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Kinetics of cellular uptake of viruses and nanoparticles via clathrin-mediated endocytosis

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Abstract

Several viruses exploit clathrin-mediated endocytosis to gain entry into host cells. This process is also used extensively in biomedical applications to deliver nanoparticles (NPs) to diseased cells. The internalization of these nano-objects is controlled by the assembly of a clathrin-containing protein coat on the cytoplasmic side of the plasma membrane, which drives the invagination of the membrane and the formation of a cargo-containing endocytic vesicle. Current theoretical models of receptor-mediated endocytosis of viruses and NPs do not explicitly take coat assembly into consideration. In this paper we study cellular uptake of viruses and NPs with a focus on coat assembly. We characterize the internalization process by the mean time between the binding of a particle to the membrane and its entry into the cell. Using a coarse-grained model which maps the stochastic dynamics of coat formation onto a one-dimensional random walk, we derive an analytical formula for this quantity. A study of the dependence of the mean internalization time on NP size shows that there is an upper bound above which this time becomes extremely large, and an optimal size at which it attains a minimum. Our estimates of these sizes compare well with experimental data. We also study the sensitivity of the obtained results on coat parameters to identify factors which significantly affect the internalization kinetics.

Keywords

nanoparticle; virus; clathrin-mediated endocytosis; random walk; vesicle formation

1. Introduction

Several biomedical applications, including targeted-drug delivery and diagnostic imaging, utilize clathrin-mediated endocytosis (CME) to deliver ligand-coated nanoparticles (NPs) to diseased tissues and cells (see review articles [1–3] and references therein). CME is also exploited by several viruses, including hepatitis C, influenza A, and dengue, to gain entry into host cells [4–8]. Understanding the details of how these nano-sized objects are taken up

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by cells via this internalization pathway is crucial for designing efficient NPs for biomedical purposes, as well as for developing strategies to inhibit cellular entry of viruses.

CME is one of the most important and best characterized forms of receptor-mediated endocytosis [9, 10]. Eukaryotic cells use this pathway to internalize nutrients, growth factors, transmembrane receptors, etc. Internalization of these molecules occurs through specialized regions of the plasma membrane called clathrin-coated pits (CCPs), which form by the assembly of a protein coat on the cytoplasmic side of the plasma membrane. The assembly of the coat—which contains clathrin, adaptors (AP-2), and several other accessory proteins—drives the bending of the membrane [11, 12], eventually leading to the formation of a cargo-containing clathrin-coated vesicle. The internalization of NPs/viruses (henceforth referred to as NPs unless stated otherwise) via CME involves two main steps. First, the formation of bonds between receptors on the cell surface and ligands on a NP anchors the particle to the plasma membrane. Second, the assembly of a CCP drives the formation of a vesicle containing the NP (see figure 1).

Several theoretical and computational models of receptor-mediated endocytosis of NPs, as well as passive wrapping of NPs by flexible membrane, have been proposed [13–18]. An assumption common to all these models is that the wrapping of the membrane around the NP is solely controlled by the formation of bonds between receptors on the membrane and ligands on the NPs. The models do not take clathrin coat assembly into explicit consideration. Since coat assembly plays a key role in CME of NPs, the above mentioned models are insufficient for understanding CME of these nano-objects.

To fill the gap, we here present a theoretical framework for studying CME of single NPs that focuses on protein coat assembly. We characterize the internalization process by the mean time between the binding of a NP to the membrane and its entry into the cell, and derive an analytical formula for this quantity. The formula contains two terms with a clear physical meaning: the first, τ_w , corresponds to the mean time that a NP spends bound to the membrane waiting for an ultimately-successful CCP assembly event (leading to the formation of a vesicle) to begin, and the second, τ_s , corresponds to the mean time required for a successful CCP assembly event, once begun, to go to completion. Our analysis of the sensitivity of these times to the parameters describing coat assembly shows that τ_w is much more sensitive to the parameter values than τ_s , and therefore could potentially be a target for improving NP design. We also study the dependence of the two times on the NP size to address the question: how the kinetics of CME of a NP is affected by the size of the particle.

2. General theory

2.1. Mean internalization time

As mentioned in section 1, we characterize the internalization process by the mean time between the binding of a NP to the membrane and its entry into the cell. We call this time the mean internalization time and denote it by τ . In order to calculate this time, like in previous models [13, 16], we assume that the binding of NP to the plasma membrane through the formation of a receptor–ligand bond is irreversible.

To derive a formula for τ , consider an ensemble of NP–receptor complexes formed on the cell membrane at time $t = 0$. Let τ_0 be the mean time required for the initiation of CCP assembly around a NP–receptor complex. CCP assembly is a reversible process which has two possible outcomes [19, 20]. One is that the assembly is successful, in which case the CCP grows in size to form a vesicle and the NP is internalized. The other possibility is that the assembly is unsuccessful, and the CCP grows only up to a certain size and then disassembles, in which case the NP fails to be internalized.

