Stochastic Model of Clathrin-Coated Pit Assembly

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ABSTRACT In recent years, fluorescence microscopy has enabled researchers to observe the dynamics of clathrin-coated pit (CCP) assembly in real time. The assembly dynamics of CCPs shows striking heterogeneity. Some CCPs are long-lived (productive CCPs); they bind cargo and grow in size to form clathrin-coated vesicles. In contrast, other CCPs (abortive CCPs) are relatively short-lived and disassemble well before reaching vesicle size. Within both populations there is significant variance in CCP lifetime. We propose a stochastic biophysical model that links these observations with the energetics of CCPs and kinetics of their assembly. We show that without cargo, CCP assembly faces a high energy barrier that is difficult to overcome. As a consequence, CCPs without cargo are almost always abortive. We suggest a mechanism by which cargo binding stabilizes CCPs and facilitates their growth. The lifetime distribution of abortive pits calculated from our model agrees well with published experimental data. We also estimate the lifetimes of productive CCPs and show that the stochastic nature of CCP assembly plays a crucial role in causing their observed wide distribution.

INTRODUCTION

Clathrin-mediated endocytosis (CME) is a major pathway for the internalization of cargo at the plasma membrane of eukaryotic cells (1–7). Cargo includes hormones, nutrients, adhesion and signaling molecules, viruses, etc. The process involves the assembly of a coat of endocytic proteins on the cytoplasmic side of the membrane, which leads to the formation of a small invagination called a clathrin-coated pit (CCP). As the coat grows in size, the CCP invaginates further, until only a narrow neck joins it to the plasma membrane. Finally, the CCP is pinched off, yielding a clathrin-coated vesicle (CCV).

In recent years, fluorescence microscopy has enabled scientists to observe the dynamics of CCP assembly in real time (8–13). In cells expressing fluorescent clathrin or adaptor protein AP-2 (typically the two most abundant proteins in the coat), CCPs appear as fluorescent spots on the surface of the cell. The dynamic behavior of these spots shows considerable heterogeneity. There exist a large number of spots that are dim and blink out rapidly. The average lifetime of these short-lived clathrin structures is < 20 s (6), and their maximal fluorescence intensity indicates that they do not contain sufficient clathrin to produce a CCV. These structures are called abortive CCPs. In addition, there are bright spots that correspond to productive CCPs, i.e., CCPs that eventually end up as CCVs. The lifetimes of these productive CCPs range from 30 s to > 120 s (13), and depending on the size of the CCV, the amount of clathrin in its coat ranges from 60 to 140 clathrin molecules (14).

It is currently believed that cargo plays a crucial role in deciding the fate (abortive or productive) of a CCP (6).

Abortive CCPs represent nucleation events of the coat components, which start to assemble but then break apart because they do not associate with cargo (6,9). On the other hand, productive CCPs are the ones that associate with cargo. Cargo binding stabilizes the CCPs and facilitates their growth toward vesicle formation. This relationship between cargo capture and CCV formation is supported by the observation that CCPs that contain cargo rarely abort (9). In addition, the overexpression of transferrin receptor, which is internalized by CCPs, decreases the fraction of abortive CCPs (13).

The above observations raise several questions related to the heterogeneity in CCP dynamics, namely: 1), Why are the pits that fail to bind cargo abortive? 2), How can one explain the lifetime distribution of the abortive pits? 3), How does cargo binding stabilize a CCP and facilitate its growth toward vesicle formation? 4), Why is there such large spread in the lifetimes of productive CCPs? Recently, statistical studies on CCP lifetimes were carried out to quantify the heterogeneity (13,15) and address some of these questions, but the mechanistic origin of the heterogeneity still remains unclear.

