are focused on metabolic aspects, and it is very difficult to integrate them with those models that explain, i.e., a cell reaction to a particular physical stimulus. HEMET (HEpatocyte METabolism) model/software platform [10-11] was developed, instead, directly with the idea to describe all physical-chemical and cell-cell interactions and their effects on metabolism. HEMET is a mathematical model implemented in a user friendly software for simulation of hepatic metabolism in standard condition (in a plastic multiwell placed in an incubator at 37°C with 5% of $CO_2$). At present time, the same group is developing HEMETβ, where Michaelis-Menten models for reversible reactions and for enzymatic inhibition are introduced, and hard non-linearities are eliminated.

In this work, we present ENMET (ENdothelial cell METabolism), an endothelial cells mathematical model, based on HEMET structure, with the addiction of specific endothelium characteristic. The endothelium plays an important role in the regulation of vascular function by producing a large number of biologically active substances that participate in the regulation of vascular tone, cell growth, inflammation, and thrombosis/haemostasis.

In particular ENMET cell presents responses to various shear stress levels to which is exposed by release of vasodilatator and vasoconstrictor substances, respectively NO and ET-1.

The production of these substances is linked to other metabolic pathway related to energetic

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metabolism, and modulated by cell energetic need as in HEMET model.

## 2. Model Structure

ENMET implements the entire metabolic network [12, 13], using differential equations, system theory approach and energetic consideration (availability of ATP and other high-energy molecules).

In ENMET Michaelis-Menten model for reversible reactions [14] is implemented, using the following equation (I):

$$\frac{d[P]}{dt} = -\frac{d[S]}{dt} = \frac{V_{\max d}\frac{[S]}{K_{Md}} - V_{\max i}\frac{[P]}{K_{Mi}}}{1 + \frac{[S]}{K_{Md}} + \frac{[P]}{K_{Mi}}} \quad (1)$$

where $K_{Md}$ (mM) and $K_{Mi}$ (mM) are the Michaelis-Menten reaction constants, reported respectively to direct and inverse reaction, $V_{maxd}$ (mM $s^{-1}$) and $V_{maxi}$ (mM $s^{-1}$) are the maximum catalysis rate that can be reached in direct and inverse reaction respectively. If $K_{Mi} = \infty$, product release step is irreversible. In general, equations for a generic reaction with more substrates and/or products have to be written using, i.e., King-Altman method [15], and defining the kinetic type. This approach produces a great increase of parameters not associable to $K_M$ or $K_i$, the only constants present in literature. For this reason we suppose that reactive event takes all reactants in the same instant, to generalizing equation (1) in equation (2) [16]:

$$\frac{d[P_m]}{dt} = -\frac{d[S_n]}{dt} = \frac{V_{\max}\left(\frac{\prod_n[S_n]}{K_{Md}} - \frac{\prod_m[P_m]}{K_{Mi}}\right)}{1 + \frac{\prod_n[S_n]}{K_{Md}} + \frac{\prod_m[P_m]}{K_{Mi}}} \quad (2)$$

where $K_{Md}$, $K_{Mi}$ are the mean of $K_{Md}$ and $K_{Mi}$ of each component, and where $V_{max}$ term includes the maximum reaction rate in direct and inverse reaction (in general of these rates are of the same order of magnitude), reducing the number of parameters. Competitive enzymatic inhibition is introduced in the model by equation (3):

$$\frac{d[P]}{dt} = -\frac{d[S]}{dt} = \frac{V_{\max}[S]}{K_{Md}\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (3)$$

where [I] (mM) represents competitive inhibitor concentration, and $K_i$ (mM) is the inhibition constant.

Following system theory approach it is possible to recreate entire metabolic pathway using metabolites concentrations as state variables. Considering equations (2) and (3) as elementary blocks and following block scheme algebra rules, an entire pathway can be created linking blocks in series and/or parallel manner following the basic structure of HEMET, and removing the metabolic pathways not common between the two cell types (for example, the glycogen biosynthesis), and finally adding specific endothelial cell pathways, in particular NO [17] and ET-1 [18] production, and their dependence to shear stress ( adding a shear stress generator).

ENMET equations are implemented in Simulink® environment (The MathWorks, Inc) with a user-friendly interface, and a modular structure designed to be easily expandable. All ENMET implemented metabolic pathways are: cell culture proliferation, glucose and aminoacid uptake, Glycolysis, Pentose Phosphate Pathway. Krebs Cycle, Aminoacid degradation and Urea Cycle, NADH, $FADH_2$, NADPH, ATP and Energy Function, Shear Stress generation, NO production, ET-1 secretion. Here we are going to describe metabolic pathway that are characteristic of the endothelial cells, so Shear Stress generation, NO production, ET-1 secretion.