Let P_s and $P_f = 1 - P_s$ be the probabilities that a CCP assembly process is successful or failed, respectively, and τ_s and τ_f be the mean durations of these events. Then the mean internalization time can be written as

$$\tau = P_s(\tau_0 + \tau_s) + P_f P_s(2\tau_0 + \tau_f + \tau_s) + P_f^2 P_s(3\tau_0 + 2\tau_f + \tau_s) + \dots \quad (1)$$

The first term on the right-hand side of the above equation is the contribution from the fraction (P_s) of complexes which bind to coat proteins and are internalized on the first attempt, i.e., without experiencing complete disassembly. The second term is the contribution from the fraction ($P_f P_s$) of complexes that are internalized on the second attempt, i.e., they first bind to coat proteins but fail to get internalized and then bind for the second time and are internalized. Subsequent terms can be understood in the same way.

Upon summing the series in equation (1), we find that the internalization time can be written as the sum of two terms

$$\tau = \tau_w + \tau_s. \quad (2)$$

Here τ_w is the mean waiting time defined as the mean duration between the initial attachment of a NP to a receptor on the cell membrane and the commencement of a successful CCP assembly event. This time is given by

$$\tau_w = (\tau_0 + P_f \tau_f) / P_s. \quad (3)$$

In the above equation, τ_0/P_s , is the mean cumulative time that a NP–receptor complex spends when disconnected from coat components, and $P_f \tau_f/P_s$ is the mean cumulative time a complex spends bound to CCPs that fail to grow into a complete vesicle.

In experiments studying CME of individual viral particles using TIRF microscopy, the times between viral binding and the onset of CCP formation, as well as the time between the onset of CCP formation and clathrin-coated vesicle uncoating, are measured [5–8]. These times are closely related to the times τ_w and τ_s described above. It is also worth mentioning that τ is identical to the ‘wrapping time’ calculated in previous models [13, 16, 17]. However, the wrapping mechanism based on receptor–ligand bonding considered in these models is

completely different from the coat assembly based mechanism considered in the present paper.

2.2. Coarse grained model

The formula in equation (1), giving the mean internalization time as a sum of two times, τ_w and τ_s , is simple and intuitively appealing. But the calculation of τ_w and τ_s in the case of CME is nontrivial. To obtain these quantities we adapt the approach developed in [26] and [27] to study the lifetime distribution of events corresponding to unsuccessful CCP assembly. Such CCPs typically contain no cargo and are called abortive [19, 20]. Here we briefly describe the approach and list the underlying assumptions.

The coat that forms during CCP assembly is made up of several proteins and has a complex structure. Clathrin (in the form of a three-legged, triskelion-shaped complex) is the most abundant protein in the coat. Clathrin triskelions polymerize to form a polyhedral scaffold which is linked to the membrane through adaptor proteins (typically AP-2 at the plasma membrane). The scaffold stabilizes membrane curvature, and its growth is governed by the topological constraints imposed by the intrinsic shape of a triskelion. Other proteins like epsin and amphiphysin bind to the cell membrane and impart local curvature. In addition to the structural and geometrical complexity, the temporal order in which these proteins arrive at the site of CCP assembly also plays an important role.

Modeling the spatiotemporal dynamics of such a multicomponent system is an extremely difficult task. Nevertheless, by using a coarse-grained approach different phenomena related to vesicle formation have been studied. These include the dynamical coupling between coat assembly and membrane shape change during CME in yeast [21], the dynamics of COP vesicle formation in the Golgi [22], the effects of actin forces and proteins containing Bin-Amphiphysin-Rvs (BAR) domains on endocytosis in yeast [23], and the rearrangement of clathrin-shaped polyhedral elements during vesicle budding from flat lattices [24, 25]. However, these models do not focus on the stochastic nature of the coat assembly process, and therefore cannot not be readily used to calculate our quantity of interest—the mean internalization time, τ .

We previously developed a coarse-grained description of the assembly dynamics to analyze the lifetime distribution of abortive CCPs [26]. Here we adapt that model to study endocytosis of NPs. The main idea of the model was to replace the real protein coat by a coat made up of identical units referred to as monomers (figure 2(a)). We assumed that (1) the coat made up of monomers has a bending rigidity and a spontaneous curvature, (2) the shape of the model CCP is a spherical cap, and (3) pit assembly proceeds via reversible binding of monomers on the edge of the pit. Due to coarse-graining, the finer details of the assembly process were lost, but nevertheless the model was able to capture the lifetime distribution of abortive CCPs very well [26].

The coarse-grained model discussed above allows us to describe the dynamics of CCP assembly around a NP–receptor complex as a nearest-neighbor random walk on a one-dimensional lattice containing $N+1$ sites. The kinetic scheme of such a random walk is shown in figure 2(b), where n is the number of monomers in a pit and n is the number of

monomers needed for a complete vesicle. The rate constants α_n and β_n characterize the growth and decay rates of a pit of size n , $k_0 = 1/\tau_0$ is the rate constant for binding of the first monomer to the NP–receptor complex, and k_N is the rate constant for the scission of a vesicle from the membrane. Analysis of CCP dynamics in living cells shows that scission is a fast process [19, 20]. Therefore, to keep things simple we choose $k_N = \infty$. Note that a finite scission rate can be easily incorporated in our analysis.