The formation of a CCV is a complex process. It involves a large number of structural, regulatory, and accessory proteins. These proteins are recruited to the site of vesicle assembly in a sequential manner, each protein having its specific role and times of arrival and departure. In trying to understand such complex phenomena, simple coarse-grained models whose global behavior resembles that of the complex system are very useful. They often help in identifying the key parameters/ steps that govern the observed phenomena. There are not many such models related to endocytosis. Some of the existing ones (16–18) are significant contributions toward understanding vesicle formation, but they do not address the questions mentioned above.
In this article, we present a generic model of vesicle formation and use it to address questions related to the heterogeneity in CCP dynamics. We treat the protein coat as a uniform elastic sheet made up of basic structural units that we refer to as monomers. Our model includes a kinetic scheme for the assembly of monomers to form a pit, and an expression relating the energy of a pit to its size (measured in terms of the number of monomers it contains). With these ingredients we are able to map the dynamics of CCPs onto a one-dimensional continuous-time nearest-neighbor random walk with site-dependent rate constants. Our results show that pits without cargo face an energy barrier that is difficult for them to overcome. For this reason, they are almost always abortive. We calculate the lifetime distribution of such abortive pits via kinetic Monte Carlo simulation and show that the distribution agrees well with experimental data. We then suggest an explanation of how cargo stabilizes a CCP and facilitates its growth. Finally, we estimate the lifetimes of productive CCPs and show that the stochastic nature of CCP assembly is an important cause of their wide distribution.

METHODS

In this section, we present our stochastic model of CCP assembly. As mentioned earlier, the process involves a large number of proteins. Therefore, when developing a theory of assembly, one faces the necessity to deal with multidimensional models. By introducing the concept of monomers, we are able to avoid the multidimensionality and treat the assembly process as a one-dimensional random walk. We assume that the number of monomers is equal to the number of clathrin molecules, and we envision that the monomers include endocytic proteins in such a way that, when assembled, they capture the structural properties of the real protein coat. In the manuscript, we constantly move back and forth between our model (which deals with monomer assembly) and reality (which deals with clathrin-coated pit formation). To make this distinction clear, we shall use the term “CCP” to refer to a real clathrin-coated pit, whereas the word “pit” will refer to our model of a CCP.

Energy of pit formation

We assume that the shape of a pit is a spherical cap (17–19) that can be described by two parameters: surface area, A, and curvature, \( \kappa \) (see the Supporting Material). The surface area of the pit can be related to the number of monomers in the pit, \( n \), by \( A = \lambda n \), where \( \lambda \) is the average area occupied by a monomer. In the following, \( n \) is considered to be a continuous variable. Although this assumption is reasonable at large \( n \), it may fail when \( n \) is small, in which case the discrete structure of the protein coat might have to be taken into account. The energy difference between the components of a pit in the free and assembled states can be written as

\[
\bar{E}(n, c) = 2\kappa_m \lambda n c^2 + 2\kappa_p \lambda n (c - c_p)^2 - b n + \sigma f(n, c),
\]

where

\[
f(n, c) = \frac{2\pi}{d} \sqrt{\frac{\lambda n}{\pi}} \sqrt{1 - \frac{\lambda n c^2}{4\pi}}.
\]

The origins of the terms in Eq. 1 are described below, and the parameter values are discussed in the Supporting Material. The values of all the quantities having units of energy are expressed in \( kT \), where \( k \) is the Boltzmann constant and \( T \) is the absolute temperature.

1. The first term on the right-hand side of Eq. 1 is the Helfrich energy, describing the energetic cost of bending the cell membrane (20). \( \kappa_m \) is the bending rigidity of the membrane, measured in units of energy. We assume that the spontaneous curvature of the membrane is zero.
2. The second term represents the bending energy of the protein coat. We assume that this term can also be written in the Helfrich-like form, \( \kappa_p \) and \( c_p \) are the bending rigidity and the spontaneous curvature of the coat, respectively.
3. The third term represents the binding energy. The binding-energy contribution comes from the protein-protein and protein-lipid bonds formed in the CCP. Therefore, we assume that this term is proportional to the number of monomers, \( n \), and that \( b \) is the corresponding proportionality constant measured in units of energy. This assumption implies that all the monomers of the pit are identical and in a similar environment. However, the monomers on the periphery form fewer bonds than those located deeper inside the pit. Thus the third term overestimates the binding energy. This overestimation is accounted for by the next term.
4. The fourth term is a correction to the overestimated binding energy. The correction term is proportional to \( f(n, c) \), the number of available binding sites on the periphery of a pit of size \( n \), and is similar to the line-tension energy (19,21). We determine the number of monomers at the periphery by calculating the perimeter and dividing it by \( d \), the average span of a monomer (see the Supporting Material). \( \sigma \) is a constant, measured in units of energy.

