Available online at www.sciencedirect.com

ScienceDirect

Mendeleev Commun., 2018, **28**, 140–142

Mendeleev Communications

Biodegradable liposome–chitosan complexes: enzyme-mediated release of encapsulated substances

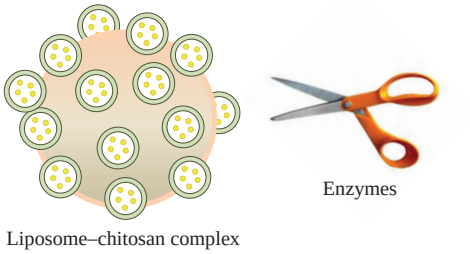
Alexander A. Yaroslavov,[a] Anna A. Efimova,*[a] Fedor D. Mulashkin,[a] Galina N. Rudenskaya[a] and Georgii G. Krivtsov[b]

[a] *Department of Chemistry, M. V. Lomonosov Moscow State University, 119991 Moscow, Russian Federation. Fax: +7 495 939 0174; e-mail: ephimova@genebee.msu.ru*

[b] *I. I. Mechnikov Research Institute of Vaccines and Sera, 105064 Moscow, Russian Federation*

DOI: 10.1016/j.mencom.2018.03.009

Multi-liposomal containers were prepared *via* electrostatic adsorption of 50 nm anionic liposomes on the surface of cationic chitosan nanoparticles (nanogels) 270 nm in diameter. The containers are stable in aqueous-salt solutions but degrade in the presence of proteolytic enzymes down to 10–15 nm particles and release water-soluble substances encapsulated into the adsorbed liposomes. Based on the kinetic data, the mechanism of the release is discussed.



In recent years, the liposomes, hollow particles with a bilayer lipid membrane separating the inner water volume from the surrounding solution, have been used as containers for encapsulation and delivery of drugs.[1–8] Multi-liposomal containers composed of dozens of liposomes with different fillings at variable ratios have been described.[9–12] Such capacious containers were prepared *via* electrostatic adsorption of anionic liposomes on the surface of cationic colloidal particles. This approach allows one to enhance drug loading and offers a simple and reliable technology for fabrication of patient-oriented multi-functional drug formulations.

Here, we report multi-liposomal containers based on chitosan nanoparticles, with the emphasis on the biodegradability of the containers in the presence of proteolytic enzymes. Chitosan is a commercially available cationic polysaccharide which can be prepared by deacetylation of chitin, the main component of the crustacean exoskeleton.[13,14] This polymer is metabolised by certain human enzymes, *e.g.* lysozyme.[15] The cationic chitosan nanoparticles, actually nanogels with controlled size and swelling, are synthesized by cross-linking of chitosan macromolecules in the presence of bi- or trivalent anions.[16,17] The obtained results seem to be promising for preparation of biodegradable multi-liposomal drug carriers.

Liposome–chitosan[†] complexes were prepared following the procedure described earlier.[21] First, anionic PC/CL^{2-} liposomes were adsorbed on the surface of chitosan nanogels in buffer solution (pH 5.5) that provided the effective (quantitative) electrostatic liposome-to-chitosan complexation. Then, pH 5.5 of the external buffer solution was changed to pH 7, while the composition of the resulting complex was preserved. For 50 nm anionic PC/CL^{2-} liposomes and 300 nm chitosan nanogels, the above procedure resulted in ~80 liposomes per one chitosan particle.[21]

Biodegradation of liposome–chitosan complex particles was initiated by addition of a proteolytic enzyme:[‡] lipase hydrolyzed ester bonds in the satellite liposomes,[22] or lysozyme hydrolyzed glycosidic bonds in the chitosan shell,[15] or Morikrase which was a mixture of enzymes capable of cleaving both ester and glycosidic bonds.[20] The degradation was controlled by measuring particle size in the complex–enzyme suspension by dynamic light scattering technique. In a control experiment without enzyme, no change in particle size was observed within 96 h (4 days) after complex preparation [Figure 1(*a*), curve *1*].