For the kinetic scheme in figure 2(b), the quantities P_s and P_f entering into equation (3) are the probabilities that a random walk, starting from site $n = 1$, eventually reaches sites $n = n$ and $n = 0$, respectively, and the times τ_s and τ_f are the mean durations of the two processes (which formally are conditional mean first-passage times [28, 29]). Analytical expressions for these quantities are given by [28]

$$P_s = \frac{\Psi_1}{\Psi_1 + \sum_{m=1}^N \Phi_m}, \quad P_f = 1 - P_s, \quad (4)$$

$$\tau_s = \frac{\sum_{m=1}^N \exp[-\tilde{F}(m)] \sum_{l=1}^m \Psi_l \sum_{l=m}^N \Phi_l}{\Psi_1 + \sum_{m=1}^N \Phi_m}, \quad (5)$$

$$\tau_f = \frac{\Psi_1 \sum_{m=1}^N \exp[-\tilde{F}(m)] \left(\sum_{l=m}^N \Phi_l \right)^2}{\sum_{l=1}^N \Phi_l \left(\Psi_1 + \sum_{m=1}^N \Phi_m \right)}. \quad (6)$$

Here, the functions Ψ_n and Φ_n are

$$\Psi_n = \exp[\tilde{F}(n)]/\beta_n, \quad \Phi_n = \exp[\tilde{F}(n)]/\alpha_n, \quad (7)$$

where $\tilde{F}(n) = F(n)/(k_B T)$, $F(n)$ is the formation free energy for a pit containing n monomeric units, k_B is the Boltzmann constant, and T is the absolute temperature. The above formulas are obtained assuming that the forward and backward rate constants are related through detailed balance,

$$\beta_n = \alpha_{n-1} \exp[\tilde{F}(n) - \tilde{F}(n-1)], \quad n = 2, 3, \dots, N \quad (8)$$

3. Model details

To calculate the mean internalization time by means of equations (2)–(6) we need to know the rate constants α_n and β_n , as well as the free energy of pit formation, $F(n)$. We now discuss these quantities.

3.1. Rate constants

A pit grows by the addition of a monomer to its edge, which has multiple binding sites. We assume that all the binding sites are identical and the monomer can bind to any one of them with equal probability. Keeping this in mind, we choose the forward rate constants to be

$$\alpha_n = \gamma f(n, N), \quad n = 1, 2, \dots, N-1, \quad (9)$$

where γ is a kinetic parameter, and the function $f(n, N)$ gives the number of available binding sites on the edge of a pit of size n . Using the approximation that the shape of a pit is a spherical cap, this function can be written as

$$f(n, N) = \rho \sqrt{n(N-n)/N}, \quad (10)$$

where ρ is a dimensionless parameter whose value is chosen to be 2 (see the appendix).

The rate constant β_1 describes the dissociation constant of the first monomer. This rate constant is unknown, and we treat it as a free parameter. As shown later, β_1 plays an important role as it significantly affects the mean internalization time, our main quantity of interest. Other dissociation rate constants, β_n , $n = 2, 3, \dots, N$, are given by equation (8).

3.2. Free energy of pit formation

The formation free energy of a pit made of n monomers and having a curvature c can be written as [22, 26]

$$F(n, c) = 2\kappa_m \lambda n c^2 + 2\kappa_p \lambda n (c - c_p)^2 - \epsilon_b n + \sigma f(n, c). \quad (11)$$

The first term in the right-hand side is the Helfrich energy [30] describing the energetic cost of bending the cell membrane assuming that its spontaneous curvature is zero. The second term represents the bending energy of the protein coat. The third term represents the effective binding energy of a monomer to the coat, and the fourth term is the line tension energy. The parameters κ_m and κ_p are the bending rigidities of the cell membrane and the coat, respectively, c_p is the spontaneous curvature of the protein coat (in the absence of the membrane), ϵ_b is the effective monomer binding energy, i.e., the difference between the binding energy and entropic cost of immobilizing the monomers, σ is the edge-energy constant, and λ is the average area occupied by a monomer.

