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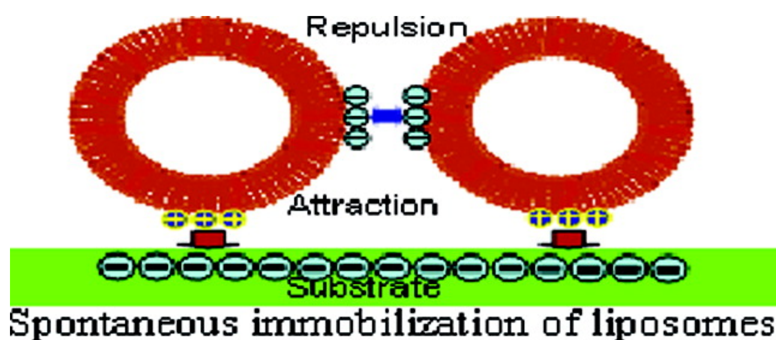

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Spontaneous Immobilization of Liposomes on Electron-Beam Exposed Resist Surfaces

Jong Min Kim,[†,§] Ho Sup Jung,[†] Jong Wan Park,[†] Tetsuo Yukimasa,[‡] Hiroaki Oka,[‡] Hea Yeon Lee,*[,†] and Tomoji Kawai*[,†]

Contribution from the Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan, Matsushita Electric Industrial Co., Ltd., Advanced Technology Research Laboratories, 3-4 Hikaridai, Seika-cho, Soraku-gun, Kyoto 619-0237, Japan, and School of Bionics, Tokyo University of Technology, Katakura, Hachioji, Tokyo 192-0982, Japan

Received June 29, 2004; E-mail: hylee@sanken.osaka-u.ac.jp; kawai@sanken.osaka-u.ac.jp

Abstract: We have found an interesting immobilization technique of liposomes on electron-beam exposed resist surfaces. The immobilized liposomes have been visualized by the atomic force microscope and have shown well-organized three-dimensional physical structures, in which the liposomes maintain their shapes and sizes similar to those of the original design in prepared solution. The immobilization is based on a strong static charge interaction between the resist surface and the liposomes. Further experiments show that very strong charge in the surfaces produces the firm immobilization of the liposome. We believe these findings can be related to various liposome applications such as drug delivery system, electrochemical or biosensors, and nanoscale membrane function studies.

1. Introduction

Liposomes (lipid vesicles) have attracted great scientific interests as models for biological membranes.[1] An understanding of membrane structure and the function of molecules within the membrane bilayer is pertinent to issues such as drug delivery across a membrane and numerous cellular regulatory processes.[2] The observation and analysis of liposomes depend on various optical techniques such as a light scattering method, a spectroscopic method, and a fluorescence microscopy method for investigating the dynamics and structures.[1,2] Recent reports have shown nonpertubational optical detection of liposomes is possible using an optical trapping method.[3] Though it is preferable to investigate liposomes in the absence of any surface perturbation, in the conventional optical techniques, the observation of subwavelength distance is ideally difficult because of far-field diffraction limit.[4] Therefore, the detection of subwavelength sized liposomes or subwavelength distance investigation in large liposomes requires a different technique. Currently, the best magnified observation comes from high-resolution surface microscopy techniques though immobilization procedures are still required. Therefore, if the three-dimensional liposome structures similar to those in liquids can be maintained in the surface, the critical problem concerned with the immobilization will not prohibit the analysis; thus, the surface techniques can also provide fluent information for the analysis. A freeze-fracture electron microscopy method is useful to detect immobilized liposomes on surfaces with high magnification but cannot offer a direct elucidation of various liposome-related function because it requires sample freezing and modification.[5]

The atomic force microscope (AFM) is now a well-known tool for observing surface-related physical or chemical properties with atomic scales.[6] Numerous reports have shown that the dynamics related to samples are observable in various environments using the AFM. In addition, the imaging mechanism endows the AFM with a unique tool for manipulating a single molecular order.[7] These merits imply the potential capabilities of the AFM for liposome-related studies in a nanoscale.

