Cellular Electrode for Antitumor Drug Screening

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Mouse leukemia L1210, a well established tumor model, can be immobilized near the surface of a dissolved O_2 probe and its O_2 uptake can be monitored continuously following the addition of various chemicals such as Mechlorethamine HCl, an antitumor agent. The output current of the O_2 electrode changed as a function of time under various experimental conditions. The dynamic response of the electrochemical sensor was determined as a function of antitumor agent concentration. A preliminary kinetic model was developed to describe the biosensor behavior.

Introduction

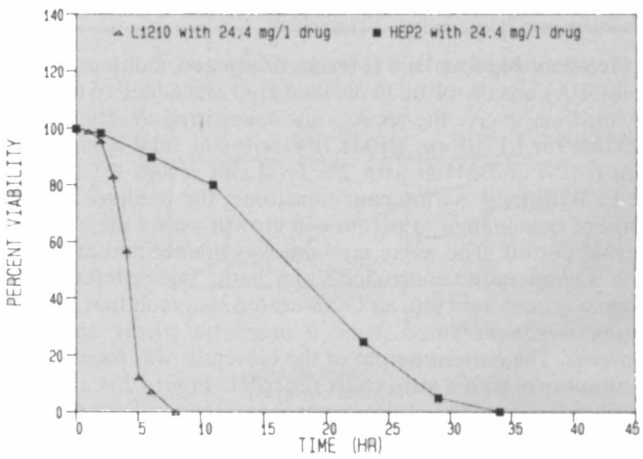
Screening programs are laboratory procedures designed to discover new biologically active compounds that possess unique and useful characteristics. The first antitumor screening program, developed in the mid-thirties by Shear et al [*11*], used laboratory mice as animal models. Antitumor activity was evaluated following injection of the test compound into mice infected with a transplantable tumor such as—sarcoma 180 (S180), adenocarcinoma 755 (Ca755), and leukemia 1210 (L1210) [*3*]. Because animal models are insensitive and expensive, it is necessary to develop *in vitro* test systems to pre-screen anticancer agents that are predictive of clinical activity. These tests can pare down the number of samples for subsequent *in vivo* screening. The present *in vitro* procedures consist of adding the test compound to a petri dish containing cultures such as L1210, S180, or human HeLa cells. After several days of incubation, cell toxicity and growth inhibition are assessed microscopically. But, these *in vitro* methods are generally very time consuming, labor intensive, and have the added difficulty of maintaining sterility during the long incubation period.

Biosensors have the potential to decrease the time necessary to complete such screening procedures, and to increase the screen sensitivity and selectivity. Biosensors have been shown to be effective in measuring the concentrations of various chemical compounds including antimicrobial agents. Karube et al [*6*] developed a biosensor consisting of a yeast membrane attached to an oxygen electrode that was used to monitor the effects of nystatin on the O_2 uptake of a yeast population. It was found that the rate of current increase was proportional to antibiotic concentration. A single determination required less than 40 minutes to complete, considerably shorter than the standard agar diffusion methods. Simpson and Kobos [*9*] also developed a biosensor to determine the tetracycline concentrations of pharmaceutical preparations. Their method was based on the inhibition of respiratory CO_2 production by an *Escherichia coli* suspension as measured potentiometrically using a CO_2 sensor.

This type of research can be extended to the use of mammalian cells as the testing cells. The objective of this research was to develop a biosensor using mammalian cells to evaluate various antitumor activities of several known drugs. This was accomplished by immobilizing mammalian cells to the surface of a dissolved O_2 electrode, inserting the probe into a solution containing the test compound, and monitoring the probe output. An increase output current indicated an inhibition of the cellular O_2 uptake rate. The basis of this change is the selective effect antitumor drugs have on various cancerous tissue. Figure 1 compares the effect of mechlorethamine HCl, a known antitumor agent, to two different cell lines in cell suspension. Notice the kinetics are different for these two different cell types. The Human larynx carcinoma (Hep-2) cell line shows a much slower response to the drug than the mouse leukemia line. The development of a biosensor for antitumor screening takes advantage of these differences.