Addition of lipase to a suspension of the liposome–chitosan complex eventually decreased the particle size from 550 nm down to ~270 nm within 96 h (4 days) [Figure 1(*a*), curve *2*]. Since the latter size is close to the size of the initial chitosan particles before complexation with liposomes, we can conclude that lipase selectively hydrolyzed ester bonds in lipid molecules but did

[†] Phosphatidylcholine (PC) and diphosphatidylglycerol (cardiolipin, CL^{2-}) from Avanti, doxorubicin (Dox) from VeroPharm (Russia) and 5(6)-carboxyfluorescein (CF) from Sigma-Aldrich were used as received. Chitosan was obtained *via* alkaline deacetylation of chitin[18] with a degree of polymerization of 200, while a degree of chitin deacetylation was of 85 mol%. The chemical structures of compounds are shown in Figure S1 (see Online Supplementary Materials).

Small unilamellar PC/CL^{2-} liposomes, including the liposomes loaded by NaCl, or CF or Dox, were prepared by the standard sonication technique (see Procedure S1, Online Supplementary Materials). The molar ratio of the anionic CL^{2-} headgroups $\nu_{CL} = 2[CL^{2-}]/(2[CL^{2-}] + [PC])$ was 0.1. In all experiments, freshly prepared liposomes with an average hydrodynamic diameter of 40–50 nm were used.

A chitosan nanoparticle suspension was prepared *via* mixing equal volumes of aqueous solution of chitosan (0.005 M) and aqueous solution of sodium sulfate (0.005 M) and further dialysis of the mixture with 10^{-2} M acetate buffer (pH 5.5).[19] Dynamic light scattering measurements showed the mean diameter of the ionically cross-linked chitosan nanoparticles of 270 ± 25 nm (see Procedures S2, Online Supplementary Materials).

[‡] The biodegradation of the liposome–chitosan complex was initiated by addition of a Morikrase proteolytic complex from the hepatopancreas of the Kamchatka crab *Paralithodes camchatica*[20] (Trinita, Russia), or enzyme lipase (Serva), or enzyme lysozyme (Sigma-Aldrich) (see Procedure S3, Online Supplementary Materials).

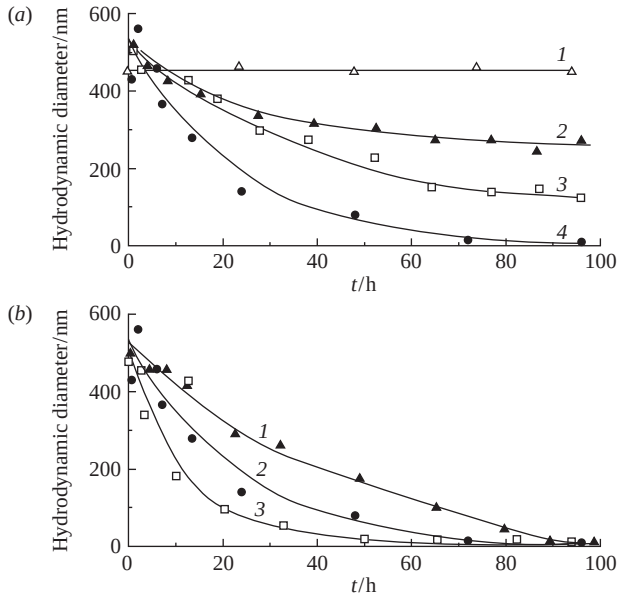


Figure 1 Time dependence of particles size of (*a*) liposome–chitosan complex at 20 °C (*1*) without enzyme, after addition of (*2*) lipase, (*3*) lysozyme and (*4*) Morikrase proteolytic complex; (*b*) Morikrase proteolytic complex at (*1*) 4 °C, (*2*) 20 °C and (*3*) 37 °C. Concentrations: lipid, 0.4 mg ml^{-1}; chitosan, 0.025 mg ml^{-1}; lipase, 0.05 mg ml^{-1}; lysozyme, 0.08 mg ml^{-1}; Morikrase, 0.05 mg ml^{-1}; 0.01 M TRIS buffer (pH 7 at 20 °C).