In our model the vesicle size is characterized by the number of monomers needed to complete a vesicle. This number, N , is related to the curvature of the pit by $4\pi/c^2 = \lambda N$. Similarly the natural curvature of the protein coat, c_p , and the natural number of monomers in the coat, N_p , are related by $4\pi/c_p^2 = \lambda N_p$. By substituting these relations into equation (11), we can write the formation free energy of a pit made of n monomers as

$$F(n, N) = E(N)n + \sigma\rho\sqrt{n(N-n)/N}, \quad (12)$$

where

$$E(N) = \frac{8\pi\kappa_m}{N} + \frac{8\pi\kappa_p}{N}\left(1 - \sqrt{\frac{N}{N_p}}\right)^2 - \epsilon_b. \quad (13)$$

3.3. Parameter values

The values of the parameters used in our calculation are summarized in table 1. The rationale behind the choices is as follows: the value of κ_m typically lies between 10 and 25 $k_B T$ [31]; we choose $\kappa_m = 20 k_B T$. The size distribution of clathrin baskets assembled *in vitro* using adaptor protein AP-2 is typically narrow and has a peak close to $d_p = 90$ nm in diameter [32].

Using the average area occupied by a clathrin molecule, $\lambda = 310 \text{ nm}^2$, and the relation $\lambda N_p = \pi d_p^2$, we find that a 90 nm basket would have approximately 80 clathrin triskelions, which we take to be the natural coat size, i.e., $N_p = 80$. The value of $\lambda = 310 \text{ nm}^2$ was estimated using the relation between diameters of clathrin baskets of different sizes and the number of clathrin triskelions they contain [33].

In [26], using experimental data on the lifetime distribution of abortive CCPs (CCPs with no cargo), we estimated the value of the effective binding energy per monomer, ϵ_b , to be approximately 5 $k_B T$. Experiments show that with cargo (present case) the binding energy increases [34]. An approximate range for its value can be determined using the following argument: for a clathrin-coated vesicle to be energetically stable, the effective binding energy has to be greater than the membrane bending energy ($8\pi\kappa_m \approx 500 k_B T$). Since a typical vesicle has 80 monomers, the binding energy per monomer should be greater than $500/80 \approx 6 k_B T$. To get an upper bound to the binding energy we consider the interaction energy between proteins containing a BAR domain, which produce membrane curvature during CME, and the cell membrane. The electrostatic binding energy between BAR domains and membrane is estimated to be approximately $15k_B T$ [35]. We choose a number between these values and set $\epsilon_b = 10 k_B T$.

The parameter, κ_p , captures the effective bending rigidity of the protein coat. This parameter, together with the natural curvature of the coat, determines the typical size of a vesicle. In [26], using the typical size of a vesicle to be 100 nm in diameter (which is somewhat larger than the average size of a basket), we estimated the value of this parameter to be approximately $\kappa_p = 200 k_B T$; here we use the same value. We choose the edge energy constant $2\sigma = 5 k_B T$ [22] and the length of a receptor ligand bond to be $l_b = 15 \text{ nm}$ [36]. Parameters γ and ρ have the same values as in [26]: $\rho = 2$, $\gamma = 0.18 \text{ s}^{-1}$.

4. Quantities of interest

Next we look at the dependences of the quantities of interest on the NP size and the parameter β_1 . When drawing these dependences we relate the number of monomers in a vesicle, N , to the NP diameter, d_{NP} , by the relationship

$$N = \pi(d_{NP} + 2l_b)^2/\lambda, \quad (14)$$

where l_b is the length of the receptor–ligand bond between the NP and the cell membrane. Note that this assumes that there is only one NP inside a vesicle, as illustrated in figure 3.

4.1. Free energy of pit formation

As mentioned earlier, the energetics of coat formation (see equation (12)) plays an important role in determining the fate of the coat assembly process (successful or failed). The dominant term in that equation is $E(N)$. The function $E(N)$, given in equation (13), contains the membrane bending energy per monomer (the first term on the right-hand side), which opposes coat assembly, and the energy gained due protein coat formation per monomer (the sum of the second and third terms on the right-hand side), which favors coat assembly. Figure 4 shows the dependence of these energies and $E(N)$ on the NP size.

The membrane bending energy per monomer (the dashed curve in figure 4) decreases monotonically with d_{NP} . The energy gained due to coat formation (the dashed–dotted curve) attains a minimal value when the vesicle has the same curvature as the natural curvature of the protein coat (90 nm). Using the relation between NP and vesicle sizes we find that such a vesicle would carry a 60 nm NP. The total energy attains a minimum at $d_E^* \approx 68$. An analytical formula for this size can be found by solving the equation $E(N)/N = 0$, which leads to

$$d_E^* = (1 + \kappa_m/\kappa_p)\sqrt{\lambda N_p/\pi} - 2l_b. \quad (15)$$

The vertical dashed lines at $d_{\min} \approx 46$ nm and $d_{\max} \approx 105$ nm show the NP sizes at which $E(N) = 0$. At these sizes, the factors favoring and opposing coat assembly balance out. The lower size is mainly determined by the competition between the membrane bending energy and the coat binding energy, whereas the upper size is determined by the coat bending energy and coat binding energy. Analytical expressions for $d_{\min}$ and $d_{\max}$ can be obtained by solving the equation $E(N) = 0$, which leads to

$$\begin{aligned} \frac{d_{\min}}{d_p} &= \frac{1 - \sqrt{(1 + k'_m)\epsilon'_b - k'_m}}{(1 - \epsilon'_b)} - \frac{2l_b}{d_p}, \\ \frac{d_{\max}}{d_p} &= \frac{1 + \sqrt{(1 + k'_m)\epsilon'_b - k'_m}}{(1 - \epsilon'_b)} - \frac{2l_b}{d_p}, \end{aligned} \quad (16)$$

where $\kappa'_m = \kappa_m/\kappa_p$, $\epsilon'_b = N_p \epsilon_b/8\pi\kappa_p$, and $d_p = \sqrt{\lambda N_p/\pi}$.

