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When the nucleation of a stable crystalline phase directly in a supersaturated old phase is greatly retarded, the crystal nuclei might nucleate within faster-forming particles of an intermediate phase. Here we present a theoretical investigation of the kinetics of this two-step nucleation of crystals and derive general expressions for the time dependence of the number of crystals nucleated within the particles of the intermediate phase. The results reveal that crystal nucleation can be strongly delayed by the slow growth of the particles and/or by the slow nucleation of the crystals in them. Furthermore, the linear part of the time dependence of the number of nucleated crystals is determined by the formation rate of the intermediate particles. This is in contrast with the one-step nucleation of crystals when this linear part is determined by the rate of crystal nucleation directly in the old phase. Criteria are proposed for distinction between the one- and two-step nucleation mechanisms, based on the supersaturation dependence of the delay time for nucleation. The application of the theoretical approach to the analysis of experimental data on the nucleation of crystals and other ordered aggregates of protein and other soluble materials is discussed. © *2005 American Institute of Physics*. [DOI: 10.1063/1.1943389]

I. INTRODUCTION

Materials of increasing complexity are crystallized for various applications from various media, but, in particular, from solutions. Crystals of proteins are grown for the purposes of structural biology;[1] large organic molecules are crystallized in pharmaceutical processes.[2] The formation of crystals and other ordered aggregates of proteins is of further interest, because they are often associated with debilitating and deadly diseases: anemia,[3,4] cataract,[5] and others. A common feature of the solutions of such large molecules is that at ionic strengths >0.1 M, where the characteristic Debye length of the electrostatic interactions is comparable (or, at higher ionic strengths, shorter) than the surface roughness of the molecules, the range of interactions between the solute molecules is largely determined by the *solvent* molecular size and is significantly shorter than the size of the *solute* molecules.[6] As a result liquid–liquid (L–L) phase separation[7–10] in such solutions is metastable with respect to the solution–crystal equilibrium.[11–14]

The complexity of the phase behavior allows for a complex dynamics of the formation of new crystalline phases at all stages of the phase transformation.[15] Over the years, the problem of the formation of the nuclei (the smallest clusters of the new crystalline phase that are capable of spontaneous overgrowth) has emerged as particularly challenging.[16] Crystal nucleation is hard to describe theoretically and difficult to quantify experimentally even in simple cases of pure substances with well-understood interactions. For systems with complex phase diagrams, general considerations invoking the Oswald rule of stages suggest that if a metastable phase exists, it may be involved in the process of formation of the stable crystalline phase. More rigorous simulation and theory have predicted that the metastability of the dense liquid phase with respect to the crystalline phase may provide for a novel mechanism of the solution-to-crystals phase transition.[17–22] According to this mechanism, the nucleation of the crystalline nuclei proceeds in two steps: a droplet of a dense liquid forms, within which a crystalline nucleus appears due to the ordering of a certain number of molecules.[10,19,23,24] This mechanism has been shown to apply for two cases (i) where the liquid phase is metastable with respect to the crystals, but has lower free energy than the low-concentration solution, e.g., Refs. 12 and 25, and (ii) where the dense liquid phase is doubly metastable with respect to both crystals and low-concentration solution and only exists as small disordered fluid clusters of limited lifetime,[15,26] e.g., Refs. 23, 24, and 27–29.

While the original theoretical works suggested that the existence of the dense liquid precursor might lower the nucleation barrier and enhance the rate of crystal nucleation by many orders of magnitude, they did not analyze the statistical and kinetic relations associated with the two-step nucleation mechanism and did not provide the criteria for comparison with the experimental data. The objective of the study presented here is to derive a general expression for the

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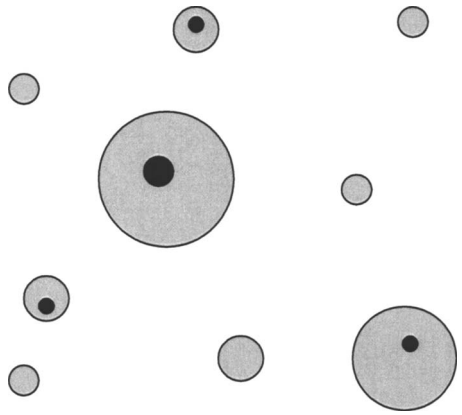

FIG. 1. Two-step nucleation by the mononuclear mechanism: only one crystal (shown black) nucleates within a particle of an intermediate phase (shown gray).

time dependence of the number of crystals formed during two-step nucleation and to apply this expression to two particular cases. For both cases, we only consider stationary nucleation of protein crystals in steadily forming droplets of an intermediate protein phase. While below we imply that the precursor is a liquid and the nucleating final phase is a crystal, the formalism that we develop is applicable to all cases of sequential nucleation of one phase within an intermediate phase, which may be liquid, gel, ramified, or even another crystalline polymorph.[30] Another important example of potential application of the results of this work is the formation of sickle cell hemoglobin polymers, an ordered solid phase, which underlies the deadly sickle cell anemia:[3,31] emerging evidence suggests that a two-step mechanism might apply to the polymer nucleation.