not attack the chitosan core. In contrast, addition of lysozyme to the complex resulted in formation of 120 nm particles in the suspension [Figure 1(*a*), curve *3*] that indicated an appreciable contribution of the chitosan core hydrolysis in the complex. Finally, in the presence of Morikrase, the complex particle size decreased down to 10–15 nm [Figure 1(*a*), curve *4*]. This size was much smaller than that of both initial components: 250–300 nm for chitosan particles and 40–50 nm for liposomes. Evidently, such deep degradation of the liposome–chitosan complex might arise from the combined action of the Morikrase enzymes capable of cleaving lipids and chitosan. The above results reveal the enzyme-induced biodegradation of the liposome–chitosan complex to small fragments which can be easily eliminated from the human body. Further experiments were performed with the most active catalyst, Morikrase.

As expected, the rate of complex biodegradation rose up if the temperature in the mixed complex-Morikrase suspension was increased [Figure 1(*b*)]. Morikrase degraded the liposome–chitosan complex down to 10–15 nm particles within 96 h at 4 °C (curve *1*), 72 h at 20 °C (curve *2*) and 48 h at 37 °C (curve *3*).

After that, we prepared liposomes with an encapsulated water-soluble substance, adsorbed them on the chitosan nanogel surface and studied the kinetics of the encapsulated substance release in the course of the liposome–chitosan complex biodegradation. Three types of liposomes were used: loaded with NaCl solution, or carboxyfluorescein (CF) solution, or antitumor antibiotic doxorubicin (Dox) solution. The release of NaCl increased the conductivity of the surrounding solution; the release of CF and Dox was accompanied by recovery of their fluorescence partially quenched inside liposomes[22–24] (see details in Procedure S1, Online Supplementary Materials). In the control experiments, no release of the encapsulated substances was found within 3 h after liposome-to-chitosan complexation. Then, Morikrase was added to suspensions of the liposome–chitosan complexes with the encapsulated substances; the mixtures were thermostated for 15 h at three temperatures: 4, 20 and 37 °C. The kinetics of release is presented in Figures 2 and 3. At 4 °C, the release began in 4–6 h and ended 10–15 h after Morikrase addition (curve *1*); at 20 °C, the release started in 3–4 h and ended in 8 h (curve *2*), while at

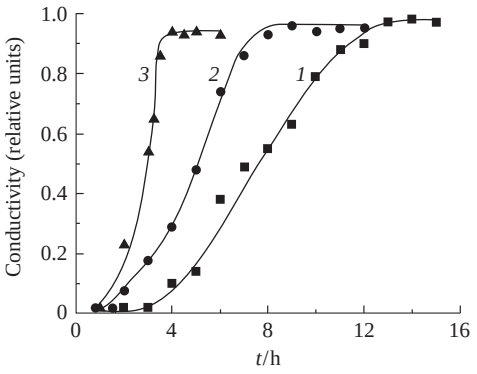


Figure 2 Time dependence of the relative conductivity of (NaCl-loaded liposome)–chitosan complex suspension after Morikrase addition at (*1*) 4 °C, (*2*) 20 °C and (*3*) 37 °C. Concentrations: lipid, 0.4 mg ml^{-1}; chitosan, 0.025 mg ml^{-1}; Morikrase, 0.05 mg ml^{-1}; 0.01 M TRIS buffer (pH 7).

37 °C, a discernible leakage of the encapsulated substances was detected in 2–3 h and come to the end in 4–5 h (curve *3*).

