Anisotropy in Shape and Ligand-Conjugation of Hybrid Nanoparticulates Manipulates the Mode of Bio–Nano Interaction and Its Outcome

Xiaoyou Wang, Li Lin, Renfa Liu, Min Chen, Binlong Chen, Bo He, Bing He, Xiaolong Liang, Wenbing Dai, Hua Zhang, Xueqing Wang, Yiguang Wang, Zhifei Dai,* and Qiang Zhang*

In an attempt to manipulate the biological features of nanomaterials via both anisotropic shape and ligand modification, four types of nanoparticulates with good morphological stability are designed and engineered, including hybrid nanospheres, nanodiscs, and nanodiscs with edge modification or plane modification of octa-arginine (R8) sequence. It is found that the R8 modification anisotropy can trigger huge differences in the endocytosis, intracellular trafficking, and even tissue penetration of nanoparticulates. From plane modification to edge modification of R8, the maximum increase in cell uptake is up to 17-fold, which is much more significant than shape anisotropy alone. On the other hand, six types of different cell lines are investigated to simulate biological microenvironment. It is demonstrated that the maximum difference in cell uptake among six cell lines is 12-fold. Three main driving forces are found to contribute to such bio–nano interactions. Based on the findings of this study, it seems possible to manipulate the biointeraction mode of nanomaterials and its output by regulating their anisotropy in both shape and ligand modification.

1. Introduction

The bio-nano interaction is one of the most fundamental scientific issues at the interface of nanotechnology and life science, and is often involved in the studies of biomaterials, tissue engineering, imaging, diagnostics, therapeutics, device, medication, even biology, pathology, immunology, and so on. For instance, the rational design of nanocarriers for molecular imaging and targeted therapy requires an understanding of the mechanism in terms of the interaction between nanocarriers and cells/tissues. The capability to manipulate the bio-nano interaction may help improve the diagnostic or therapeutic efficacy as well as the biocompatibility.

Biomimetic cerasomes, a type of silicon-lipid hybrid nanospheres, have drawn much attention because of its better biocompatibility than silica nanoparticles and its higher morphological stability than conventional lipid vectors. Bicelles are a fascinating category of membrane bilayer nanodiscs. The hybrid bicelles (HBs), made of a long-chain cerasome-forming lipid (CFL) and a short-chain phospholipid, are the further outgrowth of bicelles. Similar to cerasomes, this type of novel nanodiscs possesses higher stability, which makes them a perfect model for the study of bio-nano interaction. Such nanodiscs can also serve as a powerful tool to deliver a variety of cargoes ranging from proteins and oligonucleotides to chemicals, thanks to the combined merits of lipid vesicles, mixed micelles and silicon nanoparticulates.

Concerning the shape effect on the biointeraction with nanoparticulates, reports can be found in recent ten years. The general conclusion is that nanoparticles with larger aspect ratios (elongated nanoparticles) at the contact area are taken up in larger frequency and in faster rate. Specific aspect ratios may give rise to various other phenomena, e.g., frustrated phagocytosis, where the phagocytes spread on the particles instead of complete phagocytosis. Only few studies involve the nanodiscs, including the comparison between hydrogel nanodiscs and nanorods. The shape effect of nanomaterials on their interaction with tissue is hardly found. One study reported that the size and shape of cell-penetrating peptide TAT-modified polymer micelles affected their cell entry. However, there are currently no literatures about the impact of ligand positions on the interaction between nanoparticulates and cell/tissue.

Although the ligand modification is a highlighted aspect of nanomedicines, the membrane fluidity of lipid-based
nanoparticulates may impede the precise control of ligand stability. The use of CFLs can tackle the problem via the crosslinked siloxane network formed on the surface of nanoparticles.\cite{17,18} Cell-penetrating peptides (CPPs) can translocate across cell membranes and even the endo-lysosomal membranes via their strong interactivity with phospholipid membranes.\cite{19,20} It has been shown that CPPs own the exceptional capacity to mediate the cellular transport of an increasing library of nanoparticulate such as quantum dots, liposomes, and polymeric dendrimers.\cite{21} Although CPPs have gained much attention and even been tested in the clinical trials,\cite{22} there are nowadays no studies found on the CPP-modified nanodiscs.

Generally, the features of both nanocarriers and target biological systems may have crucial impacts on the bio-nano interactions. We believe that anisotropic functionalization with CPPs may further regulate the biointeraction of nanodiscs on the basis of their shape anisotropy, while phagocytic or endocytic nature of different types of cells may also play a key role in such a process. Here, we hypothesize that all the factors including shape anisotropy, functionalization anisotropy and phagocytic/endocytic natures of cells are not only involved in the same bio-nano interaction in different patterns and degrees, and there will be some type of crosstalk among them. The clarification of their impacts on such a process will certainly help us to manipulate the interaction modes as well as their outcomes.