II. PHYSICAL MODEL

We consider the physical model, schematically illustrated in Fig. 1. At a given moment $t=0$ an old phase (gas, liquid, or solid) of macroscopically large volume V (m^3) becomes supersaturated with respect to a stable crystalline phase. Direct (i.e., one-step) nucleation of crystals in the old phase is infinitely retarded with respect to the generation of particles of an intermediate phase (liquid or solid), which appears randomly at a rate $j(t)$ ($m^{-3}\,s^{-1}$). The intermediate-phase particles grow radially according to the law

$$r(t) = (Gt)^m, \tag{1}$$

where r (m) is the effective radius of a particle, G ($m^{1/m}\,s^{-1}$) is the growth constant, and $m>0$ is the growth exponent (e.g., $m=1/2$ or 1 for growth controlled by diffusion or interface transfer, respectively). When the particles become sufficiently large, nucleation of crystals at a rate $j_c(t)$ ($m^{-3}\,s^{-1}$) begins in them.

We also assume that just one crystal can nucleate within one particle. Different physical mechanisms can underlie this assumption: if the growth rate of the first crystal nucleus is very fast, the entire material in the particle will turn into a crystal before a second nucleus can form; if the formation of a crystal nucleus changes the chemical potential of the remaining noncrystalline material in the particle, in a way that may prevent crystallization. This assumption leads to the so-called mononuclear mechanism,[16] according to which the number of nucleated crystals is equal to the number of particles in which crystals have formed; below, we call such particles crystallized.

It is clear that when the nucleation of the crystals occurs solely within the particles of the intermediate phase, the kinetics of the crystallization process becomes dependent on the kinetics of particle formation and growth. In particular, the overall formation of the crystalline phase is delayed with respect to that of the intermediate phase.[16,32] Correspondingly, within the scope of the mononuclear mechanism, at any time t the number $N_c(t)$ of crystals in the old phase is always smaller than or at most equal to the number $N(t)$ of particles of the intermediate phase that appeared until that time. To reveal how crystal nucleation is controlled by the formation and growth of the particles of the intermediate phase, below we first obtain general results for the $N_c(t)$ dependence and then apply them for two particular cases that might be important for the crystallization of proteins and other large molecules in solution or for the formation of other ordered aggregates of such materials.

III. GENERAL RESULTS

With the assumption that two-step nucleation follows the mononuclear mechanism, the number of nucleated crystals is equal to the number of particles containing a crystal, Fig. 1. For that reason, at time t the number N_c of crystals nucleated by this mechanism within a fixed number N_0 of particles of equal constant volume v_0 (m^3) is given by[16,33]

$$N_c(t) = N_0\left\{1 - \exp\left[-\int_0^t j_c(t')v_0 dt'\right]\right\}. \tag{2}$$

This formula is a generalization of the expression[16,33,34]

$$N_c(t) = N_0[1 - \exp(-J_c v_0 t)] \tag{3}$$

in which J_c ($m^{-3}\,s^{-1}$) is the time-independent value of j_c, i.e., the stationary rate of nucleation of crystals in the particles.

To employ Eq. (2) to the case of growing particles, we use a time-dependent particle volume $v(t',t)$, where $t' \leqslant t$ is the moment of particle formation. With this definition, particles formed first (at $t'=0$) are the largest and nucleation of crystals in them is most likely. Conversely, particles formed last (at $t'=t$) have zero volume and crystals cannot nucleate within them. Equation (2) also shows that the probability of nucleation of crystals N_c/N_0 becomes larger with the increase of the volume of the particles of the intermediate phase. Thus, more generally, Eq. (2) becomes

$$N_c(t) = N_0\left\{1 - \exp\left[-\int_0^t j_c(t')v(t',t)dt'\right]\right\}, \tag{4}$$

where, according to Eq. (1), at time t the volume v of a particle formed at time t' is given by

$$v(t',t) = cr^d = cG^{md}(t-t')^{md}. \tag{5}$$

Here $d=1$, 2, 3 is the dimensionality of growth and c (m^{3-d}) is the shape factor of the growing particles; for values of c for different geometries see Table I. We note that with $d=0$

TABLE I. Dimensionality d and shape factor c for nongrowing particles with volume v_0 (m^3), for growing needles with constant cross-sectional area A_0 (m^2), for growing disks or square prisms with constant thickness H_0 (m), and for growing spheres or cubes.