4.2. Probability of successful CCP assembly

Next we consider the probability of successful CCP assembly, P_s , given in equation (4). Figure 5 shows the dependence of this probability on the NP size for different values of the parameter β_1 . The dependence of P_s on the NP size can be understood by considering the energy function shown in figure 4. In regions where $E > 0$, pit assembly is energetically unfavorable and P_s is negligibly small. In contrast, in the middle region where $E < 0$, pit assembly becomes energetically favorable, and P_s rises sharply. Interestingly, sizes of several viruses which are internalized via CME, including dengue virus (40–60 nm), semliki forest virus (50–70 nm), reovirus (60–80 nm), and influenza A (80–120 nm) roughly fall in this range where P_s is relatively high.

The maximum value of P_s decreases with increasing β_1 , which is not surprising since a higher value of β_1 means a higher dissociation rate of the NP–receptor complex from the first monomer. As discussed later, using the data on internalization kinetics of viruses, we estimate the value of β_1 to be somewhere between 2 and 8 s^{−1}. Note, for this range of β_1 the maximum value of P_s is small, which means that a virus makes many failed attempts ($1/P_s$) before being internalized.

The probability of successful CCP assembly becomes negligibly small for NPs smaller than $d_{\min}$ or larger than $d_{\max}$. Thus, these sizes correspond to the smallest and largest NPs that can be internalized via CME. The lower estimate, $d_{\min} \approx 46$ nm, cannot be compared with experimental data since our model assumes that there is only one NP per vesicle, whereas in reality small NPs may enter CCPs in clusters [37] or along with other cargo molecules. Nevertheless, our estimate of the smallest vesicle size of 76 nm, obtained using the relation $d_V = d_{NP} + 2l_b$ (see figure 3), compares very well with the size of smallest clathrin-coated vesicles observed in experiments [38]. Our estimate of $d_{\max} \approx 105$ nm is somewhat smaller than the reported value of 200 nm for NPs [39, 40]. We comment on the possible reasons for this discrepancy in section 5.

4.3. Mean waiting time, and mean time for successful CCP assembly

The mean internalization time, τ , is the sum of two terms (see equation (2)): the mean waiting time τ_w , and the mean time for successful CCP assembly, τ_s . Figure 6 shows the dependences of these two times on NP size, calculated using equations (3)–(6). We focus on the region between $d_{\min}$ and $d_{\max}$, where the probability P_s is not too low. We found that τ_s changes very little when we vary β_1 . Therefore, we show only the curve for $\beta_1 = 5$ s^{−1}. The trend of τ_s can be understood by considering the dynamics of coat assembly. As seen in figure 6, the time τ_s attains a minimum at $d_s \approx 55$ nm (shown by the arrow). For this value of the NP size, $E \approx -3 k_B T$ (see figure 4) and, hence, according to the condition of detailed balance, $\alpha_H/\beta_n \approx 20 \gg 1$. This implies that during the endocytosis of a NP of size d_s , the growth of the coat proceeds with a low probability of monomer dissociation. Thus, τ_s is determined by the rate of arrival of a new monomer. When the NP diameter is slightly larger than d_s the inequality $\alpha_H/\beta_n \gg 1$ is still true, but the number of monomers needed to wrap a NP increases and, hence, τ_s increases with NP size. As the NP size approaches $d_{\min}$ or $d_{\max}$,

the rate constants α_n and β_n become comparable. Consequently, during coat assembly the dissociation of monomers becomes more frequent, which causes τ_s to increase markedly. Values of τ_s predicted by our model fall in the same range as the experimentally measured times needed for clathrin-coated vesicle formation [20], as well as the τ_s values for viruses which are internalized via CME (given in table 2).

Figure 6 also shows the mean waiting time, $\tau_w = (\tau_0 + P_f \tau_f)/P_s$, for different values of parameter β_1 . In contrast to τ_s , the time τ_w changes significantly with the variation of β_1 . To calculate τ_w we need the values of τ_0 and β_1 , both of which are not known. To make a reasonable choice for these parameters we use the fact that during the assembly process the average growth rate of a CCP is between one and two triskelions per second [19]. This means the time interval between the arrival of triskelions is approximately 1 s. We assume that the CCP initiation time is slightly longer than the clathrin arrival time but much shorter than τ_s , and choose $\tau_0 = 5$ s. Then, to get a range for β_1 , we make use of the fact that, in the case of viruses, the magnitude of experimentally-determined times τ_w and τ_s are comparable (see table 2). Our analysis shows that the two times have similar values when β_1 falls in the range $2\text{--}8\text{ s}^{-1}$ (see figure 6). Moreover, we find that the numerator in the formula for the mean waiting time, $\tau_0 + P_f \tau_f$, is small compared to τ_s , and depends weakly on the NP size. Thus, the dependence of τ_w on the NP size is mainly determined by the factor $1/P_s$ (see equation (3)).

