On the Role of Assembly Kinetics in Determining the Structure of Clathrin Cages

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ABSTRACT The process of clathrin self-assembly into closed shells with the Fullerene structure is investigated. It is argued that the shell size distribution is governed by the kinetics of assembly and depends on the rate of growth controlled by the free clathrin concentration. The particularly abundant small structures—the "tennis ball" and the "hexagonal barrel"—are found to have a certain unique property that makes them ubiquitous in the process of slow growth. A thermal ratchet-type mechanism of the coated vesicle assembly on the membrane is proposed, and possible experimental tests are suggested.

INTRODUCTION

Clathrin is a protein that plays an important role in vesicular trafficking by providing a mechanism of forming vesicles out of external or internal membranes (for a review, see Steer and Heuser, 1991; Anderson, 1991; Brodsky, 1988; Kirchhausen, 1993; Pearse and Robinson, 1990; Smythe and Warren, 1991; Schmid, 1992; Keen, 1990). This process has been described (Hauser and Evans, 1980) as formation of a curved "coated pit" on the membrane, which evolves into a "coated vesicle" with the clathrin forming an exterior scaffolding network. The complete clathrin coat has the structure of a fullerene (Fowler et al., 1992), i.e., the vertices are 3-coordinated and all of the faces are either pentagons or hexagons. For such a structure to form a closed shell it must have exactly 12 pentagonal faces (as required by Euler theorem; Coxeter, 1989), whereas the number of hexagons, n_h, and hence the size of the coat can vary. For example, the coated vesicles in neurons are 70–90 nm in diameter, whereas the coated vesicles from other tissues range from 70 to 150 nm (Kirchhausen, 1993). The unusual overall variability of the structure, along with the observation of specific small regular coats (Crowther et al., 1976), is not easily explained in terms of conventional equilibrium considerations. However, we shall argue that the existing data can be qualitatively understood in terms of kinetics of assembly. Under the conditions of growth the complete coats are much more stable than the incomplete ones and hence fall out of polymerization equilibrium. As a result, the distribution of complete coats is not Gibbsian. Rather, the occurrence of a given structure is determined by the number and the probability of various ways to assemble it. The present investigation provides a simple illustration of this possibly common mechanism determining the structure of the "self-assembled" macromolecular complexes that are ubiquitous in biology.

A few more facts. The assembly of clathrin on the cell membrane is mediated by the cytoplasmic domains of the surface receptors and so-called adaptor proteins (Pearse and Robinson, 1990; Keen, 1990). The adaptors are presumed to target the vesiculation process to specific receptors—a role essential in protein sorting and trafficking. The clathrin/adaptor assembly must also supply the energy (Anderson, 1991) needed to shape the membrane into a vesicle, as it appears (Schmid, 1992) that this process does not require ATP until the actual pinching off of the vesicle. (The required energy may be estimated crudely to be $8\pi\sigma$, where σ is lipid membrane curvature stiffness (Helfrich, 1985); assuming that $\sigma \approx 0.5 \times 10^{-12}$ erg yields a total curvature energy on the order of 300 k_BT, independent of the radius of the vesicle.) ATP, however, is required for uncoating (Patzer et al., 1982). Under appropriate conditions (Crowther et al., 1976; Keen et al., 1979) clathrin can self-assemble in the bulk (i.e., without the membrane) into "cages" with the same fullerene structure but no vesicle inside. This assembly occurs at low pH, low ionic strength (but in the presence of Mg or Ca), without adaptor proteins and at physiological pH with adaptors (Zaremba and Keen, 1983). As found by Zaremba and Keen in the latter case, the assembled cages form a narrow distribution peaked near 80 nm diameter, whereas in the former case the distribution is broad: 70–130 nm. Most of the small (80 nm) cages appear to have the "hexagonal barrel" (HB) or "tennis ball" (TB) structures (with $n_h = 8$) originally described by Crowther, Finch, and Pearse (Crowther et al., 1976).