2. Results and Discussion

2.1. Fabrication and Characterization of Nanoparticulates

For the proof-of-concept, the HBs were engineered here from a long-chain proamphiphilic organoalkoxysilane of $N\{N\{3$ triethoxysilyl)$propylsucinamoyl\}$dihexadecylamine (CFL) and a short-chain phospholipid of 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) as a model hybrid bicollear nanodisc. To further modulate the bio-nano interaction and elicit the impact of anisotropic shape and ligand modification, the CPPs of octa-arginine sequence (R8) were conjugated with short alkyl chain (C8-R8) and long alkyl chain (C18-R8), respectively, followed by modifying the HBs around the edge by C8-R8 or on the plane (both top and bottom surfaces) by C18-R8. The obtained edge- and plane-modified hybrid bicollear nanodiscs were designated as EHB and PHB, respectively (Figure 1). The site-specific modifications of CPPs were based on the site-specific distribution of long- and short-chain lipids in the bicelles. As demonstrated before, long- and short-chain lipids position at planes and edges, respectively.\cite{6,23,24} The spherical cerasomes which consisted of only $N\{N\{3$ triethoxysilyl)$propylsucinamoyl\}$ dihexadecylamine were also prepared.\cite{25} The peak of Si–O–2Si in Fourier transform infrared (FTIR) spectra was found for all nanoparticulates (Figure S1, Supporting Information).

The HB, EHB, and PHB were then examined by transmission electron microscopy (TEM) and atomic force microscope...

---

Figure 1. Schematic illustration of engineering, structure, and shape of cerasome, HB, EHB, and PHB, respectively, as well as the manipulation of biological features by the anisotropy in shape and ligand modification through modulating the bio-nano interaction mode. In the preparation of HB, the ratio of long-chain CFL to short-chain DHPC was 7:2 (molar ratio). C18-R8 or C8-R8 was added separately for PHB or EHB. Modification intensity of R8 was controlled by the amount of C18-R8 or C8-R8 added.
The ellipsoidal and rod-like objects in TEM images were attributed to the projections of the face-on and edge-on bicelles, respectively. It seemed that there was almost no object with other shapes, suggesting the preferential modes of face-on or edge-on interaction. The average diameters of nanodiscs were around 50 nm, which were in agreement with the measurements using dynamic light scattering (Figure 2H, and Figure S2C,D, Supporting Information). The HB showed a thickness of around 5 nm (Figure 2G), indicating that each bicelle could be regarded as a single membrane lipid bilayer. These HBs could keep their discoidal structure in dry environment, showing higher stability than conventional phospholipid bicelles. The characterization of cerasomes is summarized in Figure S2E,F (Supporting Information).

The biocompatibility of nanodiscs was evaluated by reactive oxygen species (ROS) and Methylthiazolyl tetrazolium (MTT) assay. ROS generation is one of the most frequently reported nano-related toxicities. As shown in Figure S3A (Supporting Information), PHB exhibited higher ROS signals than other three nanoparticulates on human breast cancer cell MDA-MB-231 (MM-231). The reason might be that the large surface area of R8 modification on the top and bottom of PHB promoted the bio-adhesion of nanodiscs onto the cell surface. In fact, R8 is reported to affect cell membrane and trigger bio-nano interaction, leading to higher ROS signals. Besides, PHB was also noticed to cause hemolysis at high concentration and destabilize lysosomal membrane (Figure S3B,C, Supporting Information). On the other hand, these studies showed that hybrid nanospheres and nanodiscs exhibited similar patterns in ROS generation, hemolysis and lysosomal destabilization. Finally, MTT assay on MM-231 cells indicated that there was no obvious difference in cell growth among HB, EHB, and PHB groups (Figure 2I) as the concentrations of nanoparticles increased from 1 to 250 µg mL⁻¹.