The third term is the only term that is negative, and contributes towards lowering the energy of the pit. All the other terms are positive, thereby increasing the pit energy. A similar approach has been used to analyze the energetics of clathrin basket assembly (22).

Energy variation with pit size

In Eq. 1, the pit energy is expressed as a function of two variables, \( n \) and \( c \). We assume that for a given pit size, \( n \), the optimal curvature value, \( \tau \), is the one for which the total energy of the system is minimized, i.e., \( \frac{\partial E(n, c)}{\partial c} = 0 \). Solving the equation, we get

\[
\left[ 1 + \frac{\kappa_m}{\kappa_p} - \frac{\sigma}{2b\kappa_p} \sqrt{\frac{\lambda n}{4\pi - \lambda n c^2}} \right] = c_p.
\]

It is reasonable to assume that \( \kappa_p \gg \sigma \) (see the Supporting Material), in which case the last term in the square brackets can be neglected for almost all values of \( n \) (as discussed at the end of this section). Then the curvature \( \tau = \kappa_p c_p/(\kappa_m + \kappa_p) \) is independent of \( n \). It is not known whether individual CCPs grow with constant curvature during CME. However, large changes in the curvature would require significant rearrangement of the clathrin lattice, which is quite unlikely (6). Therefore, the constant curvature approximation seems reasonable.

Upon setting the curvature of the pit equal to \( \tau \) for all values of \( n \), the energy of pit formation, \( E(n) = E(n, \tau) \), can be written as

\[
E(n) = \left[ 2\kappa_m \lambda \tau^2 + 2\kappa_p \tau (c - c_p)^2 - b \right] n + \sigma f(n, \tau) = \Phi n + \Gamma \sqrt{n(N - n)},
\]

where

\[
\Phi = 2\kappa_m \lambda \tau^2 + 2\kappa_p \tau (c - c_p)^2 - b, \quad \Gamma = \frac{\sigma}{2b\kappa_p} \sqrt{\frac{\lambda}{4\pi - \lambda \tau^2}}.
\]
\[
\Phi = 2\kappa_m \lambda c^2 + 2\kappa_p \lambda (c - c_0)^2 - b, \quad \Gamma = \frac{\lambda \sigma c}{d}, \quad N = \frac{4\pi}{\lambda c^3}.
\]

A similar functional form of energy also appears in theories describing the budding of lipid bilayers induced by intramembrane domains (19) and the formation of virus capsids (23).

The energy, \(E(n)\), plays a crucial role in our analysis, particularly in determining the lifetime distribution of abortive pits. In Eq. 5, the first term is the sum of the membrane-bending, coat-bending, and binding energies. The second term represents the edge energy. The inset in Fig. 1 shows a typical plot of the two terms, and the main panel shows the total energy for the parameter values given in the figure caption. The total energy has a maximum at \(n = n_*\), which can be considered a critical pit size; for pits of size \(n < n_*\), disassembly is more favorable than assembly, whereas for pits with \(n > n_*\), assembly is more favorable. As discussed later, in the absence of cargo, the values of \(E^* = E(n_*)\) and \(n_*\) are large (see Fig. 1), whereas in the presence of cargo, the energy is modified and the values of \(E^*\) and \(n_*\) become much smaller.

Using the definition of \(N\) from Eq. 6, it is easy to see that the last term in the square brackets in Eq. 5 is proportional to \(\sqrt{n}/(N - n)\). Due to the small prefactor, \(\sigma^2/(2d_k n) = 1/250\), the value of this term is \(<1\) even when \(n = N - 1\). Thus, our assumption is valid for all values of \(n < N\).

**Kinetics of pit formation**

We assume that a pit grows by reversible binding of monomers at the edge of the pit. Fig. 2 shows the kinetic scheme for pit assembly. The scheme maps the dynamics of CCPs onto a one-dimensional nearest-neighbor continuous-time random walk with site-dependent rate constants. We presume that once a pit reaches vesicle size, \(N\), it is pinched off, but we ignore the details of the kinetics occurring during the final steps of vesicle scission, which involve dynamin and other proteins. As mentioned earlier, the number of clathrin molecules in a CCP is typically between 60 and 140. With this in mind, in our model we keep the vesicle size fixed at \(N = 100\). Our results do not change significantly with small variations in the value of \(N\).