It has been suggested that the coat size is determined by the preferred angle (Kirchhausen and Harrison, 1981) at the vertex of the clathrin triskelion, which directly defines the radius of the coat. Below we shall argue that the size and structure of the coats are not determined by such equilibrium considerations alone and are controlled instead by the kinetics of assembly, which for slow growth conditions favors the formation of pentagonal faces over hexagons and heptagons. This bias toward the curved structures competes with membrane and clathrin rigidity, which by itself would favor the formation of flat hexagonal lattices. (The small intrinsic curvature of the triskelia in this scenario plays a role only in determining which side of the triskelion will be on the exterior.) Starting with the case of clathrin cage formation, we shall discuss the different possible regimes as

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a function of parameters and formulate an appropriate simple model of assembly. The model will be studied numerically to make a qualitative prediction of the dependence of the cage structure on the free clathrin concentration, which controls the rate of growth, and on the rigidity of the clathrin shell. We shall identify the "tennis ball" and the "hexagonal barrel" structures (Crowther et al., 1976; Zaremba and Keen, 1983) as the smallest fullerenes without three pentagons meeting at a vertex. This property makes them occur naturally in the present assembly model within a certain slow growth limit. The latter limit may plausibly be realized in the case of clathrin coassembly with adaptors (at physiological pH), offering a possible explanation for the abundance of HB and TB cages observed under those conditions (Zaremba and Keen, 1983). Finally, we address the mechanism of the coated pit formation. As an alternative to the "invagination of the hexagonal lattice" scenario (Hauser and Evans, 1980; Smythe and Warren, 1991; Jin and Nossal, 1993), we shall discuss the process in which the curving of the membrane occurs simultaneously with the growth of the clathrin-coated pit (Kirchhausen, 1993). The present analysis suggests a thermal ratchet-type (Feynman, 1966) mechanism in which the formation of each pentagonal face on the periphery of the growing pit occurs via a thermal fluctuation of the membrane local curvature, which becomes trapped by the closure of the pentagon. The size of the coated vesicles formed in such a process would depend on the rate of assembly, membrane stiffness, and temperature.

At present not much is known about the interaction between the clathrin triskelions that drive their self-assembly, except that it is likely to be electrostatic in nature. The assembly process appears to be enhanced (Zaremba and Keen, 1983) at lower temperatures, suggesting that the assembly is driven by energy rather then entropy. Fortunately, some qualitative understanding of the assembly is possible without the detailed knowledge of microscopic interactions. Let Δf be the free energy of a bound triskelion relative to a free one. It is a function of pH, which affects the distribution of the ionic strength, which controls the screening length. Whereas all of the vertices of a complete fullerene cage are equivalent in their connection with each other, the sites of an incomplete cage are not (see Fig. 1). There are interior sites with all three legs of the resident triskelion connected, which we will call "3-valent"; the "2-valent" boundary sites have two bound legs, and the "1-valent" dangling peripheral triskelions have only one leg connected to the cluster. Actually, as shown in Fig. 1 *b*, there are two types of 1-valent sites, 1 and 1′, which because of the chirality of the triskelion are inequivalent. Quite generally one expects stronger binding on the higher valent sites; for simplicity let us take $\Delta f_1 = \Delta f_2/2 = \Delta f_3/3$ and $\Delta f_{1'} > \Delta f_1$, where the valence is denoted by the subscript (note that binding corresponds to $\Delta f < 0$).