To understand the mechanism of the enzyme-mediated escape of the liposome content, the rates of encapsulated substance release and the rates of liposome–chitosan complex biodegradation were compared. (1) In all cases, the release began to be detectable only a few hours after Morikrase addition, *viz.*, from 4–6 h at 4 °C down to 2–3 h at 37 °C (see Figures 2 and 3). During this time, the size of particles in the mixed complex–Morikrase suspension changed insignificantly [see Figure 1(*b*)]. At this stage, the Morikrase enzymes apparently attacked ester bonds in lipid molecules that led to destabilization of the lipid bilayer and a leakage of substances from the liposomes, whereas the chitosan core remained nearly unchanged. The total leakage of encapsulated substances required from 10–15 h at 4 °C to 4–5 h at 37 °C (see Figures 2 and 3) and was accompanied by an additional negligible decrease in the particle size [see Figure 1(*b*)]. The leakage ended

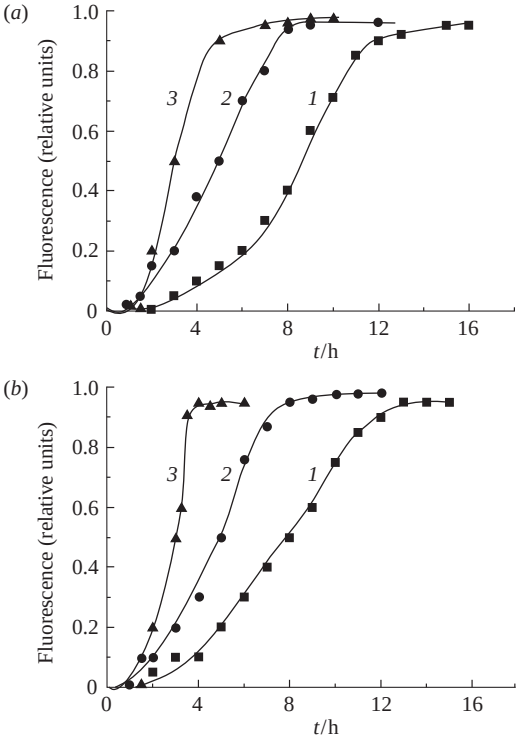


Figure 3 The time dependence of the relative fluorescence of (*a*) (CF-loaded liposome)–chitosan complex and (*b*) (Dox-loaded liposome)–chitosan complex after Morikrase addition at (*1*) 4 °C, (*2*) 20 °C and (*3*) 37 °C. Concentrations: lipid, 0.4 mg ml^{-1}; chitosan, 0.025 mg ml^{-1}; Morikrase, 0.05 mg ml^{-1}; 10^{-2} M TRIS buffer (pH 7).

after destruction of all liposomes initially involved in the complexation with chitosan but the chitosan core was not still affected by the biodegradation. (2) The biodegradation of the chitosan core started only after the destruction of liposomes, which was accelerated by increasing the temperature. (3) The rate of enzyme-mediated release did not depend on the type of encapsulated substance: a simple inorganic salt (NaCl), or a fluorescent dye (CF), or an antitumor drug (Dox).

It is of special note that in all cases, the biodegradation resulted in a complete (quantitative) release of substances from the liposomes into the surrounding solution. This fact is of particular interest for potential use of multi-liposomal chitosan-based containers for encapsulation, delivery and release of drugs.

This work was supported by the Russian Science Foundation (project no. 14-13-00255).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2018.03.009.

