Electrochemistry

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Investigation of Single-Drug-Encapsulating Liposomes using the Nano-Impact Method**

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Abstract: *Encapsulating liposomes are widely used for controlled drug delivery. We report the use of nano-impact experiments for the electrochemical attomolar quantification of the liposome load, uniquely at the single liposome level, using vitamin C encapsulated liposomes as a model. The size of the liposomes and their picomolar concentration are also determined in biological buffer in real time.*

Liposomes are very effective delivery vehicles with a bilayer structure capable of fusing with microbial membranes.[1] To date, the real-time quantification of drug content and particle characterization at the single liposome level is impossible,[2] whereas ensemble measurements average over wide populations.[2b] A recent electrochemical method detects and counts single nanoparticles,[3] where by virtue of Brownian motion individual nanoparticles randomly collide with the electrode held at a suitable potential to induce quantitative oxidation[3a] or reduction[3c] of the nanoparticle. Alternatively, mediated electron transfer can take place on the surface of impacted nanoparticles.[4] The nano-impact method sizes impacting metal and organic nanoparticles, and can quantify their doping level.[3,5]

Herein, we use a vitamin C encapsulated liposome as a model, and show how single drug-encapsulating liposomes and their contents can be characterized in biological buffer in real time at the single liposome level. The works builds on the pioneering work of Scholz and co-workers who investigated the non-Faradaic adhesion of undoped liposomes on a static mercury-drop electrode.[6] The Faradaic oxidation of ferrocene-doped oil droplets has also been reported.[4e]

First, a voltammetric method was employed to determine the amount of free vitamin C and the total amount of vitamin C in a liposome suspension (see the Supporting Information). The free and total vitamin C concentrations were determined to be 2.56 mM and 2.85 mM, respectively (Figures S1 and S2 in the Supporting Information) and, the encapsulation efficiency was estimated to be approximately 10%.

To demonstrate the oxidation of liposomal-encapsulated vitamin C, a macro glassy carbon (GC) electrode (diameter = 3 mm) was modified with liposomes and cyclic voltammograms were recorded. Figure S3 shows the first three scans of this modified electrode immersed in PBS buffer (PBS = phosphate-buffered saline). A single oxidation peak was detected around + 0.40 V versus the saturated calomel reference electrode (SCE), consistent with reported values for the oxidation of vitamin C on a glassy carbon electrode.[7] A similar oxidative peak position was measured in aqueous vitamin C solution using a glassy carbon electrode, also confirming the identity of vitamin C (Figures S1 and S2). No peak was detected on the backward scan, indicating the oxidation of vitamin C is chemically irreversible.

Next, a clean carbon microelectrode was placed in PBS buffer (100 mM) and a known concentration of dispersed liposomes were added. Under potentiostatted conditions, clear oxidative (Faradaic) current spikes at + 0.70 V versus SCE were detected (Figure 1). This potential is significantly more positive than the oxidation potential of vitamin C (as suggested by the data in Figures S1, S2, S3), ensuring the complete oxidation of vitamin C. The current spikes are attributed to the oxidation of encapsulated vitamin C when the liposome collides with the electrode. The onset of these Faradaic spikes was found to be dependent on oxidation potential and no spike was measured at lower oxidation potentials, such as + 0.10 V, confirming that the spikes result from the Faradaic oxidation of vitamin C. Another control experiment was conducted at potential + 0.70 V with no liposome in the solution and no spikes were detected, further

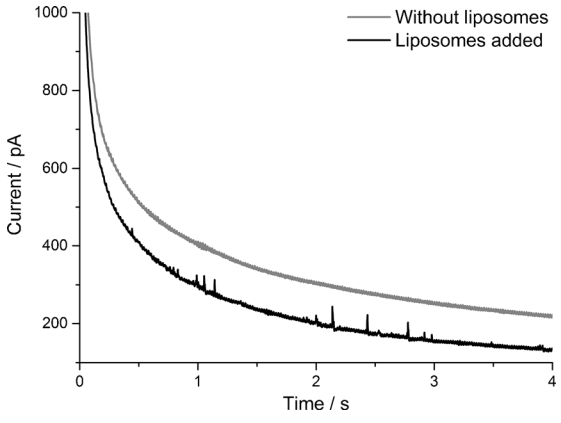

Figure 1. Chronoamperometric profiles showing oxidative Faradaic spikes of vitamin C encapsulated in single liposomes in PBS buffer (100 mM; pH 7) at 0.70 V versus SCE.