### 2.1. Shear Stress modeling

Shear stress is a mechanical stress exerted by the blood flow on the vascular wall (so it directly influences the endothelium) and depends on the strain rate in the fluid. In the case of Newtonian fluid (constant viscosity) in laminar motion the shear stress at the vessel wall ($\tau_w$) is:

$$\tau_w = \mu \left.\frac{\partial v}{\partial y}\right|_{y=0} \quad (4)$$

where μ (Pa·s) is the viscosity of the fluid, v ($m \cdot s^{-1}$) is the velocity of fluid, y (m) is the distance from the wall. A good estimation of average intensity at steady state can be obtained as:

$$\tau_w = \mu \frac{Q}{S \cdot y} \quad (5)$$

where, Q ($m^3 \cdot s^{-1}$) is the volumetric flow of fluid, S ($m^2$) is the cross-section area, y is the height of the channel (m). The shear stress was modeled with a Heaviside function, which amplitude is ShearS (Pa).

### 2.2. NO production

Nitric Oxide (NO) is one of the most powerful biochemical mediators in living organisms. It is produced from L-arginine in a reaction catalyzed by Nitric Oxide Synthase enzyme (NOS), which can be found in several isoforms [16]. In particular, we modeled the E-NOS behaviour (Endothelial-NOS) which catalyzes the following biochemical reaction (a):

$$L\text{-}Arginine + n\ NADPH + nH^{+} + mO_2 \rightarrow \rightarrow Citrulline + NO + nNADP^{+} \quad \text{(a)}$$

The vascular NO acts as potent vasodilator. This reaction depends, obviously, on substrates availability and it is influenced also by shear stress which, through various cascades of signal transduction/amplification, regulates the production of NO.

The NO production in in-vitro experiments [18] is characterized by a peak during the first hours and then it remains at a constant value. This behavior is similar to a second order system step response. After this phase NO production gradually decreases because of the lack of L-Arginine in the medium culture, the main substrate of NO synthesis. This kind of response is modeled in ENMET by a second order with complex conjugated poles system, expressed in Laplace domain according to (6):

$$G(s) = G_0 \frac{1 + \frac{s}{\omega_z}}{1 + \frac{2\chi s}{\omega_z} + (\frac{s}{\omega_z})^2} \quad (6)$$

where $\omega_z$ and $\omega_p$ are respectively the zero and the complex pole pulse, and $\chi$ is the damping factor of the complex conjugate pole. To take into account the availability of substrates, eq. (6) is included in (7) which gives the total response:

$$\frac{d[NO]}{dt} = L^{-1}\{Heaviside(s) \cdot G(s)\} \cdot [Arginine][NADPH] \quad (7)$$

where $L^{-1}$ is the Laplace anti-transform operation.

### 2.3. Endothelin-1 secretion

Endothelin-1 is a protein formed by 21 amino acids, with a vasoconstrictor effect. It is synthesized by enzyme complexes located on the cell membrane, in different passages: a pre-form (Pre-Pro-Endothelin-1) is converted to Pro-Endothelin-1, which is converted in Endothelin-1 by Endothelin Converting Enzyme (ECE). There are several works about ET-1 secretion rate at different levels of shear stress [19]. To approximate this kind of data we used a transfer function (8) with complex conjugate poles:

$$E(s) = G(Shear) \frac{1}{1 + (\frac{s}{\omega_{ET}})^2} \quad (8)$$

where the gain G depends on shear stress in accord with the lookup table of figure 1 [20], where an increase in shear stress causes a decrease in the gain of the transfer function, and $\omega_{ET}$ is the natural pulse of the response.

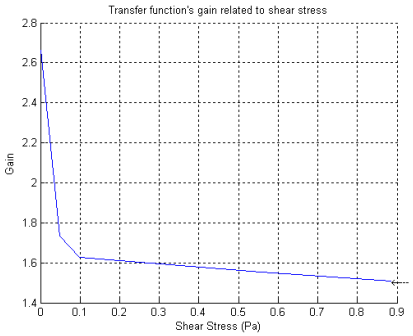


**Figure 1:look-up table that regulates the NO transfer function gain in accord to shear stress level**

The modeling strategy is based on protein synthesis of HEMET model (i.e. albumin), and it is controlled by availability of substrates and energy needs (ATP amount):

$$\frac{d[ET1]}{dt} = L^{-1}\{Heaviside(s) \cdot E(s)\} \cdot [ATP] - \alpha ET1[ET1] \quad (9)$$

