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Cerasomes containing magnetic nanoparticles: synthesis and gel-filtration chromatographic characterization

Dmitry V. Pryazhnikov,* Ol'ga O. Efanova and Irina V. Kubrakova

V. I. Vernadsky Institute of Geochemistry and Analytical Chemistry, Russian Academy of Sciences, 119991 Moscow, Russian Federation. Fax: +7 495 938 2054; e-mail: pryaznikov@geokhi.ru

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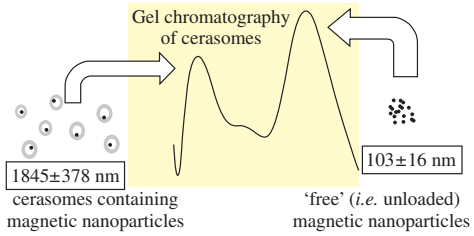
Cerasomes containing encapsulated magnetic nanoparticles were synthesized and characterized by gel filtration chromatography, elemental analysis, and photon correlation spectroscopy. Laboratory microwave system was used for the preparation of magnetic nanoparticles. The optimal synthetic conditions were revealed, and the inclusion of nanoparticles in the cerasome composition was experimentally confirmed.



Liposomal structures as a promising option to design systems for targeted and controlled delivery of drugs and biologically active substances *in vivo* are widely considered.[1–6] The drug encapsulation into liposomes allows one to administer a more local and gentle therapeutic procedure. Although the liposomes possess a number of highly attractive properties as carriers for the targeted drug delivery, they are also characterized by some disadvantages: low stability,[7,8] allergic reactions,[9] possible interactions with plasma proteins that may cause serious side effects decreasing the overall effectiveness of treatment,[10,11] *etc*. To eliminate these drawbacks, an additional chemical modification of the liposome-forming lipid molecules, *viz*. embedding a hydrophilic moiety bearing trialkoxysilyl group, has been proposed.[12,13] These groups form silica shell on the liposome surface during the hydrolysis and subsequent sol-gel process, which increases the stability as compared to conventional lipid vesicles. As a result, hybrid liposomes (so-called cerasomes) are formed.[7,8,14]

The therapeutic applications of liposomes may be further enhanced *via* a simultaneous encapsulation of both drugs and magnetic nanoparticles (MNPs) possessing superparamagnetic properties. Such hybrid systems (so-called magnetosomes)[10] along with modified nanomaterials based on MNPs[15] are able to perform several functions *in vivo*: hyperthermia of cancer cells, targeted drug delivery, opportunity to real-time control the localization of liposomes (cerasomes) in the body using MRI.

This work was aimed at the preparation of novel magnetosomes and evaluation of their storage stability, size, and gel chromatographic behavior. We reproduced the synthesis of cerasomes in two known ways,[7,13] since the data on the advantages and drawbacks of these procedures were lacking, and compared their stability during storage *in vitro* and the overall efficiency of synthesis. The stabilized MNPs with hydrophilic surface were prepared and encapsulated, and the possibility of separation of magnetosomes obtained from unencapsulated MNPs was demonstrated.

Cerasomes were synthesized using so-called cerasome-forming lipid *N*-[(triethoxysilyl)propylamidosuccinyl]dimyristyl L-aspartate as the starting material. We compared the two versions of synthesis, which are hereinafter referred as methods A and B,[†] using the gel filtration chromatography (GFC).[‡] The sorbent type and conditions of chromatography separations were selected taking into account the previously reported data.[16,17]

The temperature effect on the efficiency of GFC separation of cerasomes was investigated as the main parameter. Figure 1 shows the heights of the chromatographic peaks of cerasomes

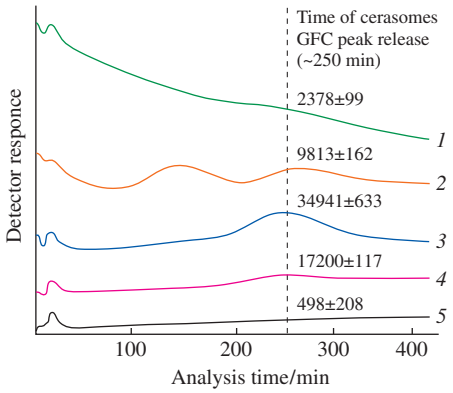


Figure 1 GFC chromatograms of cerasomes recorded at different temperatures: (*1*) 30, (*2*) 35, (*3*) 40, (*4*) 45 and (*5*) 50 °C and the peak heights measured under these conditions. The numbers indicate values of the peak heights according to the detector response (in μV).