2.2. Cellular Uptake Assay

The cell uptake or endocytosis of the bicellar nanodiscs was then investigated. In MM-231, the cellular uptake of EHB exhibited remarkable improvement with the increasing of R8 molar ratio; however, the increase of R8 modification densities led to an opposite result in PHB internalization (Figure 3A). Since the only difference of these two nanodiscs was the location of R8, the striking difference in the endocytosis between EHB and PHB was obviously ascribed to their anisotropic R8 modification. EHB might contact cell surface on its edge, so at a low modification density (0.5%), the amount of R8 molecules on EHB seemed insufficient to provide efficient adhesion to cell membrane, resulting in lower cellular uptake. As the R8 intensity increased to 1.5% and beyond, the endocytosis of EHB significantly enhanced, likely due to the unique features of edge-based biointeraction, such as large surface curvature and contact angle at the contact site. Also, during the invagination process of the cell membrane, more R8 might draw neighboring membrane closer, helping the membrane to bend and wrap EHB. In contrast, PHB might interact with the cell on its top or bottom, leading to large contact area but small surface curvature and contact angle, which might impede the
The invagination process, causing less internalization of PHB.\(^{[31]}\) The facts here were in accordance with the previous reports concerning the effect of surface curvature and contact angle on nanoparticulate uptake. Namely the particles lying parallelly to the cell membrane were less liable to be internalized.\(^{[16]}\) Generally, our findings demonstrated that anisotropic CPP modification could manipulate the endocytosis of nanodiscs likely via impacting their mode of bio-nano interaction.

The difference in the cell uptake of EHB and PHB with optimized R8 density were further compared with HB in four different cell lines (MM-231, human breast cancer cell MCF-7, human umbilical vein endothelial cell HUVEC, and murine macrophage cell RAW264.7) (Figure S4, Supporting Information). First, as seen in Figure 3B, all four cell lines exhibited increased EHB internalization and decreased PHB endocytosis compared with HB, respectively. Different cell lines showed distinct EHB/PHB uptake rates, which was consistent with their different phagocytic or endocytic functions.\(^{[32,33]}\) However, species differences could not be excluded here as the RAW264.7 is a murine cell line and the rest are human cells.

Furthermore, to address the impact of the shape anisotropy, the spherical cerasomes were compared with discoidal HB in cell internalization in four cell lines (Figure 3D). The uptake rate of HB/Cerasome could indicate the shape effect of nanoparticulate internalization, namely, a percentage higher or lower than 100% might imply more endocytosis of HB or cerasomes, respectively. As shown in Figure 3D, all the uptake ratios of HB/Cerasome exceeded 100%. This revealed that the nanodiscs were more favorable for endocytosis than nanospheres in all test cells. In fact, the internalization of nanodiscs could reach to about 2.5-fold that of nanospheres in MCF-7 cells. Second, the sequence of HB/Cerasome uptake ratio implying the effect of shape anisotropy, was found to be MCF-7, HUVEC > MM-231 > RAW264.7, and it was just contrary to the sequence

---

**Figure 3.** A) Cellular uptake of EHB and PHB with different R8 modification densities by flow cytometry in MM-231. Completely opposite changes on the cellular uptake were observed with the increase of R8 modification density, which was ascribed to the anisotropic modification of EHB and PHB. \(n = 3, ***p < 0.001, ###p < 0.001, &&&p < 0.001\) versus 0.5% group. B) The cell uptake of EHB and PHB compared to HB on different cell lines. All the cell lines showed increased EHB internalization and decreased PHB endocytosis compared with HB, and the anisotropic modification on bicelles changed the output of their biointeraction. \(n = 3, *p < 0.05, **p < 0.01, ***p < 0.001; ##p < 0.01, ###p < 0.001\) versus EHB group. C) The cell uptake of EHB compared to PHB on different cell lines. Different cell lines showed distinct EHB/PHB uptake rates, which was consistent with their different phagocytic or endocytic functions. \(n = 3, *p < 0.05, **p < 0.01, ***p < 0.001\). D) The cell uptake of HB compared to cerasome on different cell lines. Shape anisotropy and cell natures both affected the internalization of nanoparticulates. \(n = 3, ***p < 0.001\). Cellular uptake/adhesion of Rhod B-labeled HB, EHB, and PHB by MM-231 observed using CLSM at E) 37 °C and F) 4 °C. The internalization was proved to be an energy-dependent process.
of EHB/PHB ratios indicating the impact of R8-modification anisotropy. For instance, RAW264.7 cells had a biggest EHB/PHB uptake ratio but a least HB/Cerasome value. Interestingly, this phenomenon seemed to follow a regular rule: the strong-phagocytic cells were more sensitive to the change in ligand location, but relatively insensitive to the alteration in shape; the weak-phagocytic cells were the opposite. It was likely that the strong-phagocytic capacity made the shape effect less significant, and simultaneously it created an obvious synergistic effect with the enhanced bio-nano interaction via R8 edge modification, which well supported our hypothesis.