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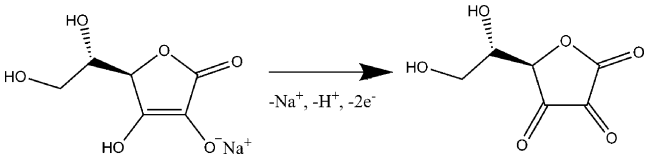
confirming that the occurrence of oxidation spikes is due to the random collisions of liposomes with the surface of the electrode and the release and oxidation of vitamin C (Scheme 1).



Scheme 1. Electrochemical oxidation of the sodium salt of vitamin C (sodium ascorbate).

A total of 198 spikes were measured from 21 chronoamperograms each of 5 seconds duration recorded at +0.70 V, corresponding to the oxidation of vitamin C encapsulated in 198 single liposomes.[8] The charge passed during oxidation of the individual liposomes was calculated by integrating the individual spikes (Figure S4). This is the first time that direct oxidation of the content of single liposomes during collision events has been observed and measured.

A quantitative analysis was next undertaken. The size distribution of the liposome D_{lip} was determined (Figure 2) to have a modal diameter of 115 nm (mean diameter = 116 ± 23 nm) by analyzing the impact spikes using Equation (1):

$$Q = \frac{D_{lip}{}^3 nF\pi C}{6} \quad (1)$$

where Q is measured charge, F is the Faraday constant, and C is the concentration of redox species in the liposome. The parameter n is the number of electrons transferred per molecule during oxidation ($n = 2$ for vitamin C[7]).

This inferred size is in good agreement with size distribution of the same batch of liposomes measured through independent dynamic light scattering (DLS) analysis (Figure S5).

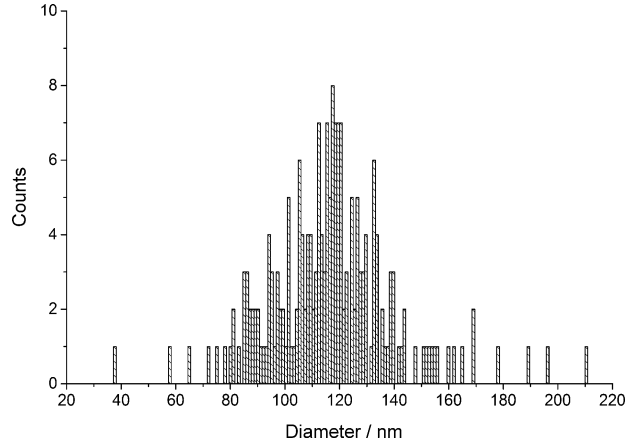


Figure 2. Size distribution of the liposomes (total: 198), showing the derived diameter of each individual liposome from the charge per current spike (Figure S4) calculated using Equation (1).

The mass of vitamin C per individual liposome was derived from the modal charge resulting from the oxidation of 198 single liposomes using Equation (2):

$$m = \frac{QM}{nF} \quad (2)$$

where m is the mass of vitamin C encapsulated in a single liposome, N is the Avogadro constant, and M is the molar mass of sodium ascorbate.

By comparing this derived mass (2.39×10^{-16} g or 1.21 attomole vitamin C per single liposome) with the theoretical estimation of vitamin C in single individual liposomes (2.24×10^{-16} g or 1.13 attomole vitamin C per single liposome), it is inferred that vitamin C is completely released from single liposomes, with a "full collapse fusion" mechanism likely dominating (Figure 3). The slightly larger mass than estimated may be tentatively attributed to a slight salt-induced aggregation of liposomes of high concentration in buffer medium during the course of experiments.[9]