Two cell lines were selected—Mouse leukemia L1210 and Human foreskin fibroblast (HFF) for this research. L1210 was chosen as the tumor model because of its consistent use in antitumor studies over the last several decades. HFF was selected on the basis of its similarity to normal human cells and served to discriminate antitumor compounds from broad

Figure 1. Drug kinetic study of mechlorethamine HCl on HEP2 and L1210.



spectrum cytotoxins. HFF is anchorage dependent, exhibits contact inhibition, and has a relatively slow growth rate compared to L1210. Extensive evaluation of any interaction between these chemicals and the various cell lines requires better understanding of their modes of action. The purpose of this paper is to demonstrate the feasibility of using these cellular electrodes to test various drug compounds.

Experimental

Cell Lines and Culture Media

Mouse leukemia L1210 was grown in suspension in 75 cm^2 tissue culture flasks at 37°C using RPMI 1640 medium with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY). Growth medium contained 50 μg/ml of streptomycin. Cells were harvested in the exponential growth phase. The human foreskin fibroblasts (HFF) were grown at 37°C in 150 cm^2 tissue culture flasks using M199 with 10% fetal calf serum (FCS). These anchorage dependent cells were trypsinized for use in the biosensor. The human epithelial (Hep-2) cell line was grown in MEM with 10% FBS. Conventional *in vitro* drug kinetic studies were carried out by adding the drug directly to the culture and then counting cell viability over time using trypan blue dye exclusion. A 4% w/v solution of kappa carrageenan (FMC Corp., Marine Colloid Div.) was prepared by adding the appropriate mass of the colloid to distilled H_2O. The solution was then autoclaved for 30 min. and stored at 55°C.

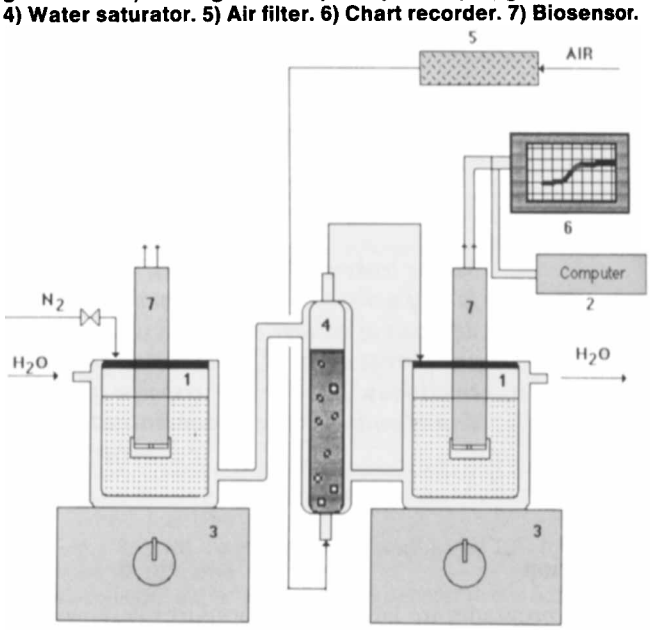
Construction of the Cellular Electrode

The dissolved O_2 sensor was constructed using the method by Johnson et al [5]. The teflon membrane placed over the silver cathode had a thickness of 0.5 mil. The diameter of the O_2 sensor was 8.0 mm. A short piece of silicone tubing was placed at the tip of the sensor and extended past the tip by about 2 mm. This protrusion served as the gel retainer for the gel-cell suspension of the biosensor. The biolayer was prepared by mixing appropriate volumes of cell suspension and gel solution to give an average cell concentration of 1.0-2.0 × 10^8 cell/ml and a gel concentration of 3.3% (W/V). The O_2 sensor was then inverted and the gel mixture added to the gel retainer at the tip of the sensor. The gel hardened within one minute, giving a mean biolayer thickness of about 1.02 mm. It is absolutely essential to consistently construct the biolayer with the same thickness and cell concentration to obtain reproducible results.