4.4. Mean internalization time

Figure 7 shows the dependence of the mean internalization time, given in equation (2), on NP size, for different values of the parameter β_1 . This is our main quantity of interest. The mean internalization time attains a minimum at a diameter which we designate to be d_{opt} (shown with arrows). The curves in figure 7 show that d_{opt} does not change much with β_1 , although the value of τ at d_{opt} changes appreciably. For example, as β_1 varies from 2 to 8 s^{-1} , the value of d_{opt} changes from 58 to 63 nm, but the value of τ at d_{opt} changes from 109 to 202 s.

From the above analysis we find that the dependence of τ on the NP size can be characterized by four quantities, namely d_{min} , d_{max} , d_{opt} and, $\tau(d_{\text{opt}})$, where the latter is the value of τ at d_{opt} . These quantities are functions of the coat parameter values, which, in principle, can change from one cell type to another. Figure 8 illustrates the dependence of these quantities on the coat parameters κ_p , N_p , and ϵ_b . Among the characteristic sizes, the maximum NP size d_{max} , shows appreciable variation whereas the other sizes show only weak dependences on the coat parameters. The value of $\tau(d_{\text{opt}})$ is most sensitive to the parameter ϵ_b . It rises rapidly as ϵ_b decreases, and becomes less than $8 k_B T$. Figures 8 (b), (d), (f) also contain plots of $\tau_s(d_{\text{opt}})$, which is the value of τ_s at d_{opt} . The weak dependence of this quantity on coat parameters shows that the increase in the value of $\tau(d_{\text{opt}})$ is mainly due to the increase in the value of τ_w at d_{opt} . Thus, similar to its sensitivity to the parameter β_1 , the time τ_w is more sensitive than is τ_s to other coat parameters.

5. Summary and discussion

In this paper we provide a theoretical framework for studying endocytosis of NPs and viruses via CME. Our model, which focuses on coat assembly, captures the stochastic dynamics of coat formation around a NP–receptor complex and the resulting probabilistic nature of the NP internalization process. Briefly, if the assembly is successful the CCP grows to form a vesicle and the NP is internalized. In contrast, if the assembly fails, then the NP–receptor complex has to wait for the initiation of a new CCP. We derive an analytical formula for the mean internalization time, defined as the mean time between the binding of a NP to the membrane and its entry into the cell. The formula has two terms; the first, τ_w , which we refer to as the mean waiting time, corresponds to the mean time a complex spends on the membrane before successful CCP assembly starts, and the second term, τ_s , is the mean duration of a successful CCP assembly event.

Experiments show that in the case of different viruses internalized via CME, the time τ_w is of the same order of magnitude as τ_s (see table 2). Based on this we infer that the probability of successful CCP assembly, P_s , is small; therefore, even though the time spent in a single failed event is relatively short, the total time in failed attempts becomes large and comparable to the time for successful CCP assembly. Analysis of the dependence of the times τ_w and τ_s on the parameters describing coat assembly shows that the former is more sensitive than the latter, because P_s depends strongly on the coat parameters. This brings forth an important point which has not emerged from previous models—the mean waiting time should be the focus of studies trying to improve the internalization efficiency of NPs.

As discussed earlier, there is an upper bound on the size of NPs which can be internalized via CME. When the diameter of the NP exceeds 105 nm, the coat has to be distorted significantly from its natural curvature in order to accommodate the NP inside a vesicle. Due to the increase in coat distortion energy, the probability of successful CCP assembly becomes very small and the mean internalization time becomes extremely large. Our estimate $d_{\max} \approx 105$ nm is smaller than the reported value 200 nm for NPs [39, 40]. There could be different reasons for this discrepancy. For example, $d_{\max}$ is sensitive to coat parameter values (see figure 8), and in our coarse-grained model the parameter values might be somewhat inaccurate. Alternatively, it could be that during internalization of larger NPs, only a partial clathrin coat, rather than a complete clathrin-coated vesicle, forms. Indeed, such fractional clathrin-containing coats are known to form around *Listeria* bacteria [47] and vesicular stomatitis virus [8]. However, in such cases actin is required for internalization [8]. The previously-mentioned theoretical models do not take the clathrin coat assembly into explicit consideration and therefore fail to capture the constraint imposed by coat energetics. In these models the upper bound is determined by a combination of factors including the binding energy, the number of receptors available on the cell membrane, and the membrane tension [13, 14, 17]. These models predict that NPs significantly larger than 200 nm can be internalized, which is inconsistent with experimental observations from [39] and [40].