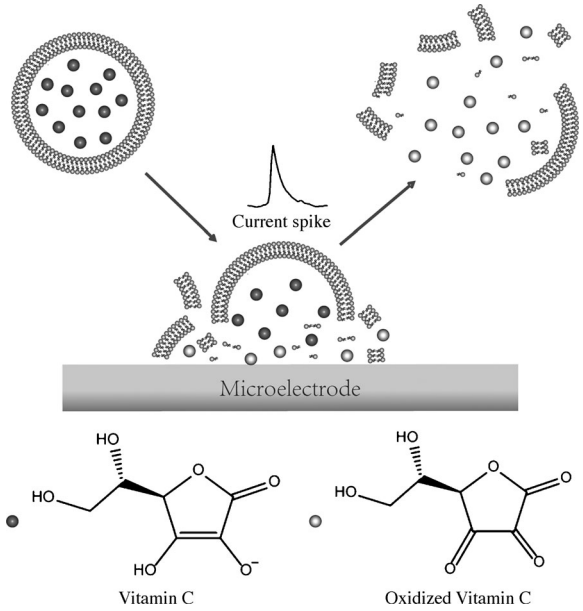


Figure 3. Representation of the "full collapse fusion" process, showing the collapse at the microelectrode during nano-impact experiments of single liposomes which encapsulate vitamin C.

The mass of encapsulated vitamin C in a single liposome was further analyzed from the spike data [Eq. (1) and (2)] and fitted assuming Gaussian distribution (Figure 4). The mean value derived from the fit is consistent with that from the distribution based on the estimation from the known size distribution from DLS (Figure S6).

Finally, the concentration of liposomes in the stock solution was determined, by comparing the total mass of encapsulated vitamin C (Figure S2) against the mass of vitamin C encapsulated per single liposome (2.39×10^{-16} g per single liposome). Taking the dilution factor into consideration, the concentration of liposomes used in the nano-impact experiments was estimated to be 42 pM. The theoret-

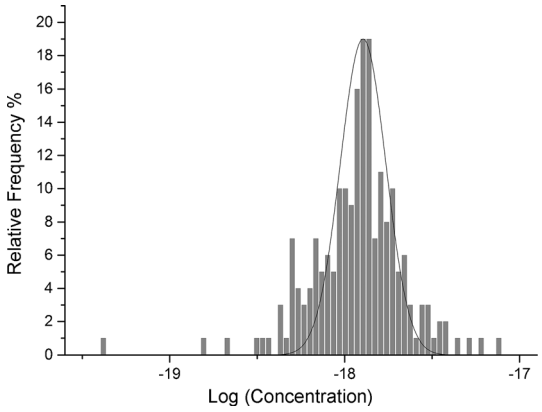


Figure 4. Histogram showing the log normal distribution of the mole amount of vitamin C encapsulated in single liposomes calculated from nano-impact experiments according to Equations (1) and (2). Concentration = mole amount of vitamin C encapsulated in a single liposome.

ical estimated frequency of current spikes was 2.4 ± 0.5 Hz, which is related to the diffusion of liposomes to the microelectrode (see the Supporting Information). The average particle-collision frequency measured from the nano-impact experiment (1.9 Hz) was in good agreement with the calculation based on approximately 100% of the impacting liposomes undergoing reaction.

The results show that by using the nano-impact method, quantitative analysis of the size of each individual liposome can be determined. Secondly, the nano-impact method can be used to quantitatively elucidate the mechanism of single-liposome fusion and release.

To summarize, using vitamin C encapsulated liposomes as a model, we have shown the use of nano-impact experiments for sizing liposomes and the attomolar-scale quantification of drug content at the single liposome level. We believe that this strategy will have major applications in the quantification of redox drugs encapsulated in single nanocarriers, such as liposomes or polymersomes. Additionally, this procedure uniquely facilitates the characterization of single liposomes within biological media, which are impossible to characterize by current methods.

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[8] Note: It is not possible to run experiments with empty liposomes of the same size for obvious reasons. The charge measured was considered to be primarily Faradaic and not capacitive as measured by Scholz for unfilled liposomes because no spikes were seen at 0.1 or 0 V (vs. SCE) where ascorbic acid is not oxidized. Note that the point of zero charge (PZC) of glassy carbon is +0.041 V [H. R. Zebardasta, S. Rogakb, E. Asselina, *J. Electroanal. Chem.* **2014**, *724*, 36 – 42]. For capacitive spikes a charge inversion is seen either side of the PZC.

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