Antitumor Drug Assay Procedure

Mechlorethamine HCl (Merck, Sharp and Dohme, West Point, PA) was dissolved in distilled H_2O and added to the assay medium to give the appropriate concentration. The assay medium for L1210 was RPMI 1640 with 5% fetal bovine serum (FBS) and M199 with 2% fetal calf serum (FCS) for HFF. With these serum concentrations, the medium is not nutrient rich enough to permit cell growth within the experimental period. The assay medium was maintained at 37°C with a temperature controlled water bath. The cellular electrode was immersed into an O_2 saturated assay solution. Saturation was maintained using a magnetic stirrer and air sparging. The current output of the biosensor was monitored continuously with a strip chart recorder. Figure 2 is a schematic diagram of the entire experimental biosensor apparatus. The gel-cell layer was replaced after each experimental trial because most assay procedures are cytotoxic in nature.

Figure 2. Schematic of biosensor experimental apparatus. Temperature controlled H_2O circulated through jacketed glassware. 1) Testing vessel. 2) Computer. 3) Magnetic stirrer. 4) Water saturator. 5) Air filter. 6) Chart recorder. 7) Biosensor.



Biosensor Dynamic Response

The following procedure was used to determine the dynamic response of a biosensor containing L1210 to a step change in O_2 concentration. The biosensor was first placed in O_2 free assay medium for approximately 15 minutes. The probe was then immediately placed in a rapidly stirring beaker of O_2 saturated medium. The dynamic response of the live cell sensor was recorded with a chart recorder or a computer. These data were used to determine the respiration rate of the immobilized cells. In a separate experiment, the response to a step change in O_2 concentration using immobilized dead cells was similarly recorded to determine the D.O. probe instrument constants and the diffusivity of O_2 in the bio-layer.

Results and Discussion

Biosensor Response to Mechlorethamine HCl

Figure 3 shows the time courses of the tumor cell L1210 biosensor to three different mechlorethamine HCl concentrations. Mechlorethamine HCl, an alkylating agent, causes breaks in replicating DNA strands [2]. In addition, it is known to have non-specific toxic effects on the tumor cell. The cellular electrode was placed into the nongrowth medium and allowed to reach steady state. At time zero the antitumor drug was added and a gradual rise in current was observed. A control experiment without drug addition was also carried out. The biosensor current output of the control remained constant for at least 76 hours.

Several trends can be seen from Figure 3. The lag time before the onset of current rise decreases as concentration increases. A correlation between drug concentration and time necessary to reach 18.2 μA, the halfway point between initial and final steady state current values, is shown in Figure 4. This plot can be used as a calibration curve to approximate

Figure 3. Biosensor response to mechlorethamine HCl.

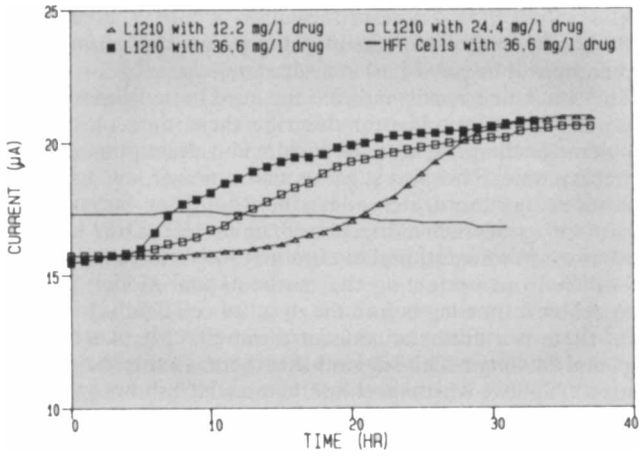
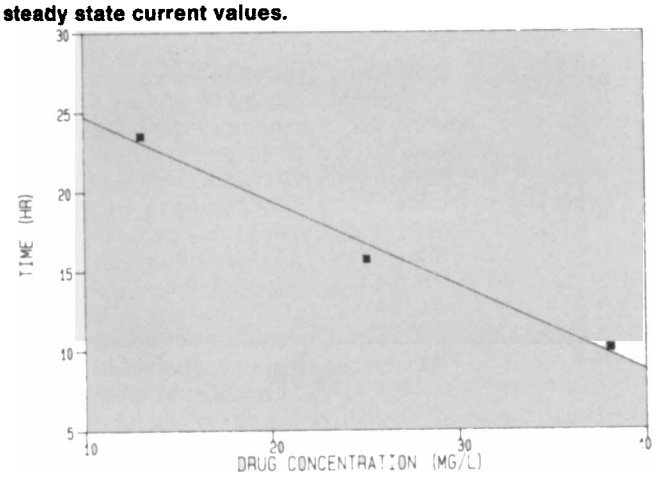