Shape	d	c
Any	0	v_0
Needle	1	$2A_0$
Disk	2	πH_0
Square prism	2	$4H_0$
Sphere	3	$4\pi/3$
Cube	3	8

and $c=v_0$, formally, Eq. (5) applies also to crystal nucleation within particles of equal constant volume v_0: then Eq. (4) passes into Eq. (2).

We can now derive a general formula for the $N_c(t)$ dependence when the number N of particles within which crystal nucleation occurs is not fixed (i.e., not equal to N_0), but changes with time in a known way. To this end, we use Eq. (4), but in a differential form, i.e., for dN_c and dN rather than for N_c and N_0. Indeed, if we consider a small number dN of particles of the intermediate phase that are formed between τ and $\tau+d\tau$, at a later time $t \geqslant \tau$ a small number dN_c of these dN particles will be crystallized by the mononuclear mechanism. Treating τ as the initial moment of crystal nucleation in the dN particles, analogous to Eq. (4) we can write

$$dN_c = \left\{ 1 - \exp\left[- \int_\tau^t j_c(t') v(t',t) dt' \right] \right\} dN. \tag{6}$$

This relation parallels the one used elsewhere[16,32] to couple the fractions of crystallized volume in two-step crystallization. We note that an equation similar to Eq. (6) was employed for describing the coverages of the successive layers in nucleation-mediated polylayer growth of crystals[35] and thin solid films.[16,36] Integrating the right-hand side of Eq. (6) from $\tau=0$ to $\tau=t$ and its left-hand side from $N_c=N_c(0)=0$ to $N_c=N_c(t)$ yields

$$N_c(t) = N(t) - \int_0^t \exp\left[- \int_\tau^t j_c(t') v(t',t) dt' \right] \times [dN(\tau)/d\tau] d\tau, \tag{7}$$

because our considerations are restricted to cases in which $N(0)=0$.

Equation (7) represents the sought general time dependence of the number of crystals appearing by the mononuclear mechanism within continuously forming and growing particles of an intermediate phase. This equation can also be used with $v(t',t)$ dependence different from that given by Eq. (5). Equation (7) reveals that N_c depends not only on the kinetics of nucleation of the crystals themselves within the particles (i.e., on j_c), but also on the formation rate dN/dt and the growth law of the particles. This general equation shows that always $N_c(t) \leqslant N(t)$, with the equality holding only in the limit of $j_c \to \infty$, i.e., in the case when a crystal instantaneously nucleates in every appearing particle. We note also that in the particular case of particles formed instantaneously at $t=0$, as required, Eq. (7) passes into Eq. (4). Indeed, in this case $N(t)=N_0$ for all $t \geqslant 0$, and the particle formation rate is given by the expression $dN/dt=N_0\delta(t)$, where δ is the Dirac delta function. Employing this expression for dN/dt in Eq. (7) and evaluating the $d\tau$ integral with the help of the general relation $\int_0^t \delta(\tau) y(\tau,t) d\tau = y(0,t)$ transforms Eq. (7) into Eq. (4) (y is an arbitrary function of τ and t).

Below, we consider in detail only the case where the number of particles increases in time according to the law[16]

$$N(t) = V \int_0^t j(t') dt', \tag{8}$$

which is valid as long as the total volume of the particles is significantly smaller than the volume V of the old phase. In this case, from Eq. (7) we obtain

$$N_c(t) = N(t) - V \int_0^t j(\tau) \exp\left[- \int_\tau^t j_c(t') v(t',t) dt' \right] d\tau. \tag{9}$$

Using Eqs. (7) and (9) allows a straightforward calculation of the number $N_p(t)$ of uncrystallized particles at time t (these are the particles within which no crystals were nucleated until that time). Since $N_p(t) \equiv N(t) - N_c(t)$, from these equations we find that

$$N_p(t) = \int_0^t \exp\left[- \int_\tau^t j_c(t') v(t',t) dt' \right] [dN(\tau)/d\tau] d\tau, \tag{10}$$

in general, and that

$$N_p(t) = V \int_0^t j(\tau) \exp\left[- \int_\tau^t j_c(t') v(t',t) dt' \right] d\tau \tag{11}$$

in the concrete case of $N(t)$ given by Eq. (8).