The chain polymerization on the 1-valent sites would occur if $c > c_1 = \exp(\Delta f_1/k_BT)$, but because of the chirality one must distinguish two regimes. For $c > c_{1'} = \exp(\Delta f_{1'}/k_BT) > c_1$, clathrin assembly occurs on all of the peripheral

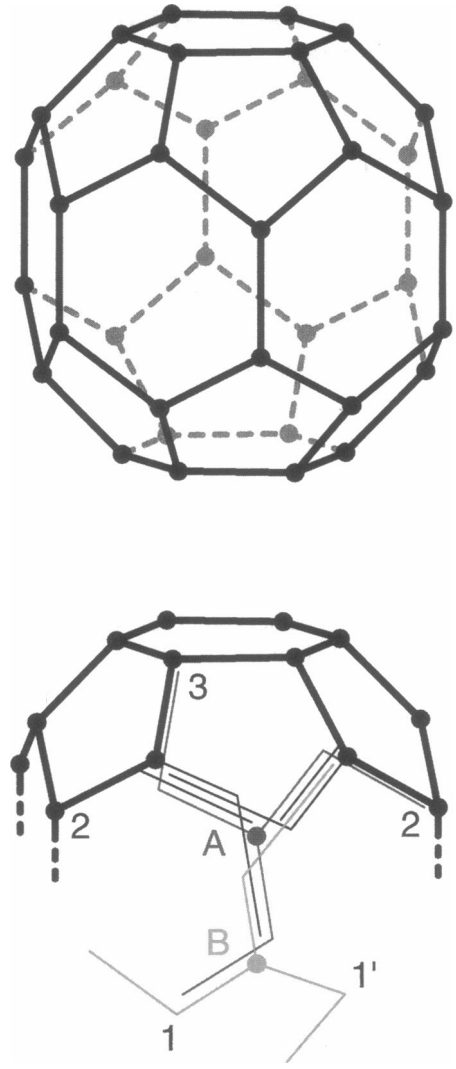

FIGURE 1 A schematic drawing of (*a*) a hexagonal barrel, (*b*) addition of a clathrin triskelion A followed by B to a growing cluster. Multiple lines along selected edges denote chains from different triskelia. The distinct clathrin sites on the incomplete shell are shown: 1 and 1′, 1-valent; 2, 2-valent; 3, 3-valent.

sites of the growing cluster. Because there are two 1-valent sites on each dangling triskelion, this will result in a branched polymer rather than a fullerene cage. On the other hand, if $c_1 < c < c_{1'}$, only one of the two sites is active and the growth is biased toward, say, "left" (see Fig. 1) turns at each fork, which favors the formation of closed faces.

For $c < c_1$ the direct polymerization on 1-valent sites is suppressed. However, the formation of a cage can proceed cooperatively by the addition of the several clathrins necessary for the completion of a new face. Because each face is closed off with an extra "bond," the free energy gain per added clathrin is $((l-1)\Delta f_1 + l\Delta f_2)/l \approx \Delta f_1\,(l+1)/l$, where l is the number of clathrins required (l depends on the local structure of the growing edge, but it does not exceed 4). Thus for $c > c_2 = \exp(5/4\ (\Delta f_1/k_BT))$, the cage assembly process has stable intermediate states and its rate is con-

trolled by the slowest step (the nucleation of the first pentagonal ring), which at low concentrations scales as c^5.

For $c < c_2$ the cages can, in principle, still be formed, at least while, $c > c_3 \approx \exp(3/2\ (\Delta f_1/k_BT))$, the free energy per triskelion is lower in the assembled state than in solution; however, the nucleation process becomes progressively higher order and the assembly becomes exponentially improbable. The complete cages, however, should remain quite stable, because the removal of a 3-valent triskelion requires a Δf_3 thermal fluctuation and is unlikely. This may explain the observed absence of clathrin exchange between the completed cages and the solution (Eisenberg, private communication). The stability of complete cages also means that their distribution cannot be an equilibrium distribution.

The measured (Crowther and Pearse, 1981) minimum clathrin density of about 0.1 μM/l for which cage assembly is observable may be identified with c_2, which results in an estimate $\Delta f_1 \approx 7.6$ kcal/M $\approx 13\ k_BT$. The estimated concentration range corresponding to cooperative polymerization growth would be $c_1/c_2 \approx 25$. These "critical" concentrations are of course strong functions of pH and ionic strength, which affect Δf.