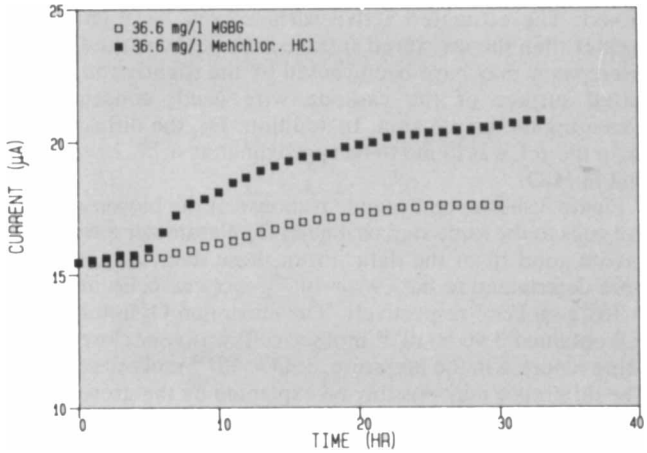
Figure 4. Mechlorethamine HCl calibration curve. Drug concentration was plotted against the time necessary for the biosensor to reach the halfway point between initial and final steady state current values.



drug concentrations. This type of correlation is similar to others found in the literature [6], [1]. The biosensor containing HFF was not sensitive to the drug agent and did not exhibit a current increase. Therefore, this method of drug detection shows Mechlorethamine HCl is selective towards L1210 tumor cells.

In a separate trial, Methylglyoxal-bis guanylhydrazone (MGBG) was added to the assay medium. There is evidence that MGBG causes the swelling of cellular mitochondria and consequently, a dysfunction in O_2 respiration [8]. A comparison of mechlorethamine HCl and MGBG is shown in Figure 5. This figure shows the differences in the dynamic responses and steady state values of these two drugs. Notice that the shapes of the two curves and the final steady state current values are different. In order to understand these variations, it is important to know the various modes of action of these drugs.

Figure 5. Comparison of mechlorethamine HCl and MGBG effects on the biosensor with L1210.

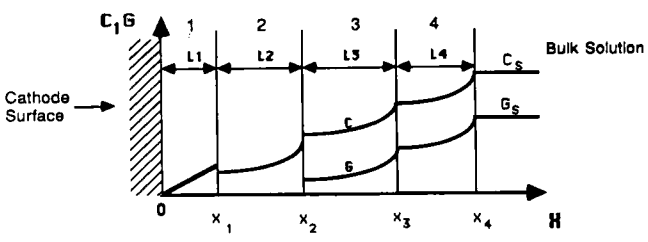


Development of Biosensor Model

To further understand what parameters govern biosensor performance, a mathematical model was developed to simulate biosensor response. A four layer model was used (Figure 6). The basis of the model is four material balances. These equations and the initial and boundary conditions are displayed in the Mathematical Model. Equation 1 is an oxygen balance in the teflon layer of the D.O. probe. Equation 2 is the oxygen balance in the gel-cell layer. The oxygen uptake rate of the cells is assumed to follow Monod growth kinetics. Equation 9 is a material balance of the antitumor drug. A simple power law assumption was used for the consumption of the drug. A power law assumption for cell death rate was also used in the viable cell balance (Equation 16). The equations were solved using the finite difference method. All parameters not experimentally determined were obtained from the literature.