† Method A was the reproduction of the known procedure.[13]

Method B was based on method A with some modifications.[7,10] The cerasome-forming lipid (10 mg) was dissolved in $CHCl_3$ (2 ml). The solvent was evaporated in a water bath at 30–40 °C for 2 h. The resulting film was dried *in vacuo* for ~30 min, dispersed in acetic acid (3%, 10 ml), and stirred vigorously for 20 min. The obtained suspension was ultrasonically treated at 45 °C for 45 min.

‡ The cerasomes were separated on a Shimadzu LC-20 Prominence liquid chromatograph using a Sephadex G-200 gel type (fraction of 40–120 μm, Pharmacia). The eluent flow through the column (inner diameter 8 mm, height of the gel layer 225 mm) was 0.021 ml min^{-1}, the volume of the dosing loop was 20 μl, and 0.01 M Na_2SO_4 was used as eluent. The choice of such small flow was due to the low mechanical resistance of Sephadex G-200. Detection was performed spectrophotometrically (250 nm).

Table 1 The effect of storage time on chromatographic behavior of the hollow cerasomes.

Storage time	The peak height/μV ($P = 0.95$, $n = 2$)[a]	
	Cerasomes prepared by method A	Cerasomes prepared by method B
3 days	4328 ± 264	40290 ± 1237
25 days	2177 ± 461	45224 ± 946

[a] P is the confidence level and n is the number of measurements.

(release time ~250 min) separating at different temperatures. It was found that the appropriate temperature range for GFC separation is quite narrow, and optimal temperature was 40 °C. This suggests that cerasomes may undergo a thermal degradation at higher temperatures, while at lower temperatures, they may be absorbed on Sephadex.

Cerasomes obtained by these two methods, both freshly prepared and after storage in a refrigerator, have been also analyzed by GFC. The heights of observed chromatographic peaks are given in Table 1.

The obtained results clearly demonstrate the difference in methods A and B affecting both the efficiency of cerasomes synthesis and their storage stability, which is due to the presence of an acidic medium (acetic acid) during vesicles formation (in method B). The acid catalyst is required for the hydrolysis of trialkoxysilane moieties in the molecules of cerasome-forming lipid. Thus, its presence in the reaction mixture allows one to obtain vesicles characterized by longer storage times (due to SiO_2 monomolecular layer on each surface of the vesicle).

Surface-modified MNPs stabilized by sodium citrate (Fe_3O_4@Na_3Cit) were prepared using microwave (MW) heating.[§] We have previously demonstrated that the application of MW-heating for nanomagnetite modification leads to a more ordered surface layer of the modifier molecules.[18–20] In the case of Fe_3O_4@Na_3Cit, we expected that the denser layer can provide a higher aggregation stability for MNPs and, consequently, the existence of more stable aqueous suspension, which is extremely important for the successful encapsulation of Fe_3O_4@Na_3Cit into the cerasomes. The MNP modification was carried out at different Na_3Cit : Fe_3O_4 ratios, pH levels, and temperatures.

To comparatively estimate the stability of obtained slurries, we monitored the relative change in the optical density (A/A_0) for slurries of samples Fe_3O_4@Na_3Cit according to the known procedure.[19,21] Particles sedimentation occurred in the cuvette of a Shimadzu UV-1800 spectrophotometer (λ = 550 nm). The used dispersions were freshly prepared and not diluted.

In the case of samples prepared at 60 °C, the sedimentation was not observed during the whole experiment time (60–70 min) [Figure 2(*a*)], while MNPs obtained at 80 °C demonstrated a significantly lower stability. Thus, the lower temperature (60 °C) can probably contribute to a more complete filling of the magnetite particle surface with the modifier molecules. The sedimentation of unmodified magnetite under the same conditions was almost completed in 3–5 min, which indicates the efficiency of the administered modification.