Next, studies under different temperature or with different method were conducted. As showed in Figure 3E and Figure S5 (Supporting Information), the confocal laser scanning microscopy (CLSM) images of MM-231 cells, MCF-7 cells, and HUVEC incubated with various nanodiscs at 37 °C suggested again the elevated endocytosis of EHB, in agreement with the flow cytomtery data. Besides, EHB and PHB exhibited greater adhesion on the biomembranes at 4 °C than HB group (Figure 3F, and Figure S5, Supporting Information), obviously owing to the R8-cell interaction. The clear differences in CLSM images between 37 and 4 °C revealed an energy-dependent process. In detail, most of labeled nanodiscs were found inside of cells in 37 °C group due to the predominant role of internalization, while most of red signals were on the cell membrane in 4 °C group resulting from the surface adhesion alone. Finally, an ImageStream imaging flow cytometer was utilized to visualize the MM-231 and human mammary fibroblast (HMF) incubated with bilayer nanodiscs (Figure S6A-C, Supporting Information). As the results, 97% of MM-231 cells were found with more than 10 fluorescent spots in EHB group, followed by HB group (60%) and PHB group (52%), indicating less PHB internalization again. Similar phenomenon was observed in HMF cell line. In short, the results of spot counts were in correlation with the uptake studies in Figure 3.

### 2.3. Cellular Uptake on Fibroblast Coculture Model

As one of the major components of tumor microenvironment, tumor associated fibroblasts (TAFs) are crucial because they can affect the growth, resistance and even metastasis of tumor. On the other hand, TAFs are the competitor of tumor cells in terms of nanoparticulate uptake. So, on the basis of studies on single cell model, a mixed 2D cell model was established here to mimic the tumor cells and neighboring fibroblasts via the coinubcation of 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) labeled MM-231 cells and 1,1′dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiD) labeled MCF-7 cells (Figure 4A–E). As seen in Figure 4E, the MM-231/HMF uptake ratio for all tested nanodiscs were below 100%, revealing more endocytosis by TAFs than tumor cells, which was consistent with some of the previous reports on the strong phagocytosis of nanoparticles by TAFs. Importantly, EHB exhibited an MM-231/HMF uptake ratio of 0.8, the highest among the four kinds of nanoparticulates and two times higher than others (p < 0.001). This explained that even in the tumor microenvironment, EHB still possessed the strongest specificity to malignant cells. Also, EHB exhibited significantly higher HMF uptake than other three types of nanoparticles (Figure 4D). So far, in all six tested cell lines, EHB showed the strongest capacity to enter cells. In general, the impact of R8 positions on the nanodisc uptake by both MM-231 cells and HMF was significant. As the HB/Cerasome uptake ratio presented in Figure S6D (Supporting Information), the shape effect on the nanoparticle endocytosis in these two cell lines was similar and not very significant.

The tests of 4 types of nanoparticulates in MM-231 stem cells not only demonstrated again the effect of shape and CPP-conjugation anisotropy on the bio-nano interaction, but also revealed the specificity of EHB to tumor stem cells (Figure 4F). By the way, the more endocytosis of EHB was in consistent with its behaviors in cytoskeleton disruption and wound healing assay (Figure S7, Supporting Information), which might be resulted from its impact on the expression of related protein after internalization.

### 2.4. Subcellular Localization

After the cell-level investigations, colocalization of nanodiscs with different organelles was next evaluated to study the effect of R8 anisotropic modifications on the pathway of intracellular transport. First, EHB showed distinct colocalization with lysosomes after 2 h incubation with MM-231 cells while both HB and PHB had less degree of overlay (p < 0.001) (Figure S5A,E). However, all three nanomaterials colocalized with lysosomes after 5 h incubation (Figure S5B). This fact, confirmed by Pearson’s coefficients in Figure 5E, revealed that EHB internalized into cells and transported to lysosomes at a faster speed than HB and PHB. The higher colocalization of EHB with late endosomes further supported this observation (Figure S6A,B). The colocalization of lysosome with HB, EHB, and PHB by imaging flow cytometer was in accordance with CLSM analysis (Figure S8, Supporting Information). As shown in Figure S5C,D,F, PHB exhibited the highest Pearson’s coefficient in ER and Golgi apparatus, and thus indicated its greater exocytosis tendency. Neither EHB nor PHB showed any significant location in recycling endosome (Figure S6C,D), and according to Figure S6E,F, PHB was more involved in caveolae-mediated endocytotic pathway, which could induce more transport to Golgi and ER. The tests of uptake inhibition by MβCD also suggested indirectly the involvement of PHB in the pathway of ER/Golgi transport (Figure S9, Supporting Information). So, it was interesting to find that PHB demonstrated a mode of ‘hard entry and easy exit’ compared with other nanodiscs. Additionally, all three nanodiscs had no apparent colocalization with mitochondria (Figure S10, Supporting Information). Together, above quantitative and qualitative studies of nano-organelle colocalization suggested that anisotropic R8 modification could also modulate the intracellular and subcellular trafficking of the nanodiscs.

