Heterogeneities in EGF receptor density at the cell surface can lead to concave up scatchard plot of EGF binding

Kapil Mayawala, Dionisios G. Vlachos, Jeremy S. Edwards*

Department of Chemical Engineering, University of Delaware, 150 Academy Street, Newark, DE 19716, USA

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Abstract The mechanism responsible for the concave up nature of the Scatchard plot of epidermal growth factor (EGF) binding on EGF receptor (EGFR) has been a controversial issue for more than a decade. Past efforts to mechanistically simulate the concave up nature of the Scatchard plot of EGF binding have shown that negative cooperativity in EGF binding on an EGFR dimer or inclusion of some external site or binding event can describe this behavior. However, herein we show that heterogeneity in the density of EGFR due to localization in certain regions of the plasma membrane, which has been experimentally reported, can also lead to concave up shape of the Scatchard plot of the EGF binding on EGFR.

Keywords: Equilibrium model; Scatchard plot; Epidermal growth factor receptor; Receptor heterogeneity; Plasma membrane; Computational cell biology

1. Introduction

The epidermal growth factor (EGF) receptor (EGFR) belongs to the family of receptor tyrosine kinases, also called as ErbB receptors. These receptors trigger a rich network of signaling pathways and regulate cell functions such as proliferation, differentiation and migration [1]. The ErbB receptors also play a key role in the genesis of several tumors including those of endometrial, breast, lung, prostate, colon, ovary, bladder, head and neck [1]. Since EGF binding represents the initial step for activating EGFR and various intracellular proteins, considerable work has been devoted to elucidating the mechanisms of ligand binding and dimerization.

The Scatchard method has been extensively used to analyze the experimental data of equilibrium EGF binding on EGFR [2–5]. In this method, the ratio of bound receptor (B) to free ligand (L) concentration is plotted as a function of bound ligand concentration, which results in a linear relation with a slope of $-K_a$ (association constant) and an x intercept as the total density of sites, R_T (in same units as B) [6]

$$\frac{B}{L} = -K_a B + K_a R_T. \quad (1)$$

However, in many situations, such as the EGF binding on EGFR, binding data plotted on a Scatchard plot exhibits nonlinear behavior with a concave up nature. In general, the curvature of the Scatchard plot has been attributed to differences in receptor affinity for the ligand and cooperative binding (see references in [3]). Concave up and concave down Scatchard plots have been related with negative and positive cooperativity in ligand binding, respectively (see references in [3]). The experimentally reported Scatchard plot of EGF binding on EGFR in intact cells exhibits a concave up shape [2,4,7,8]. The mechanism responsible for the concave up nature of the Scatchard plot has been a controversial issue. Scatchard plots generated by simulation of EGF–EGFR binding that take into account experimentally reported differences in receptor affinity (represented by K_5 and K_6 being greater than K_4 in Fig. 1), and EGF induced EGFR dimerization (represented by K_2 and K_3 being greater than K_1 in Fig. 1) show concave down curvature (see Fig. 2A, dotted line) [3–5].

Wofsy et al. [4] proposed that a necessary condition for the concave up Scatchard plot of EGF binding is that EGF binding to aggregated receptors should be negatively cooperative, i.e., ligand binding of a receptor to a dimer having one receptor already bound occurs with lower affinity than the first binding event (namely, $K_5 > K_6$ in Fig. 1). Using this negative cooperativity, Chamberlin et al. [9] and Wofsy and Goldstein [3] further showed that the receptor density is also an important aspect in determining the shape of the Scatchard plot. However, Holbrook et al. [10] later showed that a concave up Scatchard plot could be generated by considering the existence of unknown molecules that trap a small fraction of the receptors in the high-affinity state.