IV. STATIONARY FORMATION OF PARTICLES AND NUCLEATION OF CRYSTALS

An important special case of two-step nucleation of crystals is that of the stationary formation of the intermediate-phase particles in the old phase and the stationary nucleation of the crystals within the particles. Then the rates j and j_c are time independent[16] and denoting, respectively, their stationary values as J and J_c, from Eqs. (5), (8), (9), and (11) we get

$$N_c(t) = JVt - JVa \int_0^{t/a} \exp(-x^{md+1}) dx, \tag{12}$$

$$N_p(t) = JVa \int_0^{t/a} \exp(-x^{md+1}) dx, \tag{13}$$

where x is an integration variable and the parameter a (s) is given by

$$a = [(md+1)/cG^{md}J_c]^{1/(md+1)}. \tag{14}$$

Equations (12) and (13) show that asymptotically, i.e., for $t \gg a$, N_c becomes a linear function of time, and N_p reaches a maximum value $N_{p,\max}$,

$$N_c(t) = JV(t-\theta), \tag{15}$$

$$N_{p,\max} = JV\theta. \tag{16}$$

Here θ (s) is the intercept of N_c on the time axis and has the physical meaning of delay time of the process of two-step crystal nucleation. This time is given by the expression

$$\theta = a\int_0^\infty \exp(-x^{md+1})\mathrm{d}x = \Gamma[(md+2)/(md+1)] \times[(md+1)/cG^{md}J_c]^{1/(md+1)}, \tag{17}$$

where Γ is the complete gamma function. This expression shows that the slow growth (G) of the particles and/or the slow nucleation (J_c) of the crystals within them result in a longer delay of the two-step crystal nucleation. We also see that $N_{p,\max}$ contains no new information; it is merely the product of the delay time θ and the slope JV of the linear portion of the $N_c(t)$ function.

In the opposite limiting case of short times, i.e., for $t \ll a$, Eq. (12) predicts a power dependence of N_c on t. Indeed, then for the integrand in Eq. (12) we have $\exp(-x^{md+1}) \approx 1 - x^{md+1}$ and evaluating the integral yields

$$N_c(t) = [cG^{md}J_cJV/(md+1)(md+2)]t^{md+2}. \tag{18}$$

Accordingly, for $t \ll a$ the $N_p(t)$ function from Eq. (13) takes the form

$$N_p(t) = JVt - [cG^{md}J_cJV/(md+1)(md+2)]t^{md+2}. \tag{19}$$

V. APPLICATION TO TWO-STEP NUCLEATION OF PROTEIN CRYSTALS

We can now apply the above general results to two particular cases of the stationary nucleation of protein crystals in steadily forming droplets of an intermediate protein phase. When the process occurs in a supersaturated solution that is in the region below the liquid–liquid separation line in the corresponding phase diagram, the droplets represent a liquid protein phase which is stable with respect to the solution, but metastable with respect to the crystalline protein phase—then j and J are merely the time-dependent and the stationary rates of droplet nucleation in the solution. When, however, the crystallization conditions are such that the supersaturated solution is in the region above the liquid–liquid separation line in the phase diagram, the appearance of protein droplets is not mediated by nucleation and j and J do not represent nucleation rates, but, rather, the rates of nonnucleation formation of the droplets.

Let us first consider the case of droplets ($d=3$, $c=4\pi/3$) appearing at stationary rate J and having radii that increase linearly with time [then $m=1$, see Eq. (1)]. From Eqs. (12)–(19) it follows then that

$$N_c(t) = JVt - JVa\int_0^{t/a} \exp(-x^4)\mathrm{d}x, \tag{20}$$

$$N_p(t) = JVa\int_0^{t/a} \exp(-x^4)\mathrm{d}x, \tag{21}$$

where

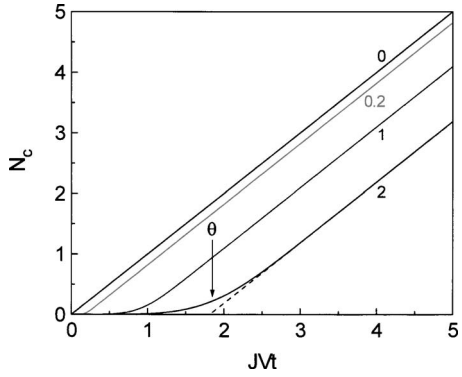

FIG. 2. Time dependence of the number of crystals: lines 0, 0.2, 1, and 2—Eq. (20) at $JVa=0$, 0.2, 1, and 2, respectively; dashed line—Eq. (25) at $JVa=2$.