Solution of the model began with the determination of A, the active surface area of the cathode and D_3, the diffusivity of O_2 in the gel layer. These parameters were determined by using the response data of the biosensor containing dead cells

Figure 6. Schematic diagram of the four layer model.



1. electrolyte layer
2. teflon membrane
3. gel-cell layer
4. mass transfer boundary layer

to a step increase in O_2 concentration. Figure 7 shows the experimental data and the computer simulation results. Through parameter estimation, a good fit of the data was obtained. The estimated active surface area, 3.19 cm^2, was greater than the measured surface area of the cathode. The discrepancy may have been caused by the slightly rough and pitted surface of the cathode wire used, consequently increasing its surface area. In addition, D_3, the diffusivity of O_2 in the gel, was found to be approximately 6.7% lower than that in H_2O.

Figure 8 shows the dynamic response of the biosensor with live cells to the same step change in O_2. Parameter estimation gave a good fit of the data. From these data, μ_{max} and k_m were determined to be 3.96×10^{-18}g-mol cm^3/cell/s and 1.47×10^{-5}g-mol/cm^3 respectively. The maximum O_2 uptake rate/cell obtained 3.96×10^{-18} mol/sec/cell, was very close to the value reported in the literature, 3.33×10^{-18} mol/sec/cell [*10*]. The difference may possibly be explained by the growth condition in which the uptake rate was measured.

Figure 9 shows the L1210 biosensor response to 36.6 mg/l of mechlorethamine HCl. The solid line is the simulation result. The best fit was obtained using: $k_s = 2.78 \times 10^{13}$, $k_d = 0.396$, and $n = 2$. The details of the parameter estimation technique will be published in a separate paper [*6*].

The simulation results indicate the need to develop a more compatible model to better describe these data. A major problem for the present is the insufficient description of the initial lag time. This also suggests that a power law assumption does not accurately describe the drug kinetics of antitumor agents such as mechlorethamine HCl. Drug kinetic studies using conventional *in vitro* methods shown in Figure 10 will help in perfecting the mathematical model. Many drugs show a time lag before the onset of cell death. In addition, there is a dramatic concentration effect between 2.44 mg/l and 24.4 mg/l. This suggests that there is a threshold concentration above which mechlorethamine HCl shows extreme toxic effects.

Figure 7. Cellular electrode dynamic response with dead cells.

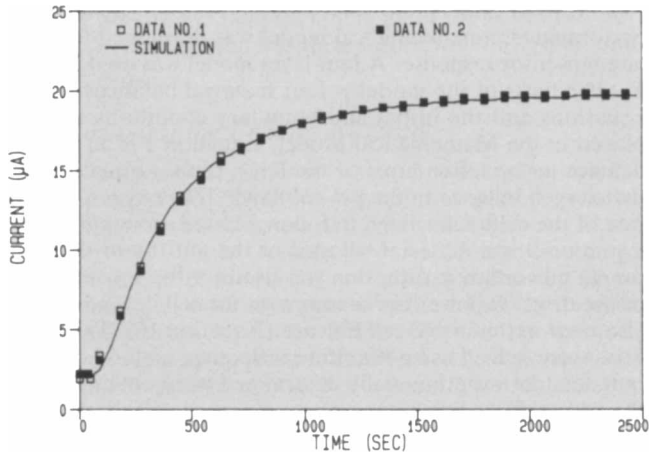


Figure 8. Cellular electrode dynamic response with live cells.