Fluorescence recovery after photobleaching (FRAP), a powerful technique for probing biomembrane, was conducted here to further investigate the bio-nano interaction. Figure 5G depicts FRAP recovery curves of 1,1′dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) labeled HUVEC after exposure to cerasome, HB, PHB, and EHB.
was found that fluorescence recovery after exposing to PHB was significantly slower than cerasome ($p < 0.05$), HB ($p < 0.01$), and EHB ($p < 0.05$) at the end of test, and there was no significant difference among other three nanoparticulates. This clearly indicated the reduction of membrane fluidity induced by PHB, in consistent with the ROS test in Figure S3 (Supporting Information). As mentioned above, PHB might firmly adhere on the cell surface via its larger contact area as well as up to 50% of active R8, which impeded the motion of biomembrane, resulting in remarkably decreased membrane fluidity. In contrast, EHB might contact with the cell membrane on its R8 modified edge, and such small contact area might lead to less restriction to cell membrane. Though EHB showed strong bio-nano interaction, it was rather localized. Generally, these findings demonstrated that effect of nanoparticulates on membrane fluidity could be affected by R8 modification anisotropy, but not significantly by shape anisotropy.

2.5. Tumor Sphere Penetration

Finally, in order to further understand the effect of shape and R8 modification anisotropy in a tissue model more closer to real tumor than a 2D cell model, a study was carried out on tumor spheroids after 12 and 24 h treatment with different nanoparticulates (Figure 7). As the results, EHB demonstrated

---

**Figure 4.** A–E) Cellular uptake of HB, EHB, and PHB by MM-231 and HMF cells in a coculture model. Blank field (BF) and fluorescent properties of A) MM-231 and B) HMF after internalization of Rhod B-labeled nanoparticles. These were representative images in 10,000 cell specimens by imaging flow cytometry. C) MM-231/HMF were stained with DiO/DiD and sorted according to their fluorescent properties. Such, a mixed 2D coculture model mimicking the tumor microenvironment was established. D) Cellular uptake of different nanoparticles by MM-231 (green) and HMF (red) in coculture model. Both EHB and PHB had 6.15% R8 modification density. E) MM-231/HMF cell uptake ratio of different nanoparticulates in coculture model. EHB exhibited the highest MM231/HMF uptake ratio among the four nanoparticulates in the mixed coculture model. $n = 3$, *$p < 0.05$, **$p < 0.01$, ###$p < 0.001$ EHB versus other three groups. F) Cell uptake of HB, EHB, and PHB in MM-231 stem cells. Both EHB and PHB had 6.15% R8 modification density. $n = 3$, **$p < 0.01$, ***$p < 0.001$. It demonstrated again the effect of shape and CPP-conjugation anisotropy on the bio-nano interaction, and also revealed the specificity of EHB to tumor stem cells.
most significant penetration among all four types of nanomaterials at two tested time points. At 12 h, compared with the few accumulation inside of tumor spheroid in cerasome, HB and PHB group, EHB exhibited strong signals in the same model. Up to 24 h, the tumor penetration of HB somehow got closer to that of EHB, but the penetration of PHB was still low, while the cerasome was the lowest. The observations here were in accordance with previous study in cell uptake (Figure 3). Therefore, the penetration of tested nanomaterials to solid tumors might be substantially improved by the anisotropic manipulation both in shape and R8 functionalization. By the way, many other factors besides endocytosis were involved in the penetration process, including exocytosis, intercellular interactions, etc., which needs further investigations.

3. Conclusion

In summary, the hybrid bicellar nanodiscs with nonmodification, edge modification, and plane modification of octa-arginine sequence were fabricated from a long-chain CFL and a short-chain phospholipid DHPC, and recorded as HB, EHB, and PHB, respectively. Their average diameters were about 50 nm, and the spherical cerasomes with similar component were also prepared. To simulate in vivo microenvironment, six types of different cell lines were investigated here. Interestingly, EHB was found to enter all tested cells most easily, transport inside of the cells most quickly, and penetrate tumor spheroids most deeply, without causing obvious damage. On the contrary, PHB exhibited much less endocytosis and tumor penetration, triggered more ROS generation, hemolysis, lysosomal destabilization and membrane fluidity reduction, and transported more to Golgi apparatus. Generally, the R8 modification anisotropy could actually impact the cellular uptake, intracellular trafficking and tissue penetration of nanomaterials, as well as the ROS generation, hemolysis, lysosomal stabilization, and membrane fluidity of cells. The shape anisotropy could also affect the endocytosis and the tissue penetration.