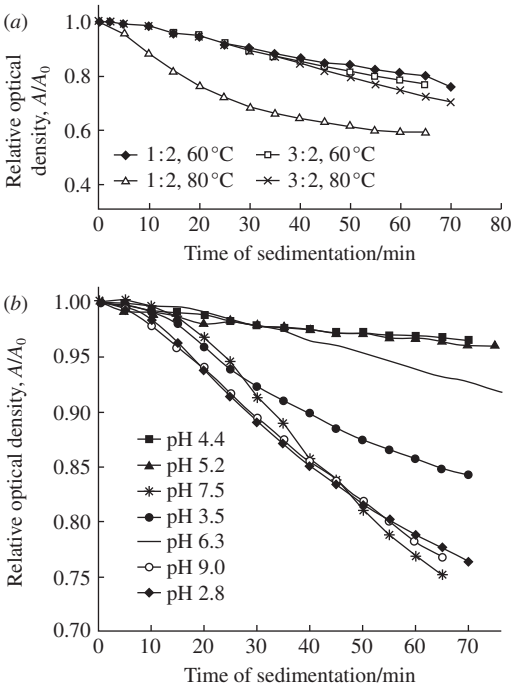


Figure 2 Sedimentation kinetics for Fe_3O_4@Na_3Cit (*a*) at different temperatures of the synthesis and primary ratios (w/w) of the components and (*b*) at different pH values of the sodium citrate solution.

The evaluation of pH effect of the Na_3Cit solution on the sedimentation of Fe_3O_4@Na_3Cit revealed a clearly defined range of pH (4.4–5.2), where particles sedimentation was minimal, while outside this range, the rate of particle sedimentation increased dramatically [Figure 2(*b*)]. This can be explained by the maximum content of mono- and bi-dissociated forms H_2Cit^- and $HCit^{2-}$ in this pH interval. These citrate forms are capable of efficient intermolecular interacting with the magnetite surface to form a negative charge on the MNP surface, thus preventing their aggregation. Under more acidic conditions, the relative content of non-dissociated form H_3Cit increases, and at more alkaline pH values, a competition between citrate and hydroxyl ions begins.

The magnetosomes were synthesized and GFC purified in the same way as the initial hollow cerasomes with some modifications introduced.[¶]

To confirm the MNP encapsulation into the vesicle cavity, the elemental analysis for iron in the fractions after magnetosomes GFC separation was additionally carried out, and the sizes of the particles present therein were determined.[††] In the first case, the fractions were continuously collected from the beginning of

[§] Solid salts $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$ (0.017 g) and $Fe(NO_3)_3 \cdot 9H_2O$ (0.035 g) were dissolved under stirring in distilled water (10 ml), from which dissolved air was removed by argon purging. The resulting solution was poured into a thick walled glass vessel. Aqueous ammonia (6 ml) was added. The mixture was stirred for 3 min using a magnetic stirrer under argon purging. The resulting precipitate was separated by a magnet and washed with distilled water three times.

The MNP modification process was carried out in a CEM Discover SP-D MW system under the focused radiation (frequency of 2.45 GHz, the maximum magnetron power of 300 W). Magnetite particles were dispersed in Na_3Cit solution (10 ml, 0.1 or 0.3%) and placed in the MW system for heating using the following parameters: the final temperature of 60 or 80 °C, power of 200 W, temperature set time of 3 min, and the heating time at the final temperature of 10 min. After cooling, the resulting product (Fe_3O_4@Na_3Cit) was separated by a magnet, washed 3 times with distilled water and redispersed in distilled water (5.0 ml). Its concentration in the resulting suspension was 2 mg ml^{-1} (based on Fe_3O_4).