The forward rate constants \(a_m, m = 1, \ldots, N - 1\), characterize the growth process and are taken to be \(a_m = k_m f(m) = \gamma f(m)\). Here, \(\gamma\) is a constant, \(k_m\) is the bimolecular rate constant of a monomer binding to an available site on the pit, \(m\) is the free monomer concentration, and \(f(m) = f(n, \Gamma)\). The value of \(f(n)\), and therefore that of \(a_m\), is maximum when the pit is close to a hemispherical surface. The rate constant \(a_N\) characterizes the scission of a vesicle from the membrane. For simplicity, we assume that \(a_N = \infty\).

When the forward rate constants, \(a_m\), are known, one can use the condition of detailed balance to find the backward rate constants, \(b_{m+1}\). This leads to the relation \(b_{m+1} = \mu f(m)\exp(E(n + 1) - E(n)), m = 1, \ldots, N - 1\) (see the Supporting Material), where \(\mu\) is an unknown constant that we consider as a free parameter. Both \(\gamma\) and \(\mu\) have units of \(s^{-1}\).

We use the above scheme to calculate the fate and lifetimes of pits by kinetic Monte Carlo simulations (see the Supporting Material). In our simulations, a pit has two possible fates: either it eventually decays in size and falls below a detection threshold (abortive pits), or it grows and reaches vesicle size \(N\) (productive pits). The inputs required to run the simulations are the values of the forward and backward rate constants \(a_m\) and \(b_m\). The rate constants depend on \(\gamma\) and \(\mu\) (whose values are not known), and the energy function \(E(n)\). We determine \(\gamma\) and \(\mu\), as well as \(b\) and \(\sigma\) appearing in the expression for \(E(n)\), by using the data on CCP lifetimes published in Loerke et al. (13) (see the Supporting Material).

**RESULTS**

**Lifetime distribution of abortive pits**

To obtain a good fit to the experimental data, we choose \(\gamma = 0.18 \text{ s}^{-1}\) and treat \(\mu, b,\) and \(\sigma\) as free parameters. As discussed later, the value of \(\gamma\) is fixed by the rate of growth of productive CCPs. For different combinations of \(\mu, b,\) and \(\sigma\), we determine the fates and lifetimes for an ensemble of pits, and then compare the lifetime distribution of abortive pits with the experimental data. Since we are fitting data on abortive CCPs, in our simulations we impose the constraint that the fraction of pits having an abortive fate should be close to unity.

We find that for \(\mu = 0.16 \text{ s}^{-1}\) and \(E(n)\), shown in Fig. 1, 1), the fraction of productive pits is approximately 1/1000, and 2), the lifetime distribution of abortive pits agrees very well with experiment (see Fig. 3). From our simulations, we find the mean lifetime of abortive pits to be 12 s, which is close to the mean lifetime of abortive CCPs calculated from the data in Loerke et al. (13) (approximately 9 s). Notice that at very small lifetimes the experimental data are nonmonotonic, whereas the model predicts monotonic behavior for the lifetime distribution. We believe that this discrepancy is due to the fact that our model treats size of pit as a continuous variable, which, strictly speaking, is valid only when the pits are sufficiently large. CCPs with short lifetimes correspond to CCPs that grow only to small sizes, and a more detailed model might be required to capture their dynamics accurately.

The inferred \(E(n)\) has a maximum, \(E^* \approx 14kT\) at a critical size \(n^* \approx 68\) monomers (see Fig. 1). By making small perturbations to \(E(n)\), we find that as long as \(E^*\) is between 12–15\(kT\) and \(n^*\) is between 65 and 75 monomers, we can fit the data reasonably well. Notice that these values were
calculated for \( N = 100 \). We repeated the same analysis for \( N = 80 \) and 120 and found \( E^* = 13kT \) and 15\( kT \), and \( n^* \approx 55 \) and 79, respectively. Thus, with increasing \( N \), the critical size increases, but the energy barrier remains approximately the same.

Along with the lifetime of an abortive pit, we can also determine its maximum size, which corresponds to the largest number of monomers the pit has during its lifetime. This quantity is similar to the size of an abortive CCP measured in terms of the number of clathrin molecules. From our simulations, we find that the average maximum size of the abortive pits is approximately 10 monomers, which is in agreement with the estimates reported in Ehrlich et al. (9).