## 3. Materials and methods

### 3.1. Cell culture

HUVEC (Human Umbilical Vein Endothelial Cells) were isolated from fresh human umbilical cord veins by enzymatic treatment with collagenase and were used between passages 2 to 8. Endothelial cells were cultured in 75-$cm^2$ flasks in M199 medium (Lonza Bioscience, Basel, Switzerland) with 50ml of fetal bovine serum (Lonza Bioscience, Basel, Switzerland), 5ml of 200mM solution of glutamine (Lonza Bioscience, Basel, Switzerland), 5ml of penicillin-streptomycin mixture containing 100 U of penicillin and 100µg of streptomycin (Lonza Bioscience, Basel, Switzerland), 10 ng/ml of endothelial growth factor (EGF) (Sigma-Aldrich, St. Louis, MO, USA), 2,5 ml of a 100µg/ml solution of hydrocortisone, in a 95% $O_2$, 5% $CO_2$ atmosphere. The cells were maintained at a density of 4-7.5x$10^5$ cells per ml by subculturing every 4 to 5 days in a 1:2 dilution in fresh medium.

### 3.2. Shear stress experiment

For this set of experiment cells between 4th and 7th passage are used. When at confluence, cells are seeded on glass slides (dimension 24 x 32 mm), treated with gelatin in an incubator for 15-20 minutes to improve cell attachment (6400 cells/$cm^2$ equivalent to 50000 cells/glass slide). About 48 hours after seeding, the

experiment starts with the assembly of the bioreactor, preventively sterilized with plasma gas and $H_2O_2$. Once assembled, the mixing chamber is filled with 25 ml of medium 199 (complete media formula without phenol red and BBE) and, then, all sensors are connected: air/$CO_2$ inlet, thermostatic system and peristaltic pump (Masterflex L/S, Cole-Palmer, Vernon Hills, IL, USA).

During experiment for NO quantitation, shear stress was set up to 9mPa, and NO concentration was evaluated at 2, 4, 6 and 24 hours. Nitric Oxide concentrations were evaluated using a colorimetric assay based on Griess reaction (CaymanChem, Ann Arbor, MI, USA) applied to medium samples.Five experiments were performed.

For ET-1 quantitation, the experiments were conducted at different flow rate of 1.5, 3, 4.5 and 6 ml/min, corresponding to shear stress values of 0.6, 0.7, 0.8, 0.9 Pa, respectively. For every shear stress level, we evaluated the cumulative concentration of ET-1 at the end of each experiment. Five experiments for each shear stress value were performed. Every experiment has duration of 24 hours, followed by cell counting and Coomassie brilliant blue stain. Once collected, samples are analyzed with an ELISA kit for Endothelin-1 (Cayman Chemical, Ann Arbor, MI USA). Metabolic activity was assessed, in both type of experiment, analyzing also glucose consumption: in particular, the glucose concentration was assessed using the Glucose Test Kit (Megazyme International Ireland Ltd.), a colorimetric assay based on glucose-oxidase and peroxidase reactions. In all cases glucose consumption is the difference between fresh medium and sample medium.

### 3.3. Implementation of developed model

The differential equations of ENMET model were implemented in the Simulink® environment and solved using stiff-Rosenbrock variable step algorithm. Parameters values, that concert metabolic pathway treated in this work, are reported in table 1. Their values are obtained from enzyme data bank [21], and from the HEMETβ model. Estimated parameters, evaluated with non-linear least squares algorithm, are reported in table 2.

Model validation was performed using maximum and mean errors, following formulas (10, 11):

$$MaxError = \max of\left(\frac{|V\exp_i - Vsim_i|}{V\exp_i}\right) \qquad (10)$$

$$MeanError = \frac{1}{N}\sum_{i}^{N}\frac{|V\exp_i - Vsim_i|}{V\exp_i} \qquad (11)$$

where $Vexp_i$ and $Vsim_i$ are experimental and simulation data, respectively.

## 5. Results and discussion

The ENMET model validation was performed using data from previous experiments carried out in a dynamic culture chamber where HUVEC cells were subjected to a laminar flow, and thus to the resulting shear stress, for the total duration of 24 hours. The various measures were taken at different times depending on the type of experiment. The validation of the model was carried out examining the distance between the simulation output and experimental data.

Cell proliferation, modeled as in HEMETβ using a modified logistic curve, explains experimental data (fig. 2), with a max error of 1%.

The glucose consumption (fig. 3) is directly correlated with cell growth. The glucose curve shows a negative derivative that increases in modulus with the time due to the increase of cell number in medium culture. The simulation curve is characterized by a max and mean error of 10% and 7% respectively.

The error is associable to the adaptation phase where the exact cell energy state is unknown, and it is not possible estimating the real cells metabolic need.