Let us now discuss the structure of the cages in more detail. The cage consisting of 12 pentagons and n_h hexagons is made up of $20 + 2n_h$ triskelions and has a diameter of approximately $46\sqrt{1 + 0.13\ n_h}$ nm (estimated from the total area of pentagons and hexagons). This estimate of the diameter assumed a spherical shell; however, for a given n_h there exist a number of isomers that differ in the arrangement of hexagons and, therefore, in their shape. For n_h not too large, the isomers have been enumerated (Fowler et al., 1992): for example, for $n_h = 4$ there are two isomers, for $n_h = 8$ there are 15 isomers, including HB and TB, and for $n_h = 20$ the buckyball is one of 1812 possible structures. As noted above, the distribution of sizes and isomers is not likely to be thermal. Instead it is determined by the kinetics of assembly, with the relative abundance of a given structure proportional to the number of ways it can be assembled, each weighed with its probability.

In the cooperative polymerization regime, $c_2 < c < c_1$, the assembly can be described in terms of the addition of closed pentagonal or hexagonal faces (assuming, in accordance with observations, that the probability of forming squares or heptagons is negligible under ordinary conditions). The different positions on the periphery of the growing cluster where a new face can be added differ in the number of triskelions, l, that must be added to make a pentagon, which varies from 3 to 0, depending on the local structure of the boundary. A hexagon, of course, always requires $l + 1$. Hence, the addition of a new face on a given site is the order l (or $l + 1$) process. The relative probability of adding a face at the position with certain l is proportional to c^l. The probability of adding a hexagon rather than a pentagon at a given position is controlled by the competition of the addition of triskelions and the closure of pentagonal rings. The ratios of probabilities of adding a pentagon and adding a hexagon are $p_5/p_6 = \Gamma c_1/c$ (where Γ is the rate of pentagon closure normalized to the rate of triskelion addition, and we have assumed tacitly that the rate of closure of hexagons is very high).

The probability of closing off a pentagonal face must depend, however, on the elastic strain introduced by it and will depend on the structure of the cluster and the position of the new face. We shall not attempt to solve for the elastic energy of the growing cage and instead introduce the relevant physics crudely by associating with each vertex a curvature energy, $\alpha\kappa$, with the local "curvature" κ depending on the three faces adjacent to the vertex. Specifically, let $\kappa(i) = (\pi/15)\ N_p(i)$, where $N_p(i)$ is the number of pentagons meeting at vertex i. Hence a three-hexagon, $N_p = 0$ vertex introduces no strain, whereas for three adjoining pentagons, $N_p = 3$, the local curvature is a maximum and so is the elastic energy cost. For each new face we can compute the total energy as a sum over its 3-valent vertices, resulting in $\Gamma = \Gamma_0 \exp(-(\alpha/k_BT)\ \Sigma\kappa(i))$, where $\Gamma_0 = \exp(-\Delta f_1/k_BT)$. This factor favors hexagons and will compete with the c_1/c factor favoring pentagons in p_5/p_6.

To implement the assembly process numerically, we represent the structure of the cage by an ordered list of faces, each face being an ordered list of (five or six) vertices. In addition, there is a list of peripheral vertices with their valence labels. Any sequential pair of 2-valent vertices defines a possible locus of face addition with its order, l, being 3 minus the number of sites intervening between the 2-valent cites. The faces are added at the loci with the relative probability $c^l/(c_1^l + c^l)$ defined so that the assembly model extrapolates sensibly into the regime of linear polymerization, $c > c_1$. The relative probability of adding a pentagon or a hexagon is as defined above. The numerical simulation was carried out using Mathematica (Wolfram, 1992).