Recently, in a mathematical model based on structural studies, Klein et al. [5] suggested that binding of EGF to a receptor dimer is independent of whether the other EGF binding site in the dimer is occupied. This study concluded that a concave up Scatchard plot could only be obtained by including an extra binding event, in which occupied EGFR dimers bind to an unidentified external site. The external site could represent receptor interactions with coated-pit regions or any cellular components involved in receptor endocytosis and turnover like coated-pit regions.

In this work, we show that heterogeneity in the surface density of EGFR can lead to a concave up Scatchard plot without considering either negative cooperativity or inclusion of external sites. It is our hypothesis that the spatial organization of membrane EGFRs exerts influence on the intracellular signaling events by modulating EGF binding.

*Corresponding author. Fax: +1 302 831 1048.
E-mail address: edwards@che.udel.edu (J.S. Edwards).

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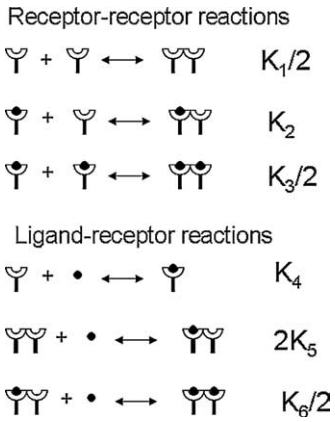

Fig. 1. Reactions considered in our model (see Appendices A and B).

2. Materials and methods

In this study, we considered EGF binding on EGFR in A-431 cells. These cells have $\sim 1.8 \times 10^6$ (N_T) receptors in a total area of $\sim 3000\ \mu m^2$ (A_T) [2,11]. The receptor–receptor and receptor–ligand reactions involved in EGF binding on EGFR are shown in Fig. 1 [4]. In our model, we considered two types of EGF binding sites on the plasma membrane: low affinity (EGFR monomer) sites and high affinity (EGFR dimer) sites. There is strong evidence based on biochemical (see review [12]) and microscopy [13,14] studies, which suggest that monomeric EGFR represents low affinity sites and dimerized EGFR exhibits high affinity sites.

Furthermore, we consider a heterogeneous density model consisting of low and high receptor density regions on the cell surface. There exists experimental evidence of spatial heterogeneity of EGFR. It has been proposed that localization of the EGFR occurs in certain regions in plasma membrane which can be lipid rafts and/or caveolae, or clathrin-coated pits after EGF binding (see reviews [12,15–17]). Therefore, in our model, we calculated the total bound ligand (B) as the sum of the number of bound ligand molecules in low receptor density (Low-D) and high receptor density (High-D) regions as described below.

Let N_T be the total number of receptors on the total cell surface area of A_T. Let f be the fraction of the receptors localized in a fraction g of the total area, which denotes the high receptor density (High-D) region of the plasma membrane, i.e.,

$$N_{\text{High-D}} = fN_T, \tag{2a}$$
$$N_{\text{Low-D}} = (1-f)N_T, \tag{2b}$$

$$A_{\text{High-D}} = gA_T, \tag{3a}$$
$$A_{\text{Low-D}} = (1-g)A_T. \tag{3b}$$

The concentration of total localized receptors (in molar units) in the high and low receptor density regions is calculated as

$$R_{T,\text{High-D}} = \left(\frac{0.55396}{0.5}\sqrt{\frac{N_{\text{High-D}}}{A_{\text{High-D}}}}\right)^3 \frac{10^{-3}}{6.023\times 10^{23}}, \tag{4a}$$
$$R_{T,\text{Low-D}} = \left(\frac{0.55396}{0.5}\sqrt{\frac{N_{\text{Low-D}}}{A_{\text{Low-D}}}}\right)^3 \frac{10^{-3}}{6.023\times 10^{23}}, \tag{4b}$$

where N is the number of receptors and A is the area in m^2 (see [5] for derivation). For a certain free ligand concentration (L) and localized receptors ($R_{T,\text{High-D}}$ and $R_{T,\text{Low-D}}$), bound ligand concentration in high and low receptor density regions ($B_{\text{High-D}}$ and $B_{\text{Low-D}}$) is calculated using the expression $RL + R_2L + 2R_2L_2$ as described in Appendix B (similar to [5]). The bound ligand concentration ($B_{\text{High-D}}$ and $B_{\text{Low-D}}$) in molar units is converted to number of bound receptors as (similar to Eq. (4))