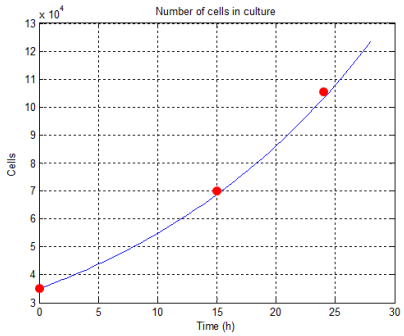


**Figure 2: Comparison between the measured (dot) and modelled (solid line) cell number density as function of time**

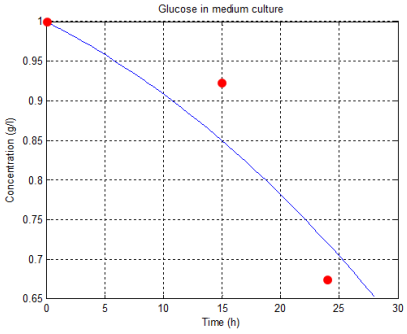


**Figure 3: Comparison between measured (dot) and modelled (solid line) glucose consumption revealed in culture medium**

Nitric oxide is characterized by extremely short life and reacts with oxygen to produce nitrite and nitrate, which analysis is an indirect assessment of NO production.

Endothelial cells release a large amount during the first few minutes after shear stress increasing, and then they reduce NO production when the steady state is reached, in accord with the curve of figure 4. This curve follows well the average trend of experimental data, and the mean error is very low (5%).

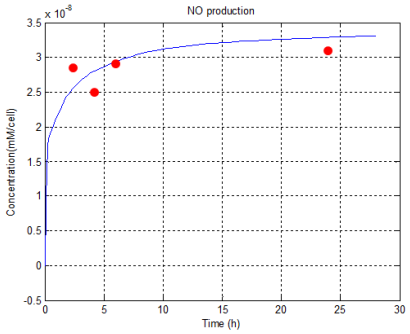


**Figure 4: Comparison between measured (dot) and modelled (solid line) NO production revealed in culture medium**

Endothelin-1 release is analyzed in a cumulative manner after subjecting cells to various shear stress levels for 24 hours. The model is in accord to experimental data, with a good level of approximation (max error of 2%) (Fig. 5).

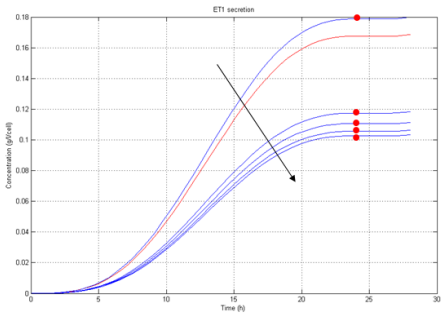


**Figure 5: Comparison between measured (dot) and modelled (solid line) ET-1 release revealed in culture medium. Measurements refer to 0 (control), 0.6, 0.7, 0.8, 0.9 Pa shear stress value.**

## 6. Conclusion

In this work we have created ENMET, an effective and versatile model of endothelial cell metabolism, starting from HEMET. The cell energy function and the modular structure derived from HEMET model are core of the model. This model is created starting from biochemical reaction, to obtain differential equations that rule cell behavior. In addiction, a shear stress dependent mechanism of vaso-active substances was implemented.

In fact ENMET can synthesize Nitric Oxide and Endothelin-1, and can modulate them to answer the shear stress which is subjected. The user friendly aspect of the HEMET model is maintained giving researchers a new instrument to evaluate endothelial cell behavior in dynamic environment for, i.e. model-based control systems for dynamic bioreactors

**Table 1: model parameters value**

| Enzyme | $V_{max}$ ENMET (mM/h) | $K_{Md}$ (mM) | $K_{Mi}$ (mM) |
|---|---|---|---|
| Arginase | 180,4608 | 11,6 | |
| ArgininoSuccinase | 570,8 | 0,12 | 3 |
| ArgSucc Synthetase | 248,4 | 0,04 | 0,028 |
| Ornithine Transcarbamylase | 1346,4 | 0,755 | 20 |
| Carbamoyl Phosphate Syntethase | 28,224 | 1,1 | |

**Table 2: model estimated parameters value**

| Parameter | Measurement unit | Value | Fractional Standard deviation |
|---|---|---|---|
| $\chi$ | | 0,125 | 0.06 |
| $G_0$ | | 337,5 | 0.10 |
| $\omega_n$ | $h^{-1}$ | 1,25 | 0.15 |
| $\omega_z$ | $h^{-1}$ | 0,25 | 0.12 |
| $\omega_{ET}$ | $h^{-1}$ | 0,25 | 0.08 |
| $\alpha_{Et1}$ | $mM^{-1}h^{-1}$ | 0,0025 | 0.20 |

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