The observation of liposomes with the AFM or surface techniques poses two traditional problems with regard to their properties.[1] The first problem is that the immobilization makes a change in the three-dimensional physical structures of liposomes. As the first problem, the inner and outer layers of liposomes are normally hydrophilic. In a general sense, interactions between liposomes and surfaces involve electrostatic and hydration forces as well as attractive contributions from long-range van der Waals forces. Various studies have supported that liposomes spontaneously form flat lipid bilayers through fusion processes on hydrophilic surfaces via such interactions.[2,8,9] Therefore, the real structure of liposomes is difficult to exist on hydrophilic surfaces because the control of the complicate

† Osaka University.
‡ Matsushita Electric Industrial Co., Ltd.
§ Tokyo University of Technology.

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mechanism is difficult. In this way, hydrophobic surfaces are ideally better, but in normal hydrophobic surfaces, individual liposome easily aggregates with each other, ultimately forming giant vesicles and aggregators in our observation. AFM investigation for liposome-related subjects has been variously reported, but almost all the research has focused on the self-assembly of lipid bilayers. For liposomes themselves, a few reports have been published but the observed dimension is still not satisfactory.[10−13] In liposome applications, the nature of the inside water pool promises the usability of liposomes to the various functional systems. Therefore, three-dimensional liposome structures similar to those in the liquid phase have to be sustained in the surfaces to investigate the dynamics related to the inside water pool. The observed height of 100-nm large unilamellar vesicles (LUVs) was 2 nm on a normal mica surface by AFM techniques.[10] In this dimension, the inside water pool is difficult to exist. Also, an AFM report for the liposome investigation has been recently published using single bilayer patches in an aqueous environment, but the obtained height of 100-nm LUVs only reached sub-20 nm (13-nm max) with complicatedly aggregated shapes.[14] These results mean the fundamental problem of the immobilization is still not overcome.

The second problem is the concentration distribution of liposomes on surfaces. The physical instabilities of liposomes render strong mixing difficult and engender the different concentration distribution on the local surface. Therefore, in our study, observing liposomes requires repeated trial and error with surface techniques.

To immobilize liposomes individually similar to the physical configuration in solution, two involved forces for the binding to surfaces and the repulsion between individual liposome are necessary. Currently, no reports have been published fulfilling such requirements.

Here, we find interesting spontaneous immobilization phenomena of liposomes on electron-beam (e-beam) exposed resist surfaces. We report those successful AFM results for the characterization.

2. Methods

2.1 Sample Preparation. Liposomes were fabricated as described previously.[15] Briefly, a lipid solution was prepared by mixing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dimyristoyl-*sn*-glycero-3-phospho-1-glycerol (DMPG), and cholesterol with a molar ratio of 10:10:1. The lyophilized solution was dehydrated by buffer solution (PBS, pH 7.4). Subsequently, five freeze−thaw cycles were applied and the lipid vesicles were extruded repeatedly to produce the POPC liposome through a polycarbonate film with 100-nm pores using an extruder device (LipoFast; Avestin Inc.).[15] Final concentration of the phospholipid was approximately 10 mM.

An e-beam lithography technique was used for the fabrication of an immobilization substrate using a standard positive polymer resist (ZEP520; Zeon Corp.). ZEP is a copolymer of chloromethacrylate and methylstyrene. It is normally used for the high-resolution e-beam lithography instead of poly-methyl-methacrylate (PMMA). Before the e-beam process, the gold layer of 100 nm had been fabricated on the Ti sublayer (10 nm) by a sputtering technique. Next, the resist was spin-coated on the whole substrate with a thickness of 300 and 150 nm before exposed to the e-beam. The e-beam exposed area was approximately 100 × 100 μm^2; various sizes of holes for testing the substrate-dependent immobilization variation were developed using the same scanning speed and line number per pixel. The total developing time was about 30−60 min depending on the developing size. The used dose was about 150 C/cm^2 using a 75 kV scanning electron microscope (ELC-2; Elionix Co. Ltd.). After the e-beam lithography, the substrates were promptly used for the immobilization experiment.