We find that the mean internalization time is shortest when the NP size is close to 60 nm, and that this optimal size is weakly sensitive to variations in the values of the coat parameters. This observation is consistent with the findings of several experiments done to

study size dependence of NP endocytosis. In these experiments different cells, NPs, and ligands were used, yet the optimal size was always found to be close to 50 nm (see table 3). Different models have been used to explain this observation. Certain models indicate that the time it takes for a NP to be wrapped by the membrane via receptor–ligand bond formation is shortest for 50 nm NPs [13]. Other models calculate the number of NPs a cell can internalize, and conclude that the number is highest for 50 nm NPs [14]. The fact that such different approaches predict the same optimal size, while interesting, makes it difficult to discriminate between the applicability of different theoretical models solely based on the predicted optimal NP size.

Some limitations of our approach are as follows. Like in other models, we assumed that NP–receptor binding is irreversible. However this assumption is valid only if the lifetime of receptor–ligand bond is much larger than the mean internalization time of a NP. Otherwise, the dissociation of the complex should be taken into consideration. Also, we do not consider in detail the initial step of CME, where multiple bonds between ligands on the NP and receptors on membrane may form. This would lead to clustering of receptors in a CCP, which is important in early stages of CCP assembly, as the binding of the cytoplasmic parts of the receptors to the coat proteins is known to stabilize the clathrin coat. In this context, it has been shown that the chances of successful CCP assembly around transferrin receptors (which are internalized via CME) increases when the receptors are clustered [48], and also that the internalization rate of virus-like particles increases when the number of ligands on the virus are increased [49]. Although, our model does not account for these factors, based on our sensitivity analysis (see figures 5 and 8), we think that increasing the number of ligands increases the probability of successful CCP assembly around the NP, leading to a decrease in the mean waiting time and the mean internalization time (as observed in [49]).

To conclude, in order to capture a complete picture of NP internalization via CME, a comprehensive theory which couples both aspects of the cellular endocytic machinery—bond formation and coat assembly—is needed. We believe our work is a step in that direction.

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Appendix.: Derivation of $f(n, N)$, as given by equation (10)

Using spherical coordinates (see figure 9), the surface area of the pit can be written as

$$A(\theta) = 2\pi R^2[1 - \cos(\theta)] = \lambda n, \quad (17)$$

where R is the radius of a sphere having the same curvature as the pit, and λ is the average area occupied by a monomer. This leads to the relation between $\cos(\theta)$ and the number of monomers, n , in the pit,

$$\cos(\theta) = 1 - \frac{\lambda n}{2\pi R^2}. \quad (18)$$

We use the above equation to infer the radius, $r(n)$, of the circular growing edge of a pit as a function of n ,

$$\begin{aligned} r(n) &= R \sin(\theta) = R \sqrt{[1 + \cos(\theta)][1 - \cos(\theta)]} \quad (19) \\ &= \sqrt{\left(\frac{\lambda n}{\pi}\right) \left(1 - \frac{\lambda n}{4\pi R^2}\right)}. \end{aligned}$$

Introducing the average linear span of the monomer, denoted by L , we find that the number of available binding sites on the periphery of the pit is

$$f(n) = \frac{2\pi r(n)}{L} = \frac{2\pi}{L} \sqrt{\left(\frac{\lambda n}{\pi}\right) \left(1 - \frac{\lambda n}{4\pi R^2}\right)}. \quad (20)$$

By changing variables from R to N using the relation $4\pi R^2 = \lambda N$, we arrive at

$$f(n, N) = \rho \sqrt{n(N - n)/N}, \quad (21)$$

where ρ is a dimensionless parameter given by $\rho = \sqrt{4\pi\lambda/L^2}$. The value $\rho = 2$ gives the correct number of available binding sites for $n = 1$.

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Figure 1.

Schematic diagram of NP internalization via clathrin-mediated endocytosis. A NP first binds to a specific cell surface receptor, forming a NP–receptor complex. The complex binds the coat proteins, and clathrin-coated pit (CCP) assembly begins. The CCP either grows to form a vesicle, in which case the NP is internalized, or grows only up to a certain size and subsequently disassembles. In such an event the NP fails to be internalized and the complex becomes free of coat proteins.

Figure 2.

(a) Coarse-grained model for the assembly of a clathrin-coated pit (CCP). In a real CCP the protein coat contains clathrin, adaptors and several other accessory proteins. In our model of a CCP the coat is made of identical monomeric units. The shape of the CCP is assumed to be a spherical cap, and the average area of a monomer is chosen to be the same as that occupied by a clathrin triskelion in a real CCP. (b) Kinetic scheme of the pit assembly shown in (a). Symbols n and N refer to the number of monomers in a pit and a complete vesicle, respectively. The rate constants α_n and β_n characterize the growth and decay rates of a pit of size n . k_0 is the rate at which the first monomer binds to the NP–receptor complex, and k_N is the rate of scission of a vesicle from the membrane.

Figure 3.

