Effect of surface charge of nanohybrids on the intracellular bacterial killing efficiency

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Research

Keywords:

Posted Date: April 8th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-21486/v1>

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Abstract

Background : Lipid polymer hybrid nanoparticles (LPHNPs) are widely investigated nanohybrid system in drug and gene delivery and also medical imaging. A knowledge of lipids-based surface engineering and its effects on the physicochemical properties of LPHNPs affect the cell – NPs interaction, consequently, influence the cytological response is in high demand.

Methods and Results : Herein, we developed a cationic and zwitterionic lipids-based surface engineering approach with antibiotics (Doxycycline or Vancomycin) loaded LPHNPs and examined the surface charge influence on the physiochemical characteristics, antibiotic entrapment, and intracellular release behaviors. Importantly, we examined the intracellular antibacterial activity of drug-loaded LPHNPs against *Mycobacterium smegmatis* or *Staphylococcus aureus* infected macrophages. Furthermore, cationic or zwitterionic lipids in LPHNP formulations improved the antibiotic loading efficiency and extended the duration of antibiotic release. In vitro particle uptake studies indicated that the cationic LPHNPs and bare nanoparticles (BNPs) were more efficiently internalized into macrophages than zwitterionic LPHNPs.

Conclusion : A play in surface charge in the formulation of the macrophage uptake and intracellular bacterial killing efficiency of LPHNPs loaded with clinical antibiotics on macrophages infected with bacteria, provided a basis for optimizing their use in biomedical applications.

Background

The treatment of intracellular bacterial infections is often challenging for clinicians, and novel therapies are needed [1]. Intracellular infections can be recurrent and difficult to treat, owing to the low availability of antibiotics in infected cells and insufficient host defenses [2]. *Mycobacterium tuberculosis*, *Salmonella typhimurium*, and *Staphylococcus aureus*[3] are the main intracellular bacteria, found inside the macrophages, where they act like a 'Trojan horse' to cause recurrent infections at secondary sites [2]. Intracellular bacterial infections typically require long-term antibiotic intake, which may lead to poor compliance and contribute to developing resistant strain [4]. Besides, most conventional antibiotics exhibit poor intracellular penetration and retention ability, which leads to a relapse of infection and antibiotic resistance [5].

Engineered nanoparticles (NPs) are helping to counteract the intracellular antibiotic delivery [6–10]. The intracellular delivery of antibiotics associated with engineered NPs offers significant advantages over free drugs, including an improved drug efficacy by protection from degradation, optimal therapeutic levels at the site of bacterial infection via sustained release, and a reduced dosing frequency, thereby minimizing drug-associated toxicity [11]. Several engineered NPs and liposomes have been evaluated for antibiotic delivery [12]. However, a low encapsulation efficiency, high burst release, cytotoxicity, and poor stability have limited their clinical success [2].

Lipid–polymer hybrid nanoparticles (LPHNPs) can overcome the issues associated with polymeric NPs and liposomes [13–16]. Compared with typical NPs, hybrid NPs exhibit improved cell interactions, higher drug loading, and prolonged drug release [15, 17–19]. We previously reported that coating of lipids of different types on the PLGA-NP core, influences the size, surface charge, therapeutics loading efficiency including gene and small molecules, and release ability [14, 17, 18]. Furthermore, the nature and charge of the NPs surface are crucial determinants of the macrophage recognition and phagocytosis mechanism [19]. Therefore, in the present study, we developed a cationic and zwitterionic lipids-based surface engineering approach with antibiotics (Doxycycline or Vancomycin) loaded LPHNPs and examined the intracellular antibacterial activity of drug loaded LPHNPs against *Mycobacterium smegmatis* (*M. smegmatis*) or *Staphylococcus aureus* (*S. aureus*) infected macrophages to determine the optimal formulation.

Results

Preparation and characterization of antibiotic loaded LPHNPs

In our previous study, we have investigated the different methods of preparation and characterization of LPHNPs. This experience helped us to significantly control the size and surface of charge of LPHNPs, which further improved the credibility and repeatability of our research [17, 18, 20]. The **Fig 1.**, outlines the schematic diagram of the experimental design. **Fig SI1** and **Table 1** displays the production of different hybrid nano-formulations of drug-loaded cationic or zwitterionic LPHNPs and non-lipid layered bare BNPs using modified emulsion solvent-evaporation process and their physicochemical characteristics [18]. The size of hybrid nanoparticles (HNPs) is considering as an essential parameