Hydrophilic Carotenoids: Surface Properties and Aggregation of Crocin as a Biosurfactant

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The surface and aggregation properties of the naturally occurring carotenoid crocin (**1**), examined through measurements of surface tension and UV/VIS absorption, have been used to determine the following parameters: critical aggregate concentration, surface-saturation concentration, molecular area, free energy of adsorption and micellation, adsorption-micellar energy relationship, equilibrium constants, and aggregate size. On structural grounds and based on the determined molecular area at the interface, the digentiobiosyl ester of the conjugated, highly unsaturated carotenoid diacid crocetin C20:7 should be classified as a bolaamphiphile. Crocin forms true monomolecular solutions in H_2O; only at rather high concentrations aggregation occurs.

Introduction. – Almost 750 naturally occurring carotenoids have been characterized, nearly all of them being hydrophobic, with the notable exception of a few diacids, sugar esters, and sulfates [1]. Generally, the solubility of carotenoid sulfates in H_2O is too low (*ca.* 0.4 mg/ml) [2] to be of practical interest. However, the diacid norbixin, for example, is water-soluble up to 5% (*colorMaker*, Anaheim, CA, USA), and the sugar ester crocin (**1**) has no saturation point in H_2O [3]. The above-mentioned compounds possess hydrophilic and hydrophobic parts that confer an amphiphilic character, manifested by surfactant activity and a tendency for aggregate formation. Interestingly, the detergent properties of naturally occurring crocin (=β,β-digentiobiosyl 8,8′-diapocarotene-8,8′-dioate; **1**), bixin, and carotenoid sulfates in general have not yet been determined, whereas the aggregation characteristics of other carotenoids with near-zero aqueous affinities have found early interest [4–6]. This academic curiosity later merged with an important commercial perspective, when the few commercialized hydrophobic carotenoids were introduced as safe food colors for soft drinks [7–9].

In this work, we report on the surface activity and aggregation properties of crocin (**1**), the disugar ester of the highly unsaturated diacid crocetin C20:7 [10]. Compound **1**, a stable ingredient making up *ca.* 25% of saffron [11–13], is, in fact, the only really highly water-soluble natural carotenoid, and the only highly unsaturated, conjugated sugar surfactant abundantly available[1]) [14]. Other carotenoid glycosides occur in

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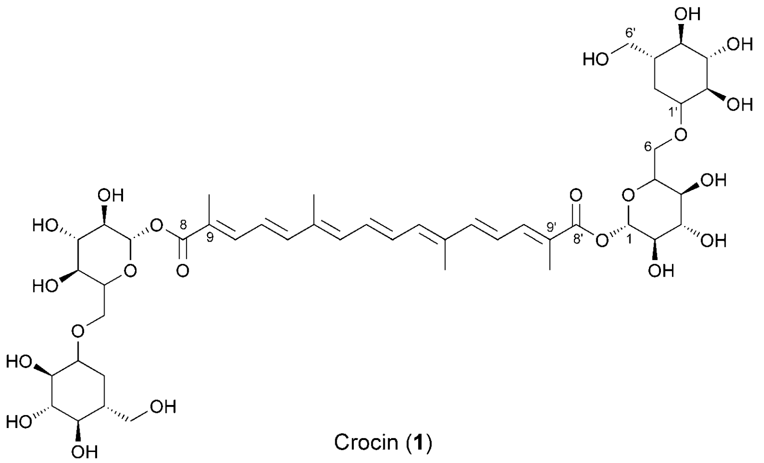
[1]) The World production of saffron in 2003 amounted to *ca.* 200 t, corresponding to an estimated 50 t of crocin (**1**).



Crocin (**1**)

much smaller amounts or are only sparingly soluble [15] [16]. Carotenoid glycosides can be synthesized, but in spite of reported high yields, the properties of these biodegradable surfactants have not been investigated [17]. Crocin (**1**) has antioxidant [18] and radical-scavenging [19] properties, acts as a singlet-oxygen ($^1O_2$) quencher [20] [21], inhibits cancer-cell growth [22] [23] and arthritis [24], and prevents neurodegenerative disorders [25]. Other crocetin glycosides are less biologically active [23].

The following results are part of recent investigations on hydrophilic carotenoids and their biological and physical properties [26–37].

Results and Discussion. – *Surface Tension and Critical Micelle Concentration.* Crocin (**1**) dissolves in H_2O to a clear, yellow solution. We did not find a saturation point up to 150 mg/ml [3]. The surface tension γ for various concentrations c of **1** in H_2O was determined by the *Wilhelmy*-plate method. A plot of γ *vs.* ln c (and γ *vs.* c) gave, at the point of discontinuity for the critical micelle (aggregate) concentration c_M of 0.8 mg/ml (0.82 mM), a γ value of 52 mN/m (*Fig. 1*). The surface excess concentration Γ, which corresponds to the concentration of molecules completely covering the H_2O surface, was calculated to be 1.4×10^{-6} mol/m^2, which corresponds to a molecule area a_m of 115 Å^2 at the interface. The thermodynamic characteristics of this solution are listed in the *Table*.

The surface excess concentration Γ for **1** was found to be constant in the concentration range of *ca.* 8–800 mg/l (constant slope dγ/d(ln c); *Fig. 1*). For most other surfactants, the concentration range between the upper and lower discontinuity amounts to 10–30%, corresponding to a much steeper slope [38]. Less-steep slopes appear to be characteristic for highly water-soluble, not easily aggregating surfactants [39] [40]. The discontinuity break of surfactants with large hydrophilic and short hydrophobic groups could represent a point of phase separation, and not of aggregate formation [41]. Yet, H-type aggregates due to card stack orientation of the polyene chains [9] [37] were detected in the UV/VIS spectrum at $c = 1000$ mg/l, slightly above c_M (*Fig. 2*). Similarly, dynamic-light-scattering (DLS) measurements indicated that mostly

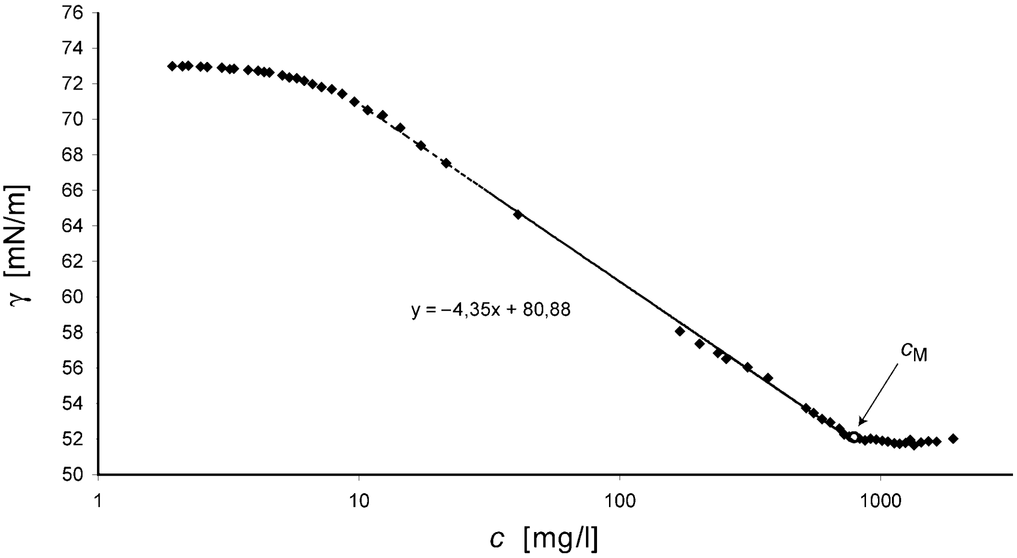


Fig. 1. *Determination of the critical micelle concentration* (c_M) *of crocin* (**1**) *in H_2O from the observed change in surface tension*. The following values were found: $c_M = 800$ mg/l (0.82 mM), $\gamma_{c_M} = 52$ mN/m.

Table. *Selected Data for Crocin* (**1**) *and Related Compounds*. For details, see text and *Exper. Part*.

Parameter	Crocin	*Cardax* [28]	C30-L[a] [27]	Bixin [43]	Isobixin [43]	Maltose-6-C12 :0 [44]
γ_{c_M} [mN/m]	52	60	57	–	–	39.0
π_{c_M} [mN/m]	21	13	16	–	–	34.0[b]
c_M [mM]	0.82	0.45 ± 0.05	1.30 ± 0.2	–	–	0.33
Γ [10^{-6} mol/m^2]	1.4	0.7 ± 0.1	4.5 ± 1	0.6[b]	5.7[b]	3.3
a_m [Å^2]	115	240 ± 30	39 ± 9	100	29	50
ΔG^0_{ag} [kJ/mol]	–17.5	-54.8 ± 0.8	-16.2 ± 0.4	–	–	–19.6[b]
ΔG^0_{ad} [kJ/mol]	–32.5	-73.6 ± 3.2	-20.1 ± 1.4	–	–	–29.8
ΔG^0_{ad-ag} [kJ/mol]	–15.0	–18.8	–3.9	–	–	–10.2[b]
K_{ag}	1200	1800	750	–	–	3000[b]
K_{ad}	450000	23000	3500	–	–	197000[b]
K_{ad-ag}	370	13	5	–	–	65[b]
AMER[c]	1.8	1.3	1.2	–	–	1.5[b]

[a] C30-Lysophospholipid. [b] Calculated from reported data. [c] $\Delta G^0_{ad}/\Delta G^0_{ag}$.

monomers and some few aggregates were present in solution at concentration below and above c_M, and that higher concentrations resulted in larger aggregates (*Fig. 3*).

The calculated value from tensiometric data ($a_m = 115$ Å^2) indicates horizontally oriented crocin molecules at the H_2O surface (*Fig. 4*), with lengths l between the 9-Me group and the O-atom at C(1) of the remote sugar of 18 Å, and between 9-Me and the corresponding O-atom of the adjacent sugar of 6.5 Å, corresponding to $a_M = 117$ Å^2, in agreement with the above value. In such an arrangement, hydration of the polar groups would not significantly increase the molecule area a_M [42]. The mol-

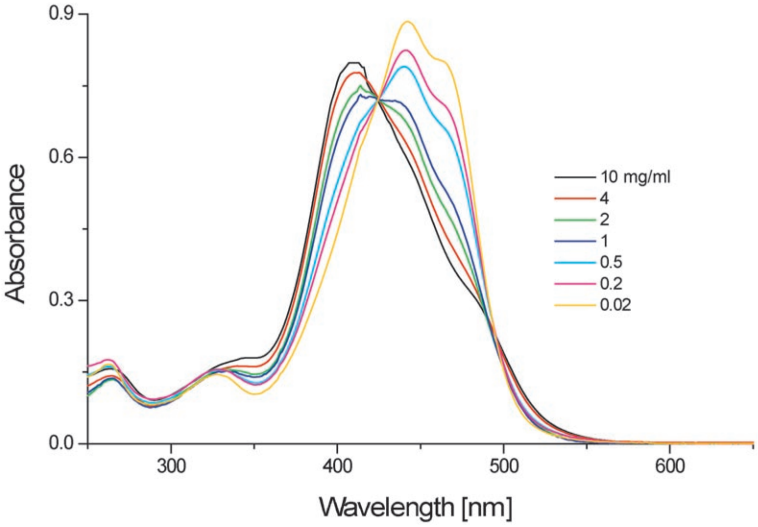


Fig. 2. *UV/VIS Spectra of crocin* (**1**) *in H_2O as a function of concentration* (colored curves; in mg/l). Upon increasing the concentration of **1**, a hypochromic shift from λ_{max} 445 to 410 nm was observed due to a change in the equilibrium between mono- and polymolecular (aggregated) forms. The equilibrium concentration (1 mg/ml) was close to the experimental c_M value (0.8 mg/l; see *Fig. 1*).

ecule area of crocin is similar to that of a flat-lying bixin, but is much larger than the one of the vertically oriented molecules C30-lysophospholipid, isobixin, and maltose-C12:0-monoester [27][43][44] (*Table*).

With c_s, c_a, and c_b denoting the concentrations of **1** at the *surface*, in the *aggregated* form, and in *bulk* solution, respectively, the values of the equilibrium constants $K_{s,b}$ ($=c_s/c_b=450000$) and $K_{a,b}$ ($=c_a/c_b=1200$) point to a high preference of crocin for surface absorption over aggregation. In addition, the relatively high equilibrium constant $K_{s,a}$ ($=c_s/c_a=370$), *i.e.*, the concentration ratio of molecules at the surface *vs.* the self-aggregated state, demonstrates a low aggregation tendency (*Fig. 5*). The adsorption-micellar energy ratio (AMER), represented by the term $\Delta G^0_{ad}/\Delta G^0_{ag}$, has been proposed as a surfactant-performance indicator [45]. AMER Values close to unity imply dense monolayer formation, enhanced aggregate concentration, and high ability in flotation, cleaning, and wetting. Compared with other carotenoid surfactants, the AMER value of crocin indicates inferior surfactant properties (*Table*).

Monomeric and Aggregated States. The UV/VIS spectra of crocin (**1**) in H_2O at concentrations below c_M were typical of the monomeric form (λ_{max} 440 nm), resembling the spectra of crocin in organic solvents (*Fig. 2*), as corroborated by DLS measurements (*Fig. 3*). The optical rotation at $c<c_M$ was not constant, with values of $[\alpha]^{25}_{289}$ of -859, -1109, and -1717 after 1, 2, and 40 min, respectively, at $c=0.18$ mg/ml. A similarly high optical rotation for **1** in H_2O has been reported (at longer wavelength) before, even below the critical micelle concentration: $[\alpha]^{21}_{644}=-1760$ ($c=1.06$ mg/ml) [46]. Unfortunately, we were not able to determine optical rotations above c_M due to very strong absorption.

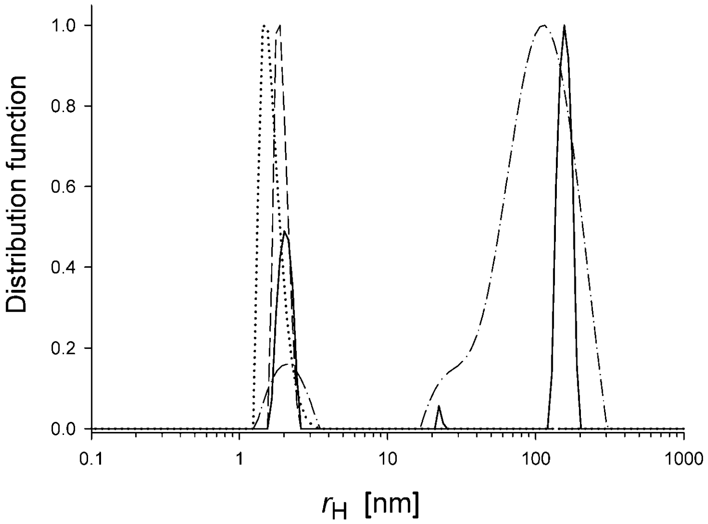


Fig. 3. *Size determination of crocin* (**1**) *in H_2O at 24° by dynamic light scattering.* The typical intensity-weighted distribution function for 0.6 mg/ml (–·–) and 4.3 mg/ml (—) of **1**, and the number-weighted distribution function for 0.6 mg/ml (···) and 4.3 mg/ml (– – –) of **1** are plotted *vs.* the equivalent hydrodynamic radius (r_H). All data were recorded 2–3 h after sample preparation. At the above concentrations, **1** is mostly present in its monomolecular form, with $r_H = 2$ nm, together with small amounts of aggregates ($r_H = 110$ and 150 nm at 0.6 and 4.3 mg/ml, resp.). The shoulder at 30 nm and the small peak at *ca.* 20 nm radius are caused by rotational motion, and do not represent particle sizes.

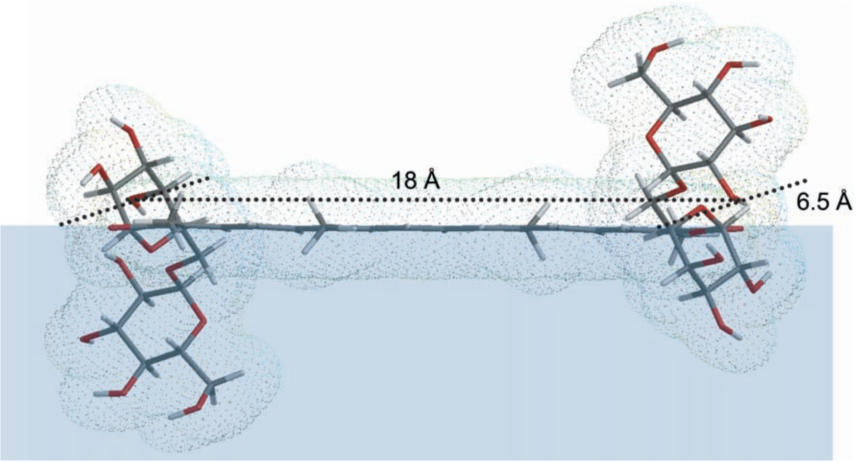


Fig. 4. *Hypothetical orientation of crocin* (**1**) *at the water surface.* The distances between the 9-Me group and the O-atom at C(1) of the opposite ($d = 18$ Å) and the adjacent sugar ($d = 6.5$ Å) correspond to a molecule area a_m of 117 Å^2 (calculated value from tensiometric measurements: 115 Å^2).

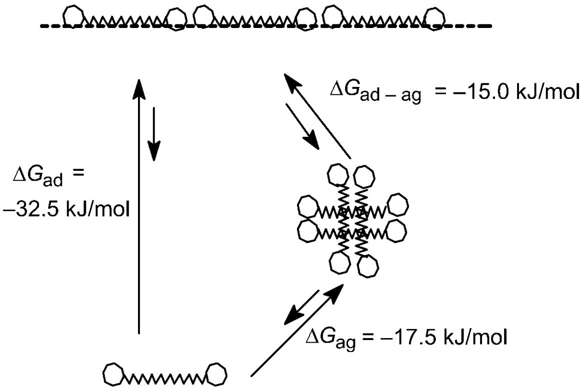


Fig. 5. *Thermodynamic cycle for surface-monolayer and aggregate formation of crocin* (**1**). ΔG^0_{ad} and ΔG^0_{ag} refer to adsorption and aggregation free-energies, resp. For the corresponding equilibrium constants *K*, see the *Table*.

Aggregate formation in H_2O was clearly indicated in the UV/VIS spectra recorded for concentrations of **1** above c_M. The absorption at λ_{max} 410 nm indicated an H-type arrangement, and a small shoulder near 480 nm pointed to some J-type arrangement ('head-to-tail' orientation) [9][37]) of the monomers in the aggregates (*Fig. 2*). To ensure that the observed changes were, indeed, caused by aggregation of crocin (**1**) in aqueous medium, we also recorded UV/VIS spectra of highly concentrated solutions of **1** (100 mg/ml) in MeOH, but could not find any changes. The detection of aggregates just after reaching the surface-saturation concentration Γ and the critical aggregation concentration c_M indicate that, for crocin, surface population and aggregation are sequential processes. In contrast, phospholipidic carotenoids tend to aggregate at concentrations as low as 5 nM [27], and aggregation and surface filling are, thus, concurrent processes. Likewise, the anionic carotenoid bolaamphiphile *Cardax* was found to aggregate and fill the surface simultaneously [28]. The different behavior of crocin (**1**) relative to the two other carotenoid surfactants is reflected by the equilibrium constants: **1** aggregates less readily, and disaggregates more readily than *Cardax* (*Table*).

The intensity and number-weighted distribution function obtained from DLS measurements of crocin (**1**) plotted *vs.* the equivalent hydrodynamic radius r_H is presented in *Fig. 3.* We found that mostly monomers with r_H of *ca.* 2 nm exist in solution, both at 0.6 mg/ml ($< c_M$) and at 4.3 mg/ml ($> c_M$). The presence of a small number of aggregates of $r_H = 110$ nm (at 0.6 mg/ml) and 160 nm (at 4.3 mg/ml) was only detected in the intensity-weighted distribution. Thus, higher concentrations gave rise to larger aggregates. We also measured the aggregate size after 24 h (data not shown). At a concentration of 0.6 mg/ml, time had no influence on aggregate size. At 4.3 mg/ml, the aggregate size increased to 180 nm. Hence, higher concentrations of **1** over longer time result in bigger aggregates.

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Experimental Part

1. *Compound Purification.* Crocin (**1**) was purchased from *Fluka*, and its UV/VIS spectrum was identical to the spectrum reported in [47]. Since commercial **1** contains considerable amounts of byproducts, probably picrocrocin and 13-*cis*-crocin (λ_{max} 325 nm) [48], it was purified by column chromatography (CC) on neutral alumina (MeOH/H_2O/MeCN 7:2:1) followed by freeze drying of the aq. fraction after evaporation of the org. solvents [49][50]. The UV/VIS spectra of such purified crocin did not show significant absorption maxima between 200 and 350 nm [49].