Relation between NP and vesicle diameters. The vesicle diameter, d_V , and the NP diameter, d_{NP} , are related by $d_V = d_{NP} + 2l_b$, where l_b is the length of a receptor–ligand bond.

Figure 4.

Average energy per monomer, $E(N)$, equation (13), (solid curve) as a function of NP size. $E(N)$ is the sum of the membrane bending energy per monomer (dashed curve) and the energy due to coat formation per monomer (dashed–dotted curve). Dashed vertical lines at $d_{min} \approx 46$ nm and $d_{max} \approx 105$ nm correspond to the NP sizes at which $E(N) = 0$. The arrow at $d_E^* \approx 68$ nm indicates the size of the NP whose carrier vesicle is energetically most stable.

Figure 5.

The probability of successful CCP assembly around a NP–receptor complex, P_s , as a function of the NP size. P_s is high in the region where $E < 0$, and pit assembly is energetically favorable. The maximum value of P_s is sensitive to the parameter β_1 , the values of which in s^{-1} are given near the curves. The dashed vertical lines are the same as in figure 4.

Figure 6.

Mean time for successful CCP assembly, τ_s (solid curve) and the mean waiting time, τ_w , (curves with symbols) for different values of β_1 , as functions of the NP size, calculated using equations (3)–(6). In contrast to the time τ_s (only the curve for $\beta_1 = 5\text{s}^{-1}$ shown), the time τ_w changes significantly with β_1 . The dashed vertical lines are the same as in figure 4. The arrow indicates the NP diameter d_s , at which τ_s is minimum.

Figure 7.

The mean internalization time, τ , given in equation (2), as a function of the NP size, for different values of β_1 . The arrows point to the NP diameters corresponding to the shortest values of the mean internalization time, which we refer to as d_{opt} . The value of d_{opt} is weakly sensitive to changes in β_1 , but the value of τ at d_{opt} varies significantly. The dashed vertical lines are the same as in figure 4.

Figure 8.

Plots of d_{min} , d_{max} (equation (16)), d_{opt} , $\tau(d_{opt})$, and $\tau_s(d_{opt})$ (calculated numerically), as functions of κ_p —the bending rigidity of the protein coat ((a) and (b)), ϵ_b —effective monomer binding energy ((c) and (d), and N_p —the natural number of monomers in the coat ((e) and (f)). Among the characteristic sizes only the maximum NP size d_{max} shows significant variation. The time $\tau(d_{opt})$ is the sum of $\tau_s(d_{opt})$ and $\tau_w(d_{opt})$. The weak dependence of $\tau_s(d_{opt})$ on coat parameters shows that the increase in $\tau(d_{opt})$ is mainly due to the increase in $\tau_w(d_{opt})$. The arrows show the parameter values used in our calculations.

Figure 9.
Spherical cap model of a pit.

Table 1.

Parameter values.

Parameter	Description	Value
κ_m	Membrane bending rigidity	$20 k_B T$
κ_p	Protein coat bending rigidity	$200 k_B T$
N_p	Number of monomers in a typical vesicle	80
ϵ_b	Binding energy per monomer	$10 k_B T$
2σ	Edge energy constant	$5 k_B T$
γ	Kinetic parameter	0.18 s^{-1}
τ_0	Average time for initiation of a CCP	5 s
k_N	Vesicle scission rate	∞
λ	Average area occupied by a monomer	310 nm^2
l_b	Length of receptor ligand bond	15 nm

Table 2.

Values of the mean waiting time, τ_w , and the mean time for successful CCP assembly, τ_s , measured for virus and virus-like particles. Internalization of these particles via clathrin-mediated endocytosis was monitored using total internal reflection fluorescence microscopy [5–8]. The acronyms IHNV and DI-T stand for infectious hematopoietic necrosis virus and defective interfering particle, respectively.

Virus	τ_w	τ_s	Ref.
Influenza A	55 s	70 s	[5]
Dengue	111 s	83 s	[6]
IHNV	120 s	120 s	[7]
DI-T	110 s	51 s	[8]

Table 3.

Summary of experiments on the size dependence of cellular uptake of NPs. NPs smaller than 200 nm are internalized via receptor-mediated endocytosis, whereas for larger NPs other internalization mechanisms are involved [39, 40]. Bold faced numbers indicate the NP size for which the cellular uptake is highest.

Cells	NPtype	NP size (diameter nm)	Ref.
B16-F10	Latex beads	50 ,100,200,500	[39]
MNNG/HOS	Metal hydroxide	50 ,100,200,350	[40]
HeLa,STO,SNB19	Gold	14,30, 50 ,74,100	[41]
HeLa	Quantum dots	5,15, ₅₀	[42]
CLL-0,HeLa	Gold	45 ,75,110	[43]
HeLa	Mesoporous silica	30, 50 ,110,170,280	[44]
A549,HeLa,MDA	Gold	15,30, 45	[45]
Caco-2	Liposomes	40 ,72,86,97,162	[46]