$$a = (3/\pi G^3 J_c)^{1/4}. \tag{22}$$

Initially ($t \ll a$), we have

$$N_c(t) = (\pi/15)G^3J_cJVt^5, \tag{23}$$

$$N_p(t) = JVt - (\pi/15)G^3J_cJVt^5, \tag{24}$$

and asymptotically ($t \gg a$), N_c and N_p are again given by

$$N_c(t) = JV(t-\theta), \tag{25}$$

$$N_{p,\max} = JV\theta, \tag{26}$$

with the delay time

$$\theta = \Gamma(5/4)(3/\pi G^3 J_c)^{1/4}. \tag{27}$$

The lines in Fig. 2 display the $N_c(t)$ dependence according to Eq. (20) at $JVa=0$, 0.2, 1, and 2. The initial parabolic and the asymptotic linear dependencies of N_c on t are clearly seen. The arrow indicates the delay time θ only at $JVa=2$; this time shortens with decreasing a, i.e., with increasing the product $(G^3J_c)^{1/4}$. At $(G^3J_c)^{1/4}=\infty$, in agreement with Eq. (27), we have $\theta=0$, because then the crystals nucleate in the droplets at the very moment of the droplet appearance. In this case the $N_c(t)$ dependence is linear for all times and takes the form $N_c(t)=N(t)=JVt$. Accordingly, there are no uncrystallized droplets and $N_p(t)=0$. Thus, although in this case no droplets can be detected in the old phase because of their instantaneous crystallization, J_c has no effect on the number N_c of nucleated crystals—this number is entirely controlled by J, i.e., by the kinetics of droplet formation.

As a second case, let us consider droplets appearing again at stationary rate J, but with equal fixed volume v_0. As noted above, in this case we have $d=0$ and $c=v_0$, so that from Eqs. (12)–(19) we obtain

$$N_c(t) = JVt - (JV/J_cv_0)[1 - \exp(-J_cv_0t)], \tag{28}$$

$$N_p(t) = (JV/J_cv_0)[1 - \exp(-J_cv_0t)]. \tag{29}$$

Initially, i.e., for $t \ll 1/J_cv_0$, N_c increases quadratically with time,

$$N_c(t) = (1/2)JVJ_cv_0t^2, \tag{30}$$

and the $N_p(t)$ dependence reads

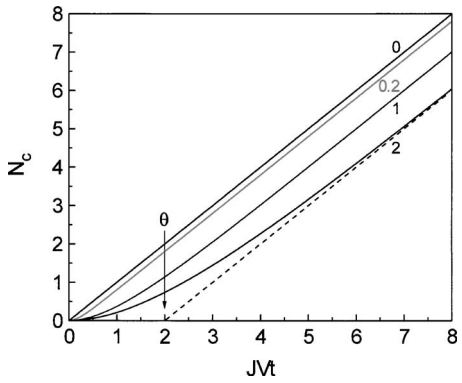

FIG. 3. Time dependence of the number of crystals: lines 0, 0.2, 1, and 2—Eq. (28) at $JV/J_c v_0=0$, 0.2, 1, and 2, respectively; dashed line—Eq. (32) at $JV/J_c v_0=2$.

$$N_p(t) = JVt - (1/2)JVJ_c v_0 t^2. \tag{31}$$

Asymptotically ($t \gg 1/J_c v_0$), N_c and N_p are again of the form

$$N_c(t) = JV(t-\theta), \tag{32}$$

$$N_{p,\max} = JV\theta, \tag{33}$$

where θ, given by

$$\theta = 1/J_c v_0, \tag{34}$$

is merely the mean time for the appearance of a crystal nucleus in a droplet.[16]

The lines in Fig. 3 depict the $N_c(t)$ dependence (28) at $JV/J_c v_0=0$, 0.2, 1, and 2. This dependence is analogous to the one in Fig. 2; N_c initially increases parabolically with time and, at longer times, becomes linear. The delay time θ (indicated by the arrow only for $JV/J_c v_0=2$) shortens with increasing $J_c v_0$ and vanishes in the limit of $J_c v_0 \to \infty$, because then the crystals nucleate in the droplets virtually at the very moment of droplet formation.