Fig. 2 presents histograms of n_h for several values of c ranging from c_2 to above c_1. As expected, the distribution shifts, with increasing c, toward larger cages. Fig. 3 shows the mean $\langle n_h \rangle$ and the variance $\langle \Delta n_h^2 \rangle = \langle n_h^2 \rangle - \langle n_h \rangle^2$ of the n_h distributions as a function of $\ln(c/c_1)$. The distribution is sub-Poisson, i.e., $\langle \Delta n_h^2 \rangle < \langle n_h \rangle$, for small c/c_1 but becomes super-Poisson, $\langle \Delta n_h^2 \rangle > \langle n_h \rangle$, in the linear polymerization regime, $c/c_1 > 1$. It must be remarked that the probabilistic local assembly rules defined above do not guarantee that the cage will be completed as a perfect fullerene: the final face may not turn out to be a pentagon or hexagon. The number of such defects increases with c/c_1 becoming quite large ($\sim$50%) in the linear polymerization regime, where the size distribution is very broad. Remarkably, in the slow growth limit the number of defects is quite small. The clathrin rigidity parameter α in Fig. 2 was chosen to make the probability of having three pentagons meeting at a vertex small. A smaller value of α makes the curvature effect weaker and shifts the distribution toward smaller n_h; a higher value of α has an opposite effect, as shown in Fig. 4.

An interesting feature of the n_h histogram for small c is the abundance of $n_h \approx 8$ cages. For example, the dominant

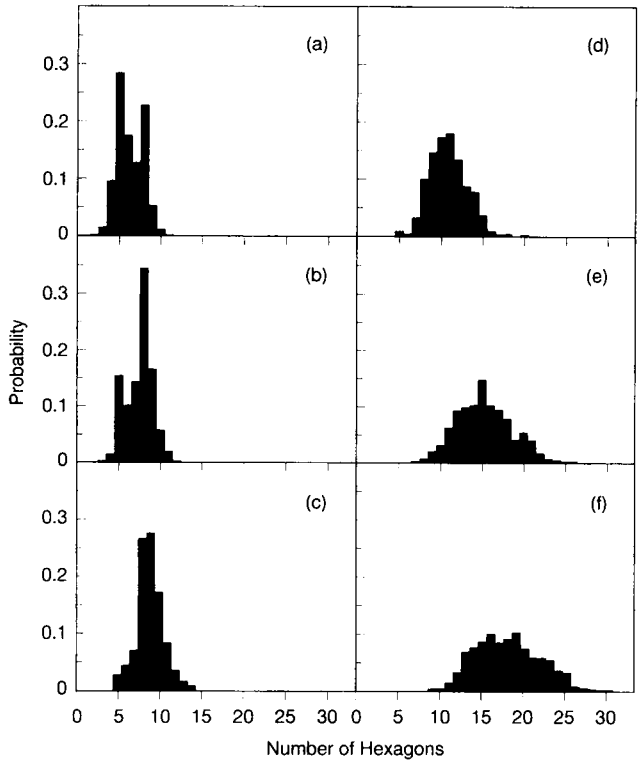

FIGURE 2 The histogram of n_h for 500 coats generated by the assembly model with $|\Delta f_1| = 13k_BT$, $\alpha = 0.85\ |\Delta f_1|$ and (*a*) $c/c_1 = 0.05$, (*b*) $c/c_1 = 0.1$, (*c*) $c/c_1 = 0.2$, (*d*) $c/c_1 = 0.4$, (*e*) $c/c_1 = 0.8$, (*f*) $c/c_1 = 1.2$. Note that *f* is in the linear polymerization regime.