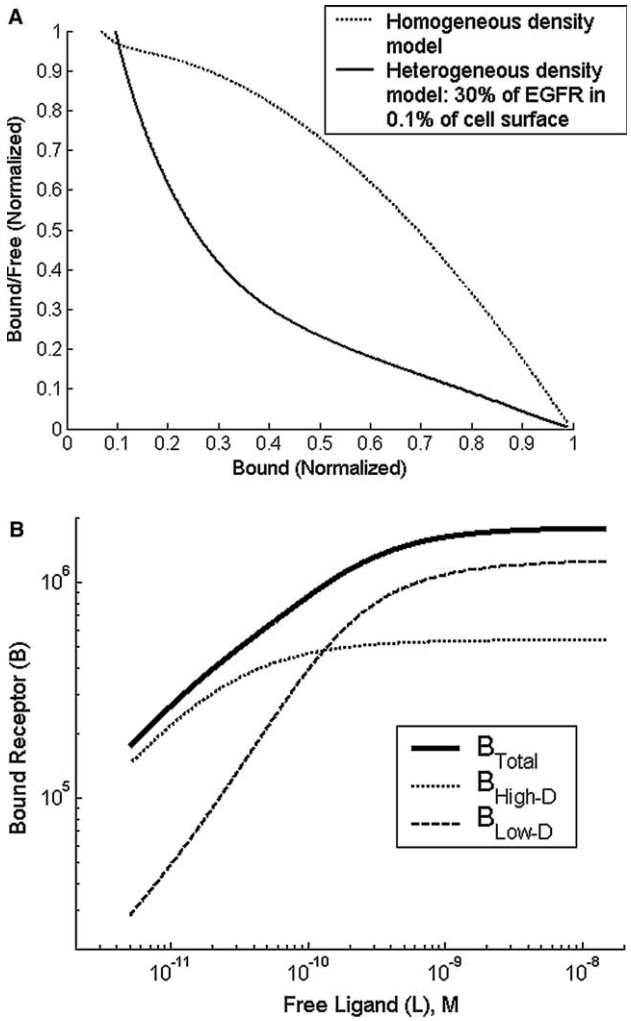

Fig. 2. (A) Scatchard plot of the EGF binding on the EGFR in A-431 cells with homogeneous ($f = g$ in Eqs. (2) and (3)) and heterogeneous surface density ($f = 0.3$ and $g = 0.001$ in Eqs. (2) and (3)) of receptors. (B) The three curves correspond to total binding (solid line), binding in high (dotted line) and low (dashed line) receptor density regions in heterogeneous receptor density model. The equilibrium parameters are: $K_1 = 100\ M^{-1}$ (in the range suggested by [18]); $K_2 = 2.5 \times 10^4\ M^{-1}$, $K_3 = 6.25 \times 10^6\ M^{-1}$ (calculated as suggested by [4]; see Appendix A); $K_4 = 4 \times 10^8\ M^{-1}$, $K_5 = K_6 = 1 \times 10^{11}\ M^{-1}$ (in the range suggested by [4,12,31,32]).

$$B_{\text{High-D}} = \left(\left(\frac{(B_{\text{High-D}} \text{ in Molar units}) \times 6.023\times 10^{23}}{1\times 10^{-3}}\right)^{1/3} \frac{0.5}{0.55369}\right)^2 \times A_{\text{High-D}}, \tag{5a}$$
$$B_{\text{Low-D}} = \left(\left(\frac{(B_{\text{Low-D}} \text{ in Molar units}) \times 6.023\times 10^{23}}{1\times 10^{-3}}\right)^{1/3} \frac{0.5}{0.55369}\right)^2 \times A_{\text{Low-D}}. \tag{5b}$$

Then, the total bound ligand in terms of number of molecules is calculated as

$$B = B_{\text{High-D}} + B_{\text{Low-D}}. \tag{6}$$

The bound ligand is calculated for varying concentrations of ligand (L) and the Scatchard plot is obtained by plotting B/L on the y-axis and B on the x-axis.