References

1 P. Burgess, P. B. Hutt, O. C. Farokhzad, R. Langer, S. Minick and S. Zale, *Nat. Biotechnol.*, 2010, **28**, 1267.
2 U. A. Budanova, I. S. Shchelik, O. O. Koloskova and Yu. L. Sebyakin, *Mendeleev Commun.*, 2016, **26**, 205.
3 M. L. Immordino, *Int. J. Nanomedicine*, 2006, **1**, 297.
4 L. Ya. Zakharova, R. R. Kashapov, T. N. Pashirova, A. B. Mirgorodskaya and O. G. Sinyashin, *Mendeleev Commun.*, 2016, **26**, 457.
5 R. Lehner, X. Wang, S. Marsch and P. Hunziker, *Nanomedicine*, 2013, **9**, 742.
6 A. V. Filippov, S. A. Kotenkov, B. V. Munavirov, A. V. Khaliullina, O. I. Gnezdilov and O. N. Antzutkin, *Mendeleev Commun.*, 2016, **26**, 109.
7 J. Lao, J. Madani, T. Puértolas, M. Alvarez, A. Hernández, R. Pazo-Cid, A. Artal and A. J. Torres, *Drug Deliv.*, 2013, 456409.
8 C. M. Knapp and K. A. Whitehead, *Expert. Opin. Drug Deliv.*, 2014, **11**, 1923.
9 A. A. Yaroslavov, A.V. Sybachin, M. Schrinner, M. Ballauff, L. Tsarkova, E. Kesselman, J. Schmidt, Y. Talmon and F. M. Menger, *J. Am. Chem. Soc.*, 2010, **132**, 5948.
10 A. V. Sybachin, O. V. Zaborova, M. Ballauff, E. Kesselman, J. Schmidt, Y. Talmon, F. M. Menger and A. A. Yaroslavov, *Langmuir*, 2012, **28**, 16108.
11 A. A. Yaroslavov, A. A. Efimova, A. V. Sybachin, S. N. Chvalun, A. I. Kulebyakina and E. V. Kozlova, *RSC Adv.*, 2015, **5**, 31460.
12 A. A. Yaroslavov, A. A. Efimova, G. N. Rudenskaya, N. S. Melik-Nubarov, I. D. Grozdova, A. A. Ezhov, S. N. Chvalun, A. I. Kulebyakina and E. V. Razuvaeva, *Mendeleev Commun.*, 2017, **27**, 299.
13 M. N. V. Ravi Kumar, R. A. A. Muzzarelli, C. Muzzarelli, H. Sashiwa and A. J. Domb, *Chem. Rev.*, 2004, **104**, 6017.
14 J. Zhang, W. Xia, P. Liu, Q. Cheng, T. Tahi, W. Gu and B. Li, *Mar. Drugs*, 2010, **8**, 1962.
15 T. Freier, H. S. Koh, K. Kazazian and M. S. Shoichet, *Biomaterials*, 2005, **26**, 5872.
16 J. Berger, M. Reist, J. M. Mayer, O. Felt, N. A. Peppas and R. Gurny, *Eur. J. Pharm. Biopharm.*, 2004, **57**, 19.
17 N. Bhattarai, J. Gunn and M. Zhang, *Adv. Drug Deliv. Rev.*, 2010, **62**, 83.
18 A. Bernkop-Schnürch and S. Dünnhaupt, *Eur. J. Pharm. Biopharm.*, 2012, **81**, 463.
19 M. M. A. Al-Remawi, *Am. J. Appl. Sci.*, 2012, **9**, 1091.
20 L. V. Liutova, M. A. Karabasova, G. V. Andreenko, G. N. Rudenskaia, V. A. Isaev and A. B. Brusov, *Voprosy Med. Khim.*, 1998, **44**, 292 (in Russian).
21 A. A. Efimova, F. D. Mulashkin, G. N. Rudenskaya, E. G. Evtushenko, V. N. Orlov, N. S. Melik-Nubarov, G. G. Krivtsov and A. A. Yaroslavov, *Polym. Sci., Ser. B*, 2018, **60**, 84 (*Vysokomol. Soedin., Ser. B*, 2018, **60**, 57).
22 A. A. Efimova, S. N. Chvalun, A. I. Kulebyakina, E. V. Kozlova and A. A. Yaroslavov, *Polym. Sci., Ser. A*, 2016, **58**, 172 (*Vysokomol. Soedin., Ser. A*, 2016, **58**, 146).
23 A. E. Zhirnov, T. V. Demina, O. O. Krylova, I. D. Grozdova and N. S. Melik-Nubarov, *Biochim. Biophys. Acta*, 2005, **1720**, 73.
24 A. A. Efimova, S. N. Kostenko, V. N. Orlov and A. A. Yaroslavov, *Mendeleev Commun.*, 2016, **26**, 99.

Received: 28th July 2017; Com. 17/5320