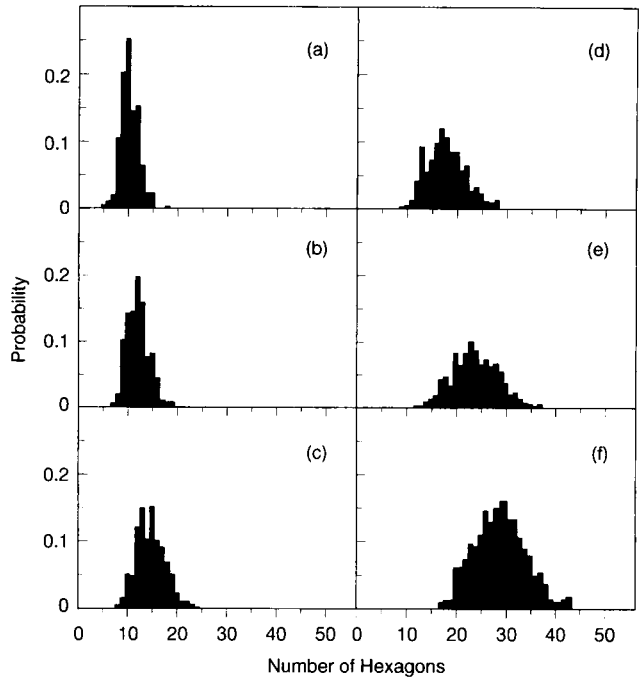

FIGURE 4 The histogram of n_h for 500 coats generated by the assembly model with $|\Delta f_1| = 13k_BT$, $\alpha = |\Delta f_1|$ and (*a*) $c/c_1 = 0.05$, (*b*) $c/c_1 = 0.1$, (*c*) $c/c_1 = 0.2$, (*d*) $c/c_1 = 0.4$, (*e*) $c/c_1 = 0.8$, (*f*) $c/c_1 = 1.2$. Note that *f* is in the linear polymerization regime. An increase in the shell rigidity parameter α compared to Fig. 2 has led to a shift of the cage distribution toward larger sizes.

structures in Fig. 2 *c* are the $n_h = 8$ TB (88%) and HB (12%) cages and the $n_h = 9$ "screwball" consisting of a linear chain of pentagons and a chain of hexagons twisted and glued together. Scaling up the values of Δf_1 and α leads, for small c/c_1, to a distribution sharply peaked at $n_h = 8$. In this limit the assembly process very nearly reduces to a simple rule: the new face is always a pentagon, except if it were to introduce an $N_p = 3$ vertex, in which case a hexagon is added instead. The former is due to the low rate of assembly and latter is due to α being large enough to suppress $N_p = 3$ vertices. It is easy to prove that the smallest fullerenes with no such vertices have $n_h = 8$ and are in fact the HB and the TB structures. Therefore they will be generated by the above assembly rule.

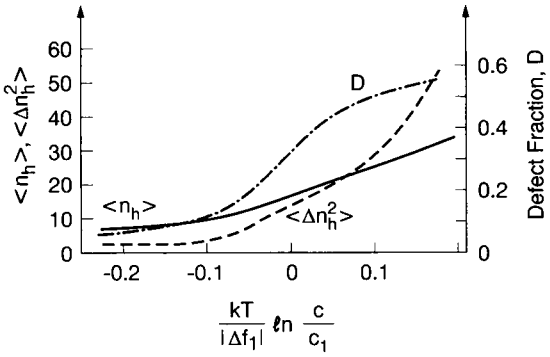

FIGURE 3 The average $\langle n_h \rangle$ (—) and the variance $\langle \Delta n_h^2 \rangle$ (- - -) of the n_h distribution as a function of lnc/c_1 (same parameter values as in Fig. 2). Fraction of defective cages, *D*.

It is natural to hypothesize that the coat assembly in the presence of adaptors (at normal pH) is in a similar limit of the cooperative polymerization regime. Because the completion of each face in that case requires the recruitment of adaptors, the nucleation process will depend on some (stoichiometry dependent) (Keen, 1991; Kirchhausen, 1993) power of adaptor concentration. This would shift the assembly process toward the slow growth limit and effect a stronger bias of the assembly kinetics toward pentagons than in the case of pure clathrin, resulting in the relative abundance of HB and TB structures. A more detailed model of clathrin/adaptor coassembly, based on the observed stoichiometry (Keen, 1991; Kirchhausen, 1993), will be discussed elsewhere.