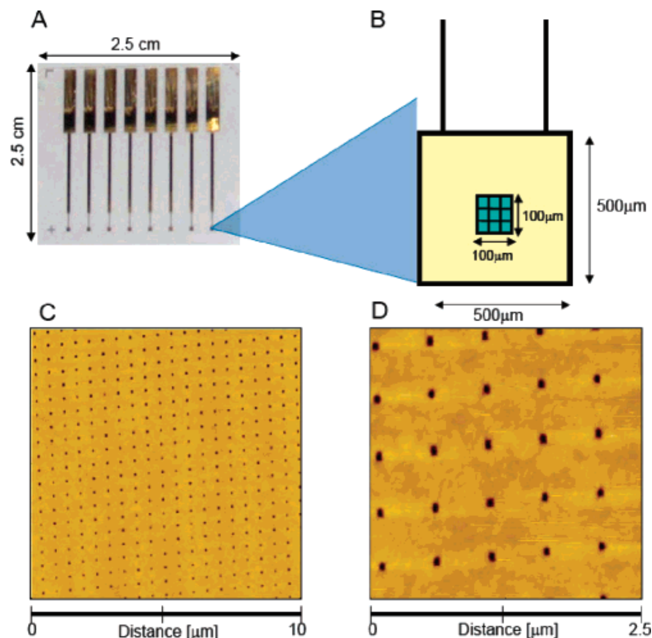

Figure 1. Schematic illustration (A, B) and AFM topographic images (C, D) for the used sample substrate. The size of the whole substrate is 2.5 × 2.5 cm^2 (A), the experimental area is 500 × 500 μm^2 (B), and the e-beam developed area is 100 × 100 μm^2 (B). (C) and (D) show the AFM images of e-beam developed areas in the scales of 10 × 10 μm^2 and 2.5 × 2.5 μm^2.

The immobilization of the liposome was performed by incubating a 100-μL portion of the liposome solution on the e-beam exposed substrate for 1 h. The substrate was washed carefully with buffer solution (PBS, pH7.4) for AFM measurements. The covering diameter of the solution was approximately 5 mm. Therefore, the 500 × 500 μm^2 experimental area (AFM imaging area, see Figure 1A, 1B) was completely incubated by the liposome solution. The AFM measurement was performed promptly after the immobilization. Before the immobilization, the size of the liposome was always confirmed by a light scattering method (See support Figure S1).

2.2 Instruments. A conventional AFM instrument (Dimension 3100, Digital Instruments, USA) was used for surface imaging. All measurements were performed using the intermittent contact mode (tapping mode) with a scanning speed of 1 Hz (512 × 512 data format) in the air. The instrument has an optical microscopic set for investigating sample surfaces. Thereby, the e-beam exposed area is found easily using the optical function. A normal tapping mode Au-coated AFM cantilever with an oscillation frequency of 150 kHz and spring constant of 4.5 N/m (μmasch, NSC12/Au) was used for all AFM imaging. For a successful mapping of the hydrophilic liposome surface, a hydrophobic modification was applied to AFM tips following a previous report using octadecyl mercaptan.[16]

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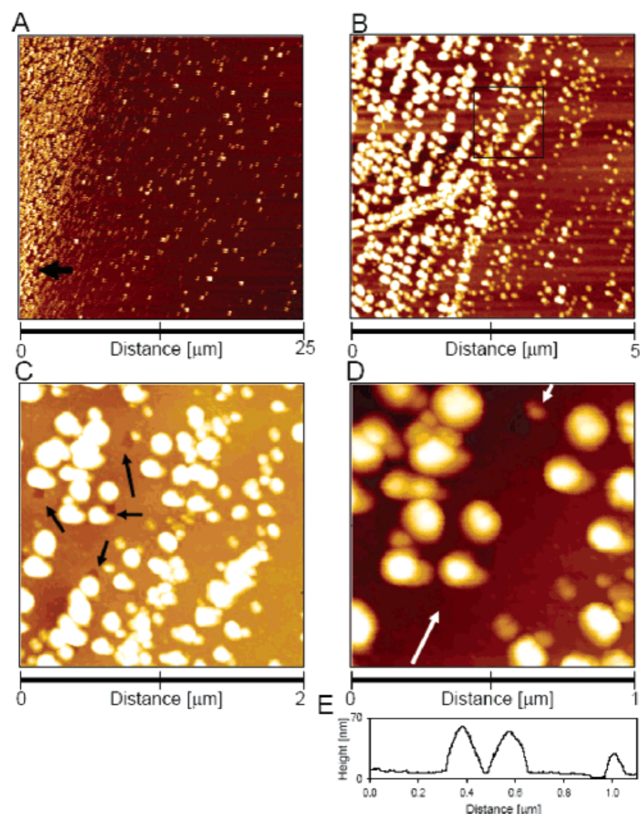