2. *UV/VIS and ORD Data.* UV/VIS Spectra of the highly concentrated crocin solns. in H_2O and MeOH (100 mg/ml) were recorded in a 0.1-cm quartz cell or as films between quartz plates (0.08–0.2 mm). The monomeric form of **1** showed a maximum absorption at λ_{max} 445 nm (0.02 mg/ml H_2O), the aggregates at 410 nm (10 mg/ml H_2O). Optical rotations were measured in H_2O with a *Perkin-Elmer 243B* polarimeter in a 10-cm cell.

3. *Critical Micelle Concentration.* Critical micelle (aggregate) concentrations c_M were determined in a conical, *Teflon*-coated vessel with a *Wilhelmy* plate on a *Krüss K100* tensiometer. Solns. of **1** (10 ml) were prepared with filtered (0.22 μm) H_2O (*Milli-Q*).

4. *Surface Parameters.* 4.1. *Surface Tension.* Surface tensions γ were measured by gradually adding H_2O to an aq. soln. of **1** with a *Metrohm 765 Dosimat*. The measurements were carried out in duplicate (for low concentrations) and in triplicate (for high concentrations) at 24°. We were not able to obtain reliable data in the range of 20–150 mg/l of **1**.

4.2. *Surface Pressure.* The surface pressure $\pi = \gamma^0 - \gamma_{c_M}$, *i.e.*, the change in surface tension caused by the substrate **1**, was calculated with $\gamma^0 = 73$ mN/m (H_2O).

4.3. *Surface Concentration.* The surface concentration was calculated as follows: $\Gamma = -1/(RT) \cdot [\mathrm{d}\gamma/(\mathrm{d}(\ln c))]_{c_M} = -1/(RT) \cdot (c\ \mathrm{d}\gamma/\mathrm{d}c)_{c_M}$; Γ in m/mol^2. When γ is measured in mN/m (=J/m^2), c in mol/m^3, R in J mol^{-1} K^{-1}, and T in K, Γ comes out in mol/m^2.

4.4. *Surface Area per Molecule.* This parameter, a_m (in Å^2), was calculated for a filled monolayer as $a_m = 10^{20}/(\Gamma N_A)$, where N_A is *Avogadro*'s constant.

5. *Dynamic Light Scattering* (DLS). Particle sizes were determined by DLS with an *ALV DLS/SLS-5022F* compact goniometer and an *ALV-5000/*E multiple τ-digital correlator (*ALV*, Langen, Germany). The light source was a 22-mW He/Ne laser (*Uniphase*, Witney Oxon, U. K.). Crocin (**1**) was carefully filtered through 0.22-μm filters. The temp. of the sample was 24°, and the scattering angle was set to 90°. Data analysis was performed with the CONTIN method available in the *ALV* software package [51]. To obtain reliable vesicle-size distributions, extended data-acquisition times were required, and the data from several runs were averaged to minimize error.

6. *Calculation of Thermodynamic Data.* The calculations were based on $c_M = 800$ mg/l (=0.82 mol/m^3), ln $c = 7.10$, $\gamma = 52$ mN/m (= 0.052 J/m^2), $T = 24°$ (297 K), and a slope dγ/dc of 0.00435. The free energy of aggregation, ΔG_{ag}, *i.e.*, the free-energy change associated with the change from a monomeric to an aggregated state, was calculated as $\Delta G^0_{ag} = -RT \ln c_M$. The free energy of adsorption, ΔG^0_{ad}, *i.e.*, the energy change associated with a molecule going from the bulk to the surface, was calculated as $\Delta G^0_{ad} = \Delta G^0_{ag} - 6.023\ \pi a_M$ (in J/mol). Equilibrium constants K for the different states were calculated as follows: *1*) Aggregated molecules/bulk monomer: $K_{ag} = \exp(-\Delta G^0/(RT)$; *2*) surface molecules/bulk monomer: $K_{ad} = \exp(-\Delta G^0_{ad}/RT)$; *3*) surface molecules/aggregated molecules: $K_{ad-ag} = \exp[-\Delta G^0_{ad} - \Delta G^0_{ag}/(RT)]$.

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