Finally, we consider the growth of a clathrin-coated pit on the membrane. Even without detailed knowledge of clathrin-adaptor-membrane interaction, one expects that there exists a range of clathrin and adaptor concentrations corresponding to the cooperative polymerization regime. As in the case of the cage assembly, the kinetics would favor the addition of pentagons but must compete with the elastic energy, which in this case should be dominated by the membrane curvature stiffness, σ. The rate of closure of

pentagonal faces would then be controlled by the rate of curvature fluctuations of the membrane, which can be crudely estimated by using the Helfrich Hamiltonian (Helfrich, 1985):

$$\tau^{-1} = \frac{\sigma}{\eta L^3} \exp\left(- \frac{2\sigma}{k_B T} \kappa \right),$$

where $L \approx 10$ nm is the characteristic size of the face, $\eta \approx 10^{-2}$ poise is the viscosity, κ is the Gaussian curvature integrated over the area of the face, which as before we shall estimate as a sum of local curvatures, $\kappa_i = (\pi/15)/N_p(i)$; at the vertices defining the face. Assuming that $\sigma \approx 0.5 \times 10^{-12}$ erg $\approx 10\ k_B T$ and estimating the characteristic frequency of the membrane fluctuations (the prefactor of the exponential above) as $\sim 5 \times 10^7\ s^{-1}$ implies a rather severe constraint on the magnitude of curvature fluctuations that occur on a realistic assembly time scale. Estimating the minimum curvature required for pentagon addition to be $\pi/3$ (the case in which there are no neighboring pentagons), we find the rate of pentagon addition to be about $0.05\ s^{-1}$. Because the addition of a pentagon next to an existing one requires a larger curvature fluctuation, its rate would be much lower: e.g., suppressed by a factor $\exp(2\sigma\Delta\kappa/k_B T) \approx 0.2 \times 10^{-3}$ (where $\Delta\kappa = 2\pi/15$ is the extra curvature due to two vertices shared by the two pentagons). The above is likely to be an underestimate for the rates; however, it does illustrate that the appearance of two pentagons next to each other would be suppressed, compared to isolated pentagons, possibly by a large factor, so that the smallest coat must be a buckyball ($n_h = 20$), the smallest fullerene with no neighboring pentagons. As in the case of the cage assembly, increasing the rate of assembly (by increasing clathrin and adaptor concentrations) would lead to larger n_h and larger size coats. An increase in the rigidity of the membrane or a suppression of curvature fluctuations due to the attachment of the membrane to a substrate would also favor the formation of hexagonal lattices (Hauser and Evans, 1980; Kirchhausen, 1993).

To summarize, we have argued above that the size distribution of clathrin cages is controlled by the rate of assembly rather than by the intrinsic curvature of the triskelia. Because the conditions of the in vitro experiments were optimized to produce small cages, one expects them to correspond to the slow assembly regime (i.e., small c/c_1). High free clathrin concentrations should lead to nonnucleated growth and large defective cages and tree-like aggregates. The broad size distribution observed experimentally (Zaremba and Keen, 1983) may be due in part to the variation of c with time. Applying the present ideas to the case of the coated pit assembly, we proposed a thermal ratchet-type mechanism (Feynman et al., 1966) in which the assembly of clathrin pentagons requires a local thermal fluctuation of the membrane. The present analysis can be confirmed or falsified by an in vitro investigation of the systematic dependence of the distribution of cage sizes on clathrin concentration and pH. Additional information about the structure of the coats for different growth conditions can be most easily obtained by collecting the statistics of N_p and the statistics of occurrence of different faces adjacent to an edge. It would also be most interesting to investigate the dependence of the coated vesicle size distribution on clathrin and adaptor concentrations as well as the membrane tension, which can be controlled in an in vitro experiment.

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