Figure 2. AFM topographic images of typical liposome immobilized patterns on the e-beam exposed surfaces in the scan areas of 25 × 25 μm^2 (A), 5 × 5 μm^2 (B), 2 × 2 μm^2 (C), and 1 × 1 μm^2. (C) and (D) are enlarged images noted as a rectangle in Figure 2B. (E) is the line profile analysis result indicated in Figure 2D.

3. Results and Discussion

Figure 1 shows schematic illustrations (a, b) and typical AFM images (c, d) of an e-beam developed substrate. A 30-nm-thick gold sublayer was formed to find easily the location of the e-beam developed area (Figure 1A, 1B). Figure 1C and 1D shows 50 × 50 nm^2 sized holes with 500-nm intervals developed by the e-beam lithography. The examined thickness of the resist ranged from 150 to 300 nm; the thickness-dependent immobilization variation was not clearly observed in this range. The surface roughness of the substrate was approximately 2 nm and was sufficient to detect the size of small unilamellar vesicles (SUV) except for the holes.

Figure 2 shows AFM topographic images of the immobilized POPC liposome around the interface between the e-beam exposed area and the undeveloped resist area. Figure 2A (25 × 25 μm^2) shows two different immobilization regions. Enlarged images (Figure 2B−2D) offer the confirmation that the highly dense liposome immobilization is located in the e-beam exposed side: small holes are visible in Figure 2C as indicated by the arrows. In addition, the size distribution of the liposome can be confirmed in Figure 2B and 2C by the interface. In the developed area, the widths of the individual liposome are 120−160 nm at full width at half-maximum (fwhm). However, the liposome size is markedly small in the undeveloped area generally sub-60 nm (see support Figure S1 for the size distribution in the prepared solution). Only giant and very largely aggregated liposomes were confirmed from AFM topographic images on the outer region more than 80 μm from the developed center, that is, the immobilization of LUVs was not confirmed (see support Figure S2). In Figure 2D and 2E, though the variation is slightly large, the normal size and height of the liposomes are confirmed as approximately 150 × 150 nm^2 and 60 nm, respectively. The three-dimensional physical structure is not perfectly ideal but is a sufficiently well-organized shape compared to various previous reports.[10,14] The confirmed volume of the liposome is similar to that of the originally designed liposome in the liquid. Figure 2A shows the immobilization pattern depicting clear evidence that the e-beam exposed area has a certain immobilization force compared with a normal resist surface (see support Figure S2). One clue is the accumulation of electrons in the resist during the e-beam lithography process.[17] Mehta et al. reported that various e-beam exposed nonconducting polymers have wide ranges of the electron accumulation. Although the liposome has both zwitterionic (POPC) and negative ionic (DMPG) chemicals, the local charge distribution of the liposome can be rearranged by a rafting process.[2,18] In the process, the landing region of the liposome on the surface can be rearranged with the positive parts of the zwitterionic POPC. Thus, the immobilization is driven by a static charge interaction between the surface and the liposomes. The rafted negative ionic DMPG leads to repulsion between each individual liposome because the landing region of the liposome is only placed to zwitterionic POPC. Therefore, the aggregation between the liposome can be reduced successfully.