3. Results and discussion

The concentration of bound ligand in each (low and high receptor density) region is $RL + R_2L + 2R_2L_2$, where the amount of localized monomer (R) and dimerized (R_2) receptors depends nonlinearly on the local density of receptors and free ligand concentration (L) (see Appendix B). Hence, heterogeneities in receptor density can change the total amount of ligand binding.

The equilibrium parameters of the reactions of Fig. 1 have been reported in a wide range [9]. Therefore, we validated our heterogeneous receptor density model at multiple sets of equilibrium parameters to show the robustness of concave up Scatchard plot with respect to model parameters. Figs. 2A and 3 show concave up Scatchard plot with two different sets of equilibrium parameters.

Fig. 2A compares our heterogeneous receptor density model with a spatially homogeneous receptor density model in terms of the Scatchard plot. The heterogeneous receptor density model curve corresponds to localization of 30% of the receptors in 0.1% of the plasma membrane. As can be observed in Fig. 2A, heterogeneities in the receptor density of the surface receptors can strongly modulate the curvature of the Scatchard plot and give rise to the concave up shape of the Scatchard plot of the EGF binding. For the concave up curve in Fig. 2A, Fig. 2B shows that the upper part of the curvature (i.e., at low ligand concentration) is generated by the binding in high receptor density regions and the lower part (i.e., at high ligand concentration) by the binding in low receptor density regions of the cell surface.

We also compared the heterogeneous receptor density model with the experimental data of EGF binding on EGFR in A-431 cells. Fig. 3 shows the comparison of the heterogeneous receptor density model (nonlinear least square fit of f and g using Gauss–Newton method in MATLAB) with the experimental data of Zidovetzki et al. [2]. The comparison suggests localization of 14.14% of EGFR in 0.17% of the plasma membrane. Furthermore, Fig. 3 also shows the traditional two site model, which assumes presence of non-interacting high and low affinity sites. The two site model does not account for receptor–receptor reactions in the plasma membrane as shown in Fig. 1 [4,5,18].

Fig. 4 shows the locus of f (fraction of receptors in high density region) and g (fraction of plasma membrane area in high density region) values that yields a concave up Scatchard plot indicated in the light gray-shaded area. To generate this locus, the Scatchard plot was checked for concavity in the dominant range of experimentally reported data (by numerically calculating the second derivative at 10% intervals in a range where the bound receptors were 15–65% of the total receptors). A different set of equilibrium parameters changes the size of shaded area as shown by the darker dashed region in Fig. 4. However, the concave up Scatchard plot is preserved. Hence, a concave up shape of the Scatchard plot is exhibited by the heterogeneous receptor density model at multiple sets of experimentally reported equilibrium parameters.

The precise details on the cause, extent and nature of the EGFR surface heterogeneity remains a controversial issue [15,19]. However, several studies have suggested the accumulation of the EGFR in high cholesterol and sphingolipids regions in the plasma membrane like caveolae and/or lipid rafts [12,15,16,20–24] to different extents which could be as small as a few percent [24] to 60% [21], and in clathrin-coated pits after EGF binding (see review [15]). The heterogeneous density model can exhibit concave up shape in almost the entire range of suggested EGFR localization in caveolae and/or lipid rafts (Fig. 4). In most cell types the size of caveolae has been suggested to occupy less than 1% of the plasma membrane [25]. The size of lipid rafts in the plasma membrane is not precisely known [16,22,26,27]. Light microscopy studies like FRET do not show the presence of rafts (see reviews [22,26]) which has been interpreted as an indication that rafts are too small to be resolved by standard light