VI. DISCUSSION AND CONCLUSIONS

The simplest case of two-step nucleation is when the rate of formation of the intermediate-phase particles is significantly faster than the rate of nucleation of crystals within them, and the particles quickly reach time-independent volume v_0 and number N_0. This is the case of instantaneous particle formation, discussed in Sec. III. The values of v_0 and N_0 may reflect stable or metastable equilibrium between the intermediate-phase particles and the old phase, as envisioned in Ref. 26. The nucleation of crystals within these instantaneously formed equisized N_0 particles would then follow Eq. (3). If the typical observation times t are much shorter than the characteristic time $1/J_c v_0$, then Eq. (3) simplifies to

$$N_c(t) = N_0 J_c v_0 t, \tag{35}$$

i.e., N_c increases proportionally to the rate of nucleation of crystals in the particles. Equation (35) indicates that analyses of $dN_c/dt = N_0 J_c v_0$ and its variations with temperature, supersaturation, solution composition, and other system parameters, such as those in Refs. 23, 24, and 37–39 provide insights, including the nucleus size and its variation with increasing supersaturation, into the nucleation of crystals within the intermediate-phase particles, the rate-determining step of the nucleation process.

In the case of steadily increasing number $N=JVt$ of intermediate-phase particles, the above results show that the delay time θ and the slope JV of the linear part of the $N_c(t)$ dependence are determined by the particle-formation rate J, growth constant G, and by the rate J_c of crystal nucleation in the particles. The time dependence of the number $N_p(t)$ of uncrystallized particles provides no additional information, because its linear part has also the slope JV, and, according to Eq. (16), its plateau value $N_{p,\max}$ is proportional to θ. Nonetheless, it is advantageous to determine in an experiment both the $N_c(t)$ and $N_p(t)$ dependencies, because the equality of the slopes of their linear parts is an unambiguous evidence for two-step nucleation.

If only the $N_c(t)$ dependence is known, it is not obvious whether the delay time θ is due to a two-step nucleation process; even in the one-step (i.e., direct) nucleation of crystals in the old phase, $N_c(t)$ may have a delay followed by a linear part.[16] In this case the delay time is not given by Eq. (17), but is the sum of the time t_n for the establishment of stationary nucleation[40,41] and the time t_g for the growth of the crystal nuclei to a detectable size,[16]

$$\theta = t_n + t_g. \tag{36}$$

To distinguish between one- and two-step nucleation from experimental data, one has to rely on the different dependencies of θ defined by Eqs. (17) and (36) on the supersaturation $\Delta\mu$ of the system ($\Delta\mu$ is the difference between the chemical potentials of the old phase and the nucleated crystalline phase). Usually t_n and t_g are relatively weak functions of $\Delta\mu$ and one expects a weak $\theta(\Delta\mu)$ dependence for one-step nucleation. In contrast, for two-step nucleation θ from Eq. (17) is expected to change considerably with $\Delta\mu$ because J_c is an exponential function of supersaturation.

An important feature of the two-step nucleation mechanism is that according to Eqs. (15), (16), and (19), the slope of the linear portions of the $N_c(t)$ and $N_p(t)$ curves is proportional to the rate J of formation of the intermediate-phase particles, while the delay time θ of the $N_c(t)$ and the plateau of the $N_p(t)$ dependencies are determined by the nucleation rate J_c of the crystals within the particles. This interpretation of the slope of experimentally obtained $N_c(t)$ curves in two-step crystal nucleation differs radically from that in the case of one-step nucleation, where the slope is proportional to the rate $J_{c,1}$ of crystal nucleation directly in the old phase.

Experimentally, it would be advantageous to obtain the $N_c(t)$ and/or $N_p(t)$ dependencies at different supersaturations $\Delta\mu$ of the system. Then J and θ can be determined as functions of $\Delta\mu$ and can be used for the verification of theories for particle formation and growth in the old phase and for crystal nucleation within the particles.

The model that we analyze here is based on assumptions that may limit its applicability. For instance, one can readily envision cases in which the particle-growth law is different from the simple power law in Eq. (1). Also, the crystals within the particles of the intermediate phase nucleate under supersaturation, which in fact varies with the particle size because of the Gibbs–Thomson effect. This effect is negli-

gible only for sufficiently large particles; thus, the assumption of a stationary rate of nucleation of crystals within the particle J_c only applies to large particles. Furthermore, it is important to consider a mechanism in which more than one crystal could appear in each particle of the intermediate phase. Accounting for this effect is needed for a more accurate description of crystallization processes in two-step nucleation.

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