Slight changes in the resist pattern make a graphical influence on the immobilization characteristics. Figure 3 shows AFM images of immobilized liposomes using a different pattern of 150 × 150 nm^2 sized holes with 250-nm intervals. The imaging area is relatively the center of the e-beam developed area. As shown in Figure 3A, the liposome is immobilized equally over the scan area of 30 × 30μm^2. In the enlarged image b, it is confirmable that the discrimination of the individual and aggregated liposomes is possible. As shown in Figure 3B, only a few liposomes are aggregated. In Figure 3C, the dimension of the individual liposome is approximately 130 × 130 × 80 nm^3, which is similar to the dimension in the liquid confirmed by the light scattering method. The slight difference in the liposome height compared with Figure 2 may be reasoned by the surface charge intensity. In the current substrate, the single hole area is 150 × 150 nm^2, which is bigger by about 10 times than that of Figure 2. It means that the e-beam exposed time of the substrate is larger by about 10 times than that of Figure 2 and means that the possibility of the more strong charge intensity on the surface because we have used the same e-beam parameters (scan speed, line-number per pixel) on the all substrate fabrication.

We performed two indirect support experiments to confirm the immobilization mechanism. In the first experiment, we used streptavidin molecules to measure the surface charge intensity, indirectly. In that experiment, changing the solution pH values allowed the control of the charge polarity of the streptavidin. In other words, the isoelectric point of the streptavidin is about 5.9. Therefore, by changing the pH value of the buffer solution to 4.2 (positive) and 7.4 (negative), the charge polarity of the streptavidin can be controlled. Thereby, the positively charged streptavidin binds strongly with the e-beam exposed resist, whereas the negatively charged streptavidin does not (see sup-

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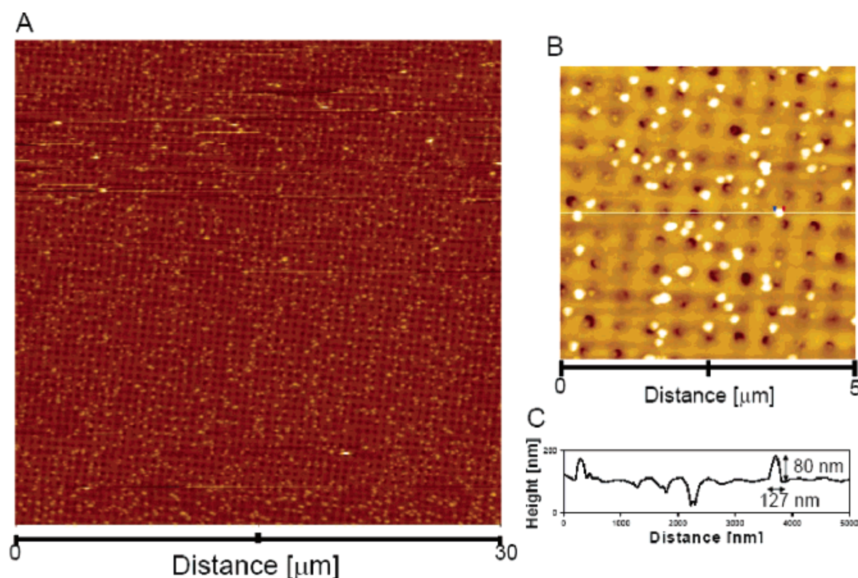

Figure 3. Liposome imaging results for the near center of the e-beam exposed area. Image scales are 30 × 30 mm^2 (A) and 5 × 5 mm^2 (B). In the line profile (C), the width and the height are obtained as approximately 150 and 80 nm, respectively. One can easily find that the heights and widths of individual liposomes are similar in all image areas (A, B).