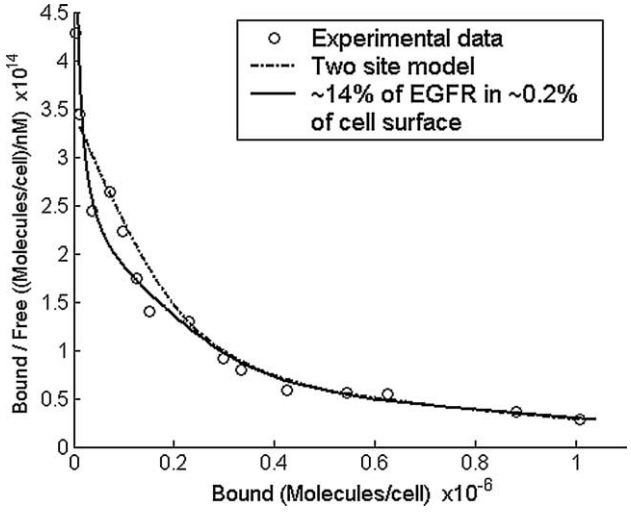

Fig. 3. Comparison of Scatchard plot from heterogeneous receptor density model ($f = 0.1414$ and $g = 0.0017$), two site model [2] and experimental data reported by Zidovetzki et al. [2]. The equilibrium parameters are: $K_1 = 2.19\ M^{-1}$ (in the range suggested by [18]); $K_2 = 1.02 \times 10^3\ M^{-1}$, $K_3 = 4.77 \times 10^5\ M^{-1}$ (calculated as suggested by [4]; see Appendix A); $K_4 = 6 \times 10^6\ M^{-1}$, $K_5 = K_6 = 2.8 \times 10^9\ M^{-1}$ (in the range suggested by [18]).

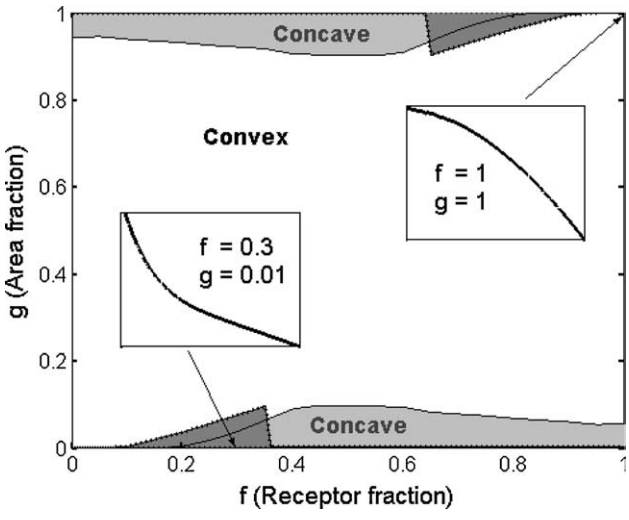

Fig. 4. Behavior of concave up shape with respect to model parameters. The gray-shaded area shows the locus of the points that give concave up shape corresponding to equilibrium parameters used in Fig. 2. The darker shaded region shows the corresponding domains of concave up shape corresponding to equilibrium parameters used in Fig. 3.

microscopy [22]. Furthermore, the size of clathrin-coated pits is suggested to be small (~2% of the cell's surface area [28]). In agreement with these suggestions of EGFR localization, our study indicates that the concave up Scatchard plot is consistent with a fraction of the EGFR being localized to small regions of the plasma membrane which may be caveolae, lipid rafts, or clathrin-coated pits (or a subfraction of them [29]) (Figs. 3 and 4).