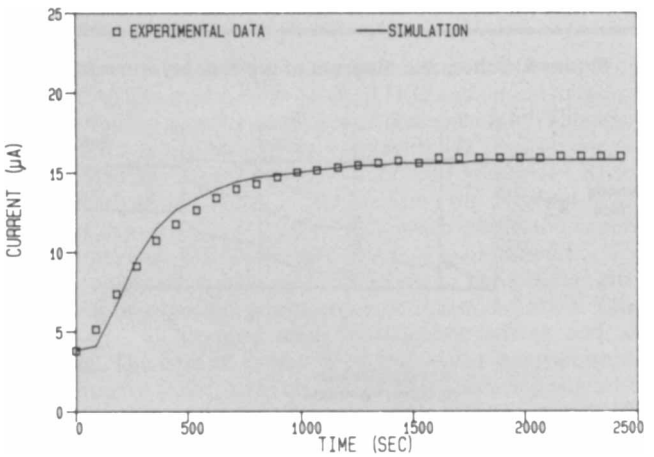


Figure 9. Simulation results of biosensor to 36.6 mg/l mechlorethamine HCl.

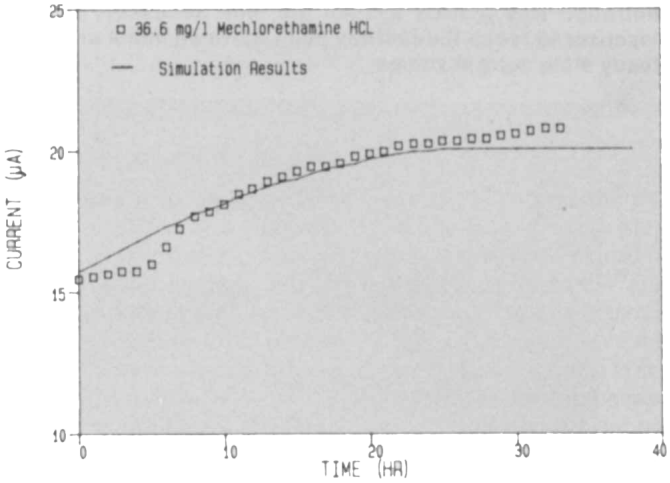
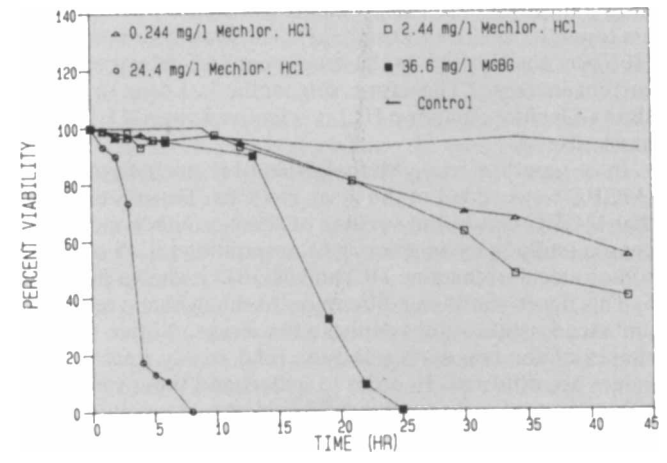


Figure 10. Drug kinetic studies of mechlorethamine HCl and MGBG on L1210.



Development of this model will help to optimize biosensor performance. By defining the parameters most important in biosensor response (biolayer thickness, cell concentration, electrode surface area, etc.) the model becomes an important tool in improving the performance of the biosensor. Under the current configuration, the biosensor does not show marked advantages in sensitivity over conventional *in vitro* screening methods. but, this biosensor method is less tedious and labor intensive to carry out. In addition, the sensor can be used as an important tool in studying drug-cell interactions. The sensor gives the researcher the ability to continuously monitor the physiological aspect of a living cell; something that cannot be done using conventional techniques. Also, by observing the dynamic response of the sensor to various drugs one can compare the similarities and differences of various drugs based on the dynamic behavior of the biosensor current output. This introduces a whole new concept for studying drug-cell interactions.