Importantly, the HB/Cerasome and EHB/PHB uptake ratio could indicate the impact of shape and ligand-conjugation anisotropy on the endocytosis of nanoparticulates, respectively. With the change from nanospheres to nanodiscs and from plane- to edge-modified HB, the maximum increase in cell uptake could reach 10.5- and 17-fold, respectively. So, the effect of CPP-modification anisotropy on bio-nano interaction was more significant than shape anisotropy. Also, different cell lines showed very different uptake levels. The maximum difference in EHB/PHB uptake ratio was 12-fold between RAW264.7 cell
PHB in the ER/Golgi transport than EHB showed in Figure 5. This finding supported the greater involvement of Cav-1. PHB with Cav-1 by CLSM. F) Pearson’s coefficient analysis of cerasome, HB, EHB, and PHB with significant distribution in recycling endosome. E) Colocalization of cerasome, HB, EHB, and PHB with recycling endosome. No significant colocalization was seen in both groups, indicating no endosomes marked by rab11 by CLSM. D) Pearson’s coefficient analysis of EHB and PHB in late endosome.

Figure 6. A) Colocalization of EHB, PHB with late endosome marked by rab7 after 45 min incubation monitored by CLSM. B) Pearson’s correlation analysis of EHB or PHB with late endosome. Greater colocalization was seen in EHB group, indicating more distribution of EHB in late endosome. \( n = 6, ***p < 0.001. \) C) Colocalization of EHB and PHB with recycling endosomes marked by rab11 by CLSM. D) Pearson’s coefficient analysis of EHB and PHB with recycling endosome. No significant colocalization was seen in both groups, indicating no significant distribution in recycling endosome. E) Colocalization of cerasome, HB, EHB, and PHB with Cav-1 by CLSM. F) Pearson’s coefficient analysis of cerasome, HB, EHB, and PHB with Cav-1. \( n = 6, *p < 0.05. \) PHB showed greater colocalization with Cav-1, suggesting its greater dependence on caveolae-mediated pathway. This finding supported the greater involvement of PHB in the ER/Golgi transport than EHB showed in Figure 5.

and HUVEC. Among the tested cell lines, the sequence of HB/Cerasome uptake ratio implying the shape effect was just contrary to the sequence of EHB/PHB ratios indicating the impact of R8-position. Interestingly, this phenomenon could be explained by the phagocytic or endocytic function of cells and followed a regular rule: the strong-phagocytic cells were more sensitive to the change in ligand location, but insensitive to the alteration in shape, and vice versa.

So far, three driving forces of bio-nano interaction were demonstrated here: shape effect, ligand modification anisotropy and the phagocytic or endocytic function of cells. It was believed that the shape and ligand anisotropy induced different surface curvature and contact angle during the invagination process. The cell phagocytic or endocytic capacity had a significant synergism with the impact of R8 edge modification. All these led to different modes of bio-nano interaction. Although the current findings are based on in vitro studies, they hint potential use in biomedical fields such as tissue engineering, diagnostic agents, and transfection reagents. Still, more applications need further verifications in vivo.

In short, it is demonstrated here that all tested factors were involved in the bio-nano interaction in different degrees via a cross-talking way. The double effect of anisotropic shape and CPP modification were very significant on the mode of bio-nano interaction as well as its outcome in the test condition. Based on our findings, it seems possible to manipulate the bio-interaction mode of nanomaterials and its output, through regulating their anisotropy in shapes and ligand modifications. Meanwhile, we also need pay attention to the related biological microenvironment consisted of different types of cells.