Our model assumes a simplified representation of the receptor density heterogeneity by dividing the plasma membrane into two domains of different receptor densities. In general, there can be multiple domains with multiple receptor densities. Furthermore, the heterogeneity at the cell population level can also contribute to the overall heterogeneity in receptor density [30]. Our model framework can be generalized by considering distributions of f and g to account for these cases. As a last note, we should point out that use of the Scatchard plot to obtain all equilibrium constant and heterogeneity parameters has no unique solution. Knowledge of such distributions of f and g, for example using microscopy in combination with biochemical and biophysical methods, will help in mathematically generating the Scatchard plot and in the estimation of equilibrium parameters.

While the analysis was done for A-431 cells, the general approach of this study shows that heterogeneities in the surface receptor density can also be a potential factor controlling the shape of the Scatchard plot for other systems as well. This equilibrium analysis shows that heterogeneities in the surface EGFR density can have a strong influence on the amount of EGF binding. Hence, spatial effects due to the heterogeneities in the surface receptor density can influence the downstream intracellular signal and should be studied in greater detail experimentally as well as computationally.

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Appendix A. Relation among equilibrium constants

Linear dependence of reactions imposes the following constraints on the equilibrium constants [3,4]

$$K_2 = \frac{K_1 K_5}{K_4}$$
$$K_3 = \frac{K_1 K_5 K_6}{(K_4)^2} \tag{A1}$$

Appendix B. Equilibrium relations for generating the Scatchard plot

$$Ka_1 = K_1/2$$
$$Ka_2 = K_2$$
$$Ka_3 = K_3/2$$
$$Ka_4 = K_4$$
$$Ka_5 = 2K_5$$
$$Ka_6 = K_6/2 \tag{B1}$$

$$Ka_1 = \frac{R_2}{(R)(R)} \Rightarrow R_2 = Ka_1(R)^2 \tag{B2}$$

$$Ka_2 = \frac{R_2 L}{(R)(RL)} \tag{B3}$$

$$Ka_3 = \frac{R_2 L_2}{(RL)(RL)} \tag{B4}$$

$$Ka_4 = \frac{RL}{(R)(L)} \Rightarrow RL = Ka_4(R)(L) \tag{B5}$$

$$Ka_5 = \frac{R_2 L}{(R_2)(L)} \Rightarrow R_2 L = Ka_5(R_2)(L) = Ka_5 Ka_1 (R)^2 L \tag{B6}$$

$$Ka_6 = \frac{R_2 L_2}{(R_2 L)(L)} \Rightarrow R_2 L_2 = Ka_6(R_2 L)(L) = Ka_6 Ka_5 Ka_1 (R)^2 (L)^2 \tag{B7}$$

The following equations show a method for calculating the bound ligand concentration (B) given ligand concentration (L) and density of receptors (R_T).

The concentration of bound ligand:

$$B = RL + R_2 L + 2R_2 L_2 \tag{B8}$$
$$= Ka_4(R)(L) + Ka_5 Ka_1 (R)^2 (L) + 2Ka_6 Ka_5 Ka_1 (R)^2 (L)^2 \tag{B9}$$

Calculation of free monomer receptor concentration (R) using the balance on the total number of receptors reads (similar to [5]):

$$R_T = R + RL + 2R_2 + 2R_2 L + 2R_2 L_2$$
$$= R + Ka_4(R)(L) + 2Ka_1(R)^2 + 2Ka_5 Ka_1 (R)^2 (L) + 2Ka_6 Ka_5 Ka_1 (R)^2 (L)^2 \tag{B10}$$

This quadratic equation ((B10)) is solved for free monomer receptor concentration (R) as follows:

$$R = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \tag{B11}$$

where

$$a = 2Ka_1 + 2Ka_5 Ka_1 (L) + 2Ka_6 Ka_5 Ka_1 (L)^2,$$
$$b = 1 + Ka_4 L,$$
$$c = -R_T.$$

The free monomer receptor concentration (R) from Eq. (B11) is substituted in the equation for bound ligand (Eq. (B9)). The bound ligand concentration (B) is calculated from Eq. (B9) at varying concentrations of free ligand (L).

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