Acknowledgements

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Notation

A active surface area of cathode, cm^2
C oxygen concentration, $gmol\text{-}cm^{-3}$
D diffusivity of oxygen, $cm\text{-}sec^{-1}$
D_t diffusivity of antitumor drug, $cm^2\text{-}sec^{-1}$
F Faraday constant, 9.65×10^4 $coul\text{-}gmol^{-1}$
G antitumor drug concentration, $gmol\text{-}cm^{-3}$
i current output, A
k_d deactivation constant, $gmol^{1-n}\text{-}cm^{3n}\text{-}sec^{-1}$ $cell^{-1}$
k_s specific kill rate, $cm^{3n}\text{-}sec^{-1}\text{-}gmol^{-n}$
k_m respiration constant, $gmol\text{-}cm^{-3}$
L thickness, cm
M number of parameters
N viable cell concentration, $cell\text{-}cm^{-3}$
n order of reaction
S_i solubility of oxygen in ith layer, $gmol\text{-}cm^{-3}\text{-}cmHg$
t time, sec
x space coordinate, cm
μ_{max} respiration rate constant, $gmol\text{-}sec^{-1}\text{-}cell^{-1}$

Subscripts

i for ith layer, 1 = electrolyte, 2 = membrane, 3 = gel-cell layer, 4 = boundary layer.
s bulk solution
t antitumor drug

Mathematical Model

Oxygen balance equations:

$$\frac{\partial C_i}{\partial t} = D_i \frac{\partial^2 C_i}{\partial x^2} \text{ for } i = 1, 2, 4 \quad (1)$$

$$\frac{\partial C_3}{\partial t} = D_3 \frac{\partial^2 C_3}{\partial x^2} - \frac{\mu_{max} C_3}{k_m + C_3} N, \; x_2 \le x \le x_3 \quad (2)$$

B.C.:

$$C_1 = 0, \text{ at } x = 0 \quad (3)$$

$$C_i/S_i = C_{i+1}/S_{i+1}, \text{ at } x = x_i \quad (4)$$

$$D_i \frac{\partial C_i}{\partial x} = D_{i+1} \frac{\partial C_{i+1}}{\partial x}, \text{ at } x = x_i, \; i = 1, 2, 3 \quad (5)$$

$$C_4 = C_s, \text{ at } x = x_4 \quad (6)$$

I.C.:

$$C_i = 0, \; i = 1, 2, 3, \text{ at } t = 0 \quad (7)$$

$$C_4 = C_s \text{ at } t = 0 \quad (8)$$

Antitumor agent balance equations:

$$\frac{\partial G_3}{\partial t} = D_{t,3} \frac{\partial^2 G_3}{\partial x^2} - K_d G_3^n N, \; x_2 \le x \le x_3 \quad (9)$$

$$\frac{\partial G_4}{\partial t} = D_{t,4} \frac{\partial^2 G_4}{\partial x^2}, \; x_3 \le x \le x_4 \quad (10)$$

B.C.:

$$D_{t,3} \frac{\partial G_3}{\partial x} = 0, \text{ at } x = x_2 \quad (11)$$

$$G_3 = G_4, \text{ at } x = x_3 \quad (12)$$

$$D_{t,3} \frac{\partial G_3}{\partial x} = D_{t,4} \frac{\partial G_4}{\partial x}, \text{ at } x = x_3 \quad (13)$$

$$G_4 = G_s, \text{ at } x = x_4 \quad (14)$$

I.C.:

$$G_i = 0, \; i = 3, 4, \text{ at } t = 0 \quad (15)$$

Viable cell balance equations:

$$\frac{dN}{dt} = -k_3 G_3^n N, \; x_2 \le x \le x_3 \quad (16)$$

I.C.:

$$N = N_0, \text{ at } t = 0 \quad (17)$$

Current Output

$$i = -4FAD_1 \left. \frac{\partial C_1}{\partial x} \right|_{x=0} = 0 \quad (18)$$

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