### Cargo binding promotes vesicle formation

In the previous section, we discussed the \( E(n) \) for abortive pits. We assume that they represent abortive CCPs, i.e., CCPs without cargo. Experiments suggest that cargo binding can change the coat parameter values of a CCP, which in turn would modify \( E(n) \).

Adaptor proteins (like AP-2) are crucial for CCP assembly (24). They bind to the cargo proteins and also act as links between membrane and clathrin molecules, which form a lattice around the CCP (14). In the presence of cargo, the affinity of AP-2 for plasma membrane, as measured by the dissociation constant, changes from \( \mu M \) to nM (25). That is, the association energy between AP-2 and the membrane increases. Stably bound AP-2 can facilitate the formation of a clathrin lattice through an increase in the effective binding strength between triskelion legs (22). Based on these observations, we assume that cargo binding to a pit increases the effective binding energy, \( b \). The energy function, \( E(n) \), can be characterized by the critical size, \( n^* \), and energy barrier, \( E^* \). We now explore how these quantities depend on \( b \).

The dependence of \( n^* \) and \( E^* \) on the coat parameter values (see Eqs. 4–6) can be determined using \( \partial E/\partial n \) at \( n = n^* \) and \( E(n^*) = E^* \). This leads to

\[
n^* = \frac{N}{2} \left( 1 + \frac{Z}{\sqrt{1 + Z^2}} \right), \quad E^* = \frac{N}{2} \left( \sqrt{1 + Z^2} + Z \right),
\]

(7)

where \( Z = \Phi/\Gamma = [2\kappa_\alpha \lambda z^2 + 2\kappa_\rho (\tau - c_\rho)^2 - b]/[|\lambda \sigma |/d] \). Fig. 4 shows plots of \( E^* \) and \( n^* \) as a function of \( b \). The arrows point to the values at which the system operates in the absence of cargo, namely, \( b = 5.5kT \), \( n^* \approx 68 \), and \( E^* \approx 14kT \). Note that a small increase in the value of \( b \) (of the order of \( kT \)) lowers \( n^* \) and \( E^* \) to very small values (see also Fig. 5). This observation suggests that the endocytic machinery operates at the borderline of stability and instability. In the absence of cargo, the energy barrier is sufficiently high so that the pits are unstable and disassembly is favored. However, a small increase in the binding energy can significantly change the energy landscape and make assembly favorable.

### Lifetimes of productive CCPs

We now discuss how the stochastic nature of CCP assembly manifests itself in lifetimes of productive CCPs. To address this question, we again make use of kinetic Monte Carlo simulations. We consider the starting situation where a pit of size \( n = 10 \) contains cargo and then calculate the time it takes for an ensemble of such pits to reach vesicle size \( N = 100 \). The presence of cargo is represented by choosing \( b = 9kT \). We deliberately choose \( b \) large enough so that \( E(n) \) decreases rapidly with \( n \). Then the backward rate constants, \( \beta_n \), become negligibly small, and the time to vesicle formation is determined only by the forward rates, \( \alpha_n \). A similar analysis can be done to

\[ \text{FIGURE 4} \quad \text{Energy barrier, } E^*, \text{ and critical size, } n^*, \text{ as a function of the binding-energy constant, } b. \quad \text{The arrows point to the operating point (values of } b, n^*, \text{ and } E^* \text{) in the absence of cargo. Note that a small increase in the value of } b \text{ results in sharp decreases in } n^* \text{ and } E^*. \]
study the lifetime distribution of vesicles having other values of \( N \).

Productive CCPs grow at an average rate of 1–2 clathrin molecules per second (9). So, in our simulations we introduce the constraint that the mean lifetime of productive pits should be approximately 70 s. The constraint is satisfied if we choose \( k = 0.18 \text{s}^{-1} \). The forward rate constants are independent of \( E(n) \), and we used the same value of \( k \) earlier in the calculation of lifetime distribution of abortive pits. Fig. 6 shows the histogram of the calculated lifetimes of productive pits. The lifetimes vary from 50 to 90 s. The large spread is the result of the stochastic nature of pit assembly. Note that in our model, the vesicle size is constant \( (N = 100) \), and that in this particular simulation, all the pits start at \( n = 10 \). However, during CME, the CCPs can grow to vesicles of different sizes, and also can incorporate cargo at different stages of maturation. Our assessment of the spread in lifetimes is therefore an underestimation. Nevertheless, it indicates that the stochastic nature of CCP assembly is an important factor contributing to the wide lifetime distribution of productive CCPs.