[¶] In the synthesis of magnetosomes (method B), the acetic acid (3%, 10 ml) was replaced by a mixture of acetic acid (3%, 9 ml) and aqueous slurry of Fe_3O_4@Na_3Cit (1 ml, pH adjusted to 5.0). In the case of GFC fractionation of magnetosomes and their additional studies, the fractions were collected using a larger column (the length of the gel layer was 105 mm, the inner diameter was 39 mm). An Easy Load II peristaltic pump (MasterFlex) was used for eluent supply, the eluent flow was 0.2 ml min^{-1}, and the sample volume was 1 ml. Detection was in the visible region (400 nm) for Fe_3O_4@Na_3Cit, and that for magnetosomes was in the UV region (250 nm).

[††] A Thermo Electron AES-ICP IRIS Intrepid Duo instrument and a Beckman Coulter Delsa Nano particle size analyzer were used for the elemental and particle size analyses, respectively.

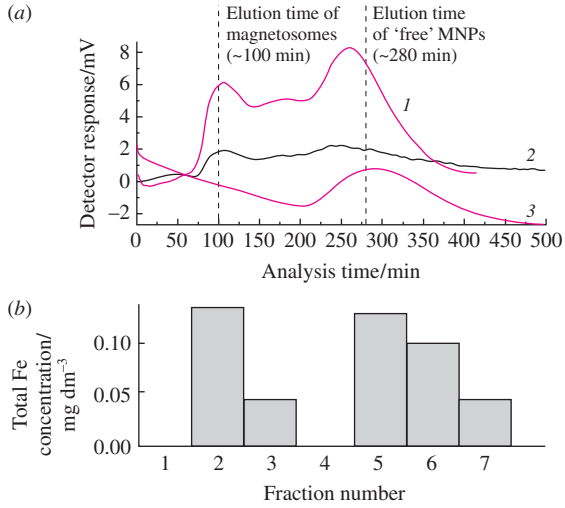


Figure 3 (*a*) GFC chromatograms of magnetosomes visualized at (*1*) 250 and (*2*) 400 nm and (*3*) citrate-stabilised MNPs visualized at 400 nm; (*b*) total iron content in the fractions after GFC fractionation of magnetosomes.

chromatography (each for 1 h; 7 fractions total). In the second case, the particle sizes were determined for two fractions sampled in the intervals of 70–160 min and 200–350 min corresponding to the GFC elution times of magnetosomes [Figure 3(*a*), chromatogram *1*].

The chromatographic experiments revealed [see Figure 3(*a*)] that a single peak of citrate-stabilised MNP, which was detected in the visible region (chromatogram *3*), was divided into 2 peaks as a result of the encapsulation (chromatogram *2*). The release times for these peaks matched with the peaks on the iron distribution histogram [Figure 3(*b*)] that could presumably confirm the presence of two types of MNP-containing objects possessing different sizes (since they have different release time during GFC-fractionation) in the prepared suspension. Consequently, this hypothesis was directly confirmed by the particle size determination in two corresponding fractions (Figure 4): 1845±378 nm (magnetosomes, the 70–160 min fraction) and 103±16 nm ('free' MNPs, the 200–350 min fraction).

Thus, cerasomes stable during storage have been successfully obtained *via* two known synthetic procedures. Optimal conditions

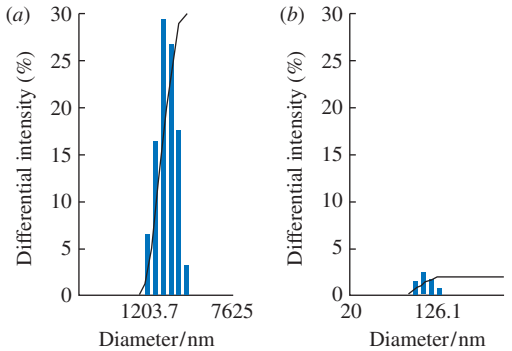


Figure 4 Particles sizes after the GFC separation of magnetosomes in fractions eluted in the interval (*a*) 70–160 min and (*b*) 200–350 min.

for GFC separation of cerasomes have been found, and their sizes have been determined. The comparative analysis demonstrated that method B was more efficient and provided more stable cerasomes, apparently, due to the interaction of acidic media with cerasome surface. Our results have also indicated that the gel chromatography is a promising approach to both preliminary fractionation of cerasomes and their isolation and purification in preparative amounts.

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