4. Experimental Section

Material and Cell Line

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC-HCl) was purchased from Shanghai Medpep Co., Ltd. MTT, aminopropyltriethoxysilane (99.9%), and amiloride hydrochloride were provided by Sigma-Aldrich. Hexadecylamine was bought from ABCR GmbH Company. Octa-arginines-C8 or Octa-arginines-C18 was provided by ChinaPeptides Co., Ltd. (Shanghai, China). DHPC and Lissamine Rhodamine B were purchased from Avanti Polar Lipids, Inc. Fluorescent probe DiO, Dil, and lactate dehydrogenase assay kit were obtained from Beyotime Institute of Biotechnology. ROS assay kit was purchased from Nanjing KeyGEN Biotech. Co., Ltd. Lysotracker, ERtracker, Mitotracker Deep Red, Caveolin-1 (Cav-1) antibody, rab7 antibody, rab11 antibody, and DiD were bought from Thermo Fisher Scientific Inc., and Golgi-tracker from Beyotime Institute of Biotechnology, respectively. All chemicals and reagents were commercially obtained and used directly without further purification.

Human breast cancer cell line MCF-7 and MDA-MB-231 (MM-231), HUVECs, murine macrophage RAW264.7 were purchased from Institute of Basic Medical Science, Chinese Academy of Medical Sciences (Beijing, China), and HMF cell was bought from Tongpai (Shanghai) Limited Company of Biology Science and Technology. MCF-7 and HUVEC were cultured in RPMI-1640 medium; MDA-MB-231, HMF, and RAW264.7 were cultured in DMEM medium; all supplemented with 10% fetal bovine serum and antibiotics (100 \( \mu \)g mL\(^{-1}\) streptomycin and 100 \( \mu \)g mL\(^{-1}\) penicillin) in 5% CO\(_2\) atmosphere at 37 °C. Cell culture media RPMI-1640, DMEM were bought from Beijing North TZ-Biotech Develop, Co. Ltd., fetal bovine serum and antibiotics were purchased from Macgene Biotech Co., Ltd. (Beijing, China). Animals (ICR mice, half male and female, 6-8 weeks), were purchased from Peking University Health Science Center, provided with free access to standard water and food and kept under SPF conditions. All the experiments were approved by the Institutional Animal Care and Use Committee of Peking University (no. LA2015156) and carried out under the principles of care and use of laboratory animals.

Preparation and Characterization of Cerasome, HB, Edge-modified HB (EHB), and Plane-modified HB (PHB): The synthesis of CFL referred to the previous literature.[9] Briefly, CFL was synthesized through condensation of 3-aminopropyltriethoxysilane and \( \text{N,N-dihe} \text{d} \text{e} \text{k} \text{a} \text{c} \text{e} \text{y} \text{s} \text{i} \text{n} \text{a} \text{m} \text{i} \text{c} \) acid,
which was synthesized by coupling dihexadecylamine and succinic anhydride. Dihexadecylamine and succinic anhydride were added into dried tetrahydrofuran (THF), dissolved upon heating, and stirred at room temperature for 1 d. THF was removed by vacuum rotary evaporator, and the crude product was dissolved by dichloromethane, then washed sequentially with 10% aqueous citric acid and saturated aqueous sodium chloride. Residual water was removed by anhydrous sodium sulfate and filtered; the solvent was removed by vacuum evaporation. Acetonitrile was then used for recrystallization. Then the product was dissolved in dry dichloromethane, added with EDC and stirred for 15 min. Then, 3-aminopropyltriethoxysilane was added, and the solution was stirred overnight at room temperature. The solvent was removed by vacuum evaporation, and the crude product was then purified eluted with ethyl acetate/dichloromethane 1:3 by column chromatography.

For cerasome or nonmodified HB, appropriate amounts of CFL or CFL and DHPC (molar ratio $= 7:2$) were dissolved by chloroform in a flask. The solvent was evaporated with vacuum rotary evaporator, and then ultrapure water was added and hydrated for 30 min at 55–65 °C. After 10 min water-bath ultrasonication, probe-type sonicator was applied on the dispersion at 30% amplitude for 5 min. The samples were kept overnight at room temperature before experiments.

For EHB or PHB, octa-arginine linked to short alkyl chain or long alkyl chain were added together with CFL and DHPC, respectively. The following procedures were similar, and octa-arginine linked to short alkyl chain would distribute together with the short-chain DHPC on the edge of the nanodisc, while octa-arginine linked to long alkyl chain would distribute together with the long-chain CFL on the top and bottom plane of the nanodisc. Nanostructures with fluorescent labeling were prepared by doping Rhodamine-Lissamine or encapsulating hydrophobic fluorescent probes such as DiD.