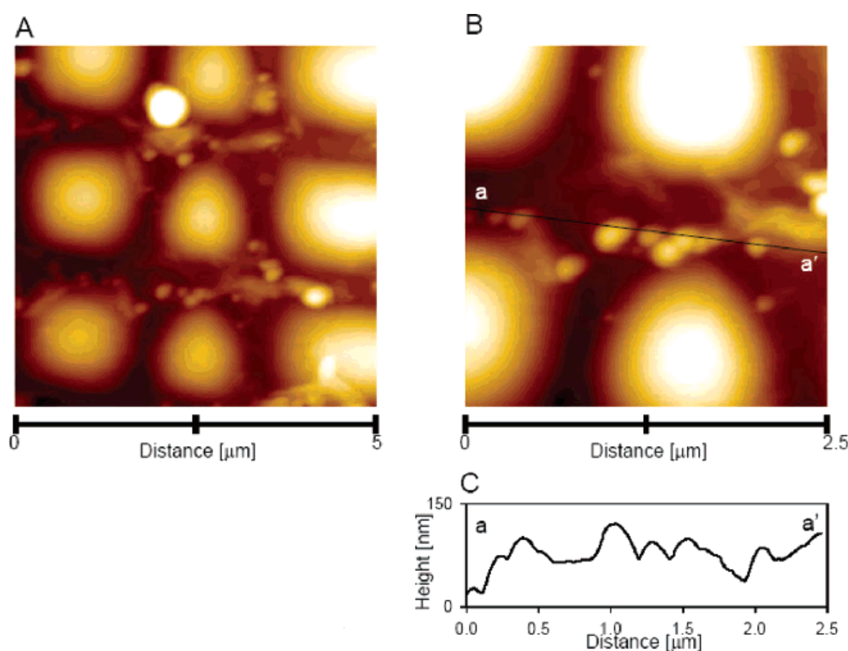

Figure 4. Typical AFM images of stearic acid mixed liposomes immobilized on an e-beam exposed substrate. Almost all the positively charged liposomes are located on e-beam developed gaps. (A) and (B) show the AFM images of 5 × 5 μm^2 and 2.5 × 2.5 μm^2, respectively. (C) shows a line profile analysis indicated as "a−a′" in Figure 4B.

port Figure S3). This can be an indirect evidence for the static charge interaction between the substrate and the liposome. More than 10 molecular order streptavidin layers, which imply the surface charge strength indirectly, could be confirmed when the positively charged streptavidin was used. This result was comparative with a similar experiment done by a mica surface as the substrate, that is, the mica is negatively charged in aqueous solution. In the case of the mica, only randomly adsorbed single molecular order streptavidin molecules were visualized with the same buffer condition (pH 4.2). Thus, e-beam exposed area has a noticeably strong charge intensity compared to that of the mica surface with more than 10-fold (see support Figure S4). Such a substrate of strong charge intensity is fundamentally necessary to immobilize the large liposome firmly.

In the second experiment, we used positively charged liposomes fabricated by adding stearic acid ($CH_3(CH_2)_{16}COOH$, 30 wt. %). Figure 4 shows typical AFM images of the immobilized liposome using the same immobilization procedure. The used substrate has a different configuration as shown in Figure 4A, that is, the resist part (bright parts) has a wider surface area than that of e-beam developed gaps. Because the liposome is composed of various chemicals of different polarity such as POPC, DMPG, and stearic acid, the liposome is easily aggregated and seemed to be more unstable than the original POPC liposome. Nevertheless, the immobilized liposomes are always located in the developed gaps; thereby, it supports the possible strong charge intensity around the gaps. Similar tendency can be confirmed

in Figure 3, that is, the location of the liposome is generally nearby the holes in Figure 3B.

In the current paper, we have shown the efficient immobilization technique of liposomes. The immobilized liposomes maintain their three-dimensional configuration similar to those in the liquid. The reproducibility of the experiment was related to the substrate age, that is, the time lapse after the fabrication. Though we have an experimental reproducibility of about 100% with a fleshly fabricated substrate, the immobilization experiment was not successful by using the substrate fabricated before three months. It seems to be explained by the leakage of the accumulated charge. The main factor for the successful immobilization is a strong static charge interaction but more careful investigation for the surface chemistry is still required in the future. Finally, we believe that the current immobilization technique is helpful not only for surface-related liposome observation but also for many liposome-related studies and applications by designing the shape and the structure of the e-beam exposed areas. Because the surface or pattern design by the e-beam is flexible, the application of the current immobilization technique will be a wide range. In addition, we have already confirmed the electrochemical activity of the immobilized liposome on the e-beam exposed resist electrode with a similar mechanism reported by Xu.[19] The dynamic changes of the individual liposome induced by the interaction with proteins are now investigated in our laboratory and will be reported elsewhere.

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Supporting Information Available: AFM imaging results for the indirect support experiments and the immobilized liposomes on the nonexposed normal ZEP resist as well as the result for liposome size measurement in liquid (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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