**DISCUSSION**

Over the years, the main method used to probe the mechanism of CCP assembly has been knock-down and knock-out experiments. In recent years, fluorescence microscopy combined with automated image analysis has appeared as a new tool. This approach is particularly useful, since it does not interfere with the assembly process significantly (unlike the knock-out and knock-down method) and thus provides information about a relatively unperturbed system. However, this approach is still in its infancy, and more accurate methods to determine the fate and lifetimes of CCPs need to be developed. An important feature that comes from these studies is the remarkable heterogeneity in CCP dynamics. CCPs have different fates, sizes, and lifetimes. Understanding the origin of the heterogeneity is crucial, as it can provide insights into the mechanism of the assembly process.

In this article, we have presented a stochastic biophysical model to address certain questions related to the fate and lifetimes of CCPs. Our aim was to keep the model as simple as possible while still being able to reproduce the key aspects of the assembly process. We focused on two basic features, namely, growth by reversible binding of free endocytic proteins, and competition between effects that favor and those that disfavor CCP formation. These characteristics are present not only during the assembly of a CCV, but also, e.g., in COP vesicle formation, caveolin-dependent endocytosis, and virus capsid assembly. Thus, our approach is fairly general and can be used as a starting point to develop other more detailed models.

The simple approach adopted here ignores some features of CCP assembly. For example, the clathrin lattice satisfies certain topological constraints, which in turn impose constraints on the curvature and the overall shape of a CCP. Our modeling of the protein coat as an elastic sheet does not account for this fact. Similarly, we accounted for cargo binding by changing the binding-energy parameter, \( b \), but cargo size and shape can lead to variations in CCP curvature as well. Currently, only limited experimental information is available on how these features affect CCP assembly.

The key points of our work are 1), the energetics of CCP formation plays a crucial role in determining the fate of a CCP, and 2), stochastic kinetics is an important factor responsible for the wide distribution of CCP lifetimes. Our results suggest that when there is no cargo in a CCP, the energy of the CCP increases with size and reaches a maximum value, \( E^* \), that is \( >10kT \), at a critical pit size, \( n^* \) (see Fig. 3). The chance that a CCP grows beyond the critical size by stochastic addition of proteins is almost zero, so CCPs without cargo are almost always abortive.

Incorporation of cargo increases the value of the effective binding energy, \( b \). Our model suggests that the endocytic machinery operates at a point where the critical size is
very sensitive to the values of \( b \), and that a small increase in \( b \) can move the CCP from a disassembly-favored state to an assembly-favored state. This point is illustrated in Fig. 5, where we show the energy profile for two values of \( b \), viz., \( b = 5.5kT \) (solid curve) and \( b = 6.5kT \) (dashed curve). We assume that the effect of cargo binding is to move a pit from one energy curve to another, as shown by the arrow. In the absence of cargo, a pit with \( n = 10 \) monomers is below the critical size, and its disassembly is favored. In the presence of cargo, the same pit is beyond the critical size, so its assembly is favored. The mechanism described above suggests that very few empty (without cargo) coated vesicles form, and that the pits with cargo end up as vesicles with high probability. Thus, in agreement with the experimental observations (9), the binding of cargo acts like a switch that determines whether a pit results in a vesicle or not.

Our model reproduces the qualitative trend of the lifetime distribution of abortive CCPs very well. When discussing the lifetime distribution of CCPs, Loerke and co-workers (13) divided the abortive CCPs into two kinetically distinct subpopulations (early and late abortive). Our model reproduces the full data set, suggesting that there is no fundamental difference between early and late abortive CCPs, i.e., the same stochastic dynamics gives rise to both.

To summarize, we have developed a model that links coated-pit formation to underlying physical processes. Despite being relatively simple, it captures essential features of CCP dynamics and provides insights that cannot be obtained through biochemical experiments alone. We believe that our model is a step forward toward better understanding the mechanistic basis of CME.

**SUPPORTING MATERIAL**

Two figures, the derivation of certain formulas appearing in the main text, Monte Carlo simulation algorithm, and references are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(12)00563-2.

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