The hydrodynamic diameters of nanoparticulates were analyzed with a 90Plus/BI-MAS DLS analyzer (Brookhaven Zeta PALS instruments). FTIR spectrum was recorded using a Varian Resolution FTIR spectrophotometer (Varian FTS 3100, USA), samples were lyophilized to prepare KBr discs. The morphologies of different nanostructures were examined by FEI TecnaiTM T20 TEM, FEI NanoSEM 430 scanning electron microscopy (SEM), and Scan Probe Microscope (SPI3800/ SPA400, Seiko Instruments Inc., contact mode). To prepare TEM specimens, nanoparticulates were deposited onto a carbon-coated copper grid, stained with uranyl acetate (4%) for 5 min, and air dried before observation. To prepare SEM specimens, nanoparticulates were deposited onto a silicon wafer for 10 min, and washed with distilled water.

### Figure 7.

Penetration into MM-231 tumor spheroids of cerasome, HB, EHB, and PHB labeled with Rhod B at 12 and 24 h and monitored by CLSM, and images were collected at the center of the spheroids. EHB exhibited most significant penetration among the four nanoparticulates at both time points, which was consistent with highest cell uptake of EHB.
water and air-dried before imaging. The AFM specimens were prepared by spreading the nanoparticulate solutions onto a fresh mica surface for 5 min. Then the specimens were washed several times with distilled water and air-dried before imaging.

Biocompatibility of Cerasomes, HB, EHB, and PHB: For MTT assay, cells were seeded in 96-well plate and incubated with different blank nanoparticulates of different concentrations, and blank medium was used as negative control. After incubation for 24 h, the viability of the cells was determined by MTT staining.

ROS assay was carried out to evaluate the nanomaterial-caused injury. MM-231 cells were cultured in glass-bottomed dishes and incubated with blank nanoparticles (500 ug mL\(^{-1}\)) for 6 h. ROS was detected with the ROS assay kit by CLSM.

Lysosomal destabilization was studied by observing the leakage of FITC-dextran into the cytoplasm. Cells were seeded on glass-bottomed dishes and cultured overnight till adherence, preloaded with 1 mg mL\(^{-1}\) FITC-dextran for 3 h, and then washed thrice with PBS. Then the cells were incubated with nanoparticulates for 24 h at 50 ug mL\(^{-1}\), washed with PBS. The release of FITC-dextran from lysosomes was observed with CLSM.

Cellular Uptake Behavior and Intracellular Distribution of Cerasomes, HB, EHB, and PHB: Flow cytometry and CLSM were performed to examine the cellular uptake of Rhodamine B labeled nanostructures. Cells were cultured in 6-well plates or glass-bottomed dishes. The cells were washed thrice and incubated with different nanoparticulates at 37 °C for 3 h for uptake examination or at 4 °C for 201 min time duration was recorded to assess the fluidity of cell membrane by CLSM. The fluorescent immediately after photobleaching was set as 100%, while the fluorescent before photobleaching was set as 100%.

The wound healing rate was assessed by ImageJ. For microfilament staining, cells were seeded into 12-well plates and incubated till reached 70%-80% confluence. Then the cells were incubated with serum-free medium overnight, and the wound was made with a 10 uL tip. The cells were washed and observed immediately as 0 h. Then the cells were incubated with nanoparticulates at 100 ug mL\(^{-1}\) for 48 h and observed. The wound healing rate was assessed by ImageJ. For microfilament staining, cells were treated with nanoparticulates at 100 ug mL\(^{-1}\) at 37 °C for 3 h, washed and fixed, and then treated with TPBS for 5 min, incubated with Rhodamine phallodin for 30 min at 37 °C, then washed and observed with CLSM.

In Vitro Penetration of Cerasomes, HB, EHB, and PHB: Tumor spheroids were prepared by hanging drop method. 20 g L\(^{-1}\) agarose was dissolved in DMEM medium at 80 °C and added to 48-well plate (200 µL per well), and after solidification, 900 µL complete culture medium was added to the wells. 20 µL cell suspension containing 500 cells was dropped onto the inside of the plate cover. After 2–3 d, the cell cluster transferred into the wells and continued to culture for 2–4 d. Tumor spheroids were incubated with fluorescent-labeled nanostructures for 12 or 24 h, washed thrice and fixed, and observed with CLSM.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
This work was supported by the National Key Research and Development Program of China (No. 2016YFA0201400), the National Natural Science Foundation of China (Nos. 81690264, 81230036, and 81421004), the National Basic Research Program of China (No. 2015CB932100), and the Innovation Team of the Ministry of Education (BMU20110263).

Conflict of Interest
The authors declare no conflict of interest.

Keywords
delivery manipulation, endocytosis, ligand-modification anisotropy, nanodiscs, shape anisotropy

Received: January 22, 2017
Revised: March 18, 2017
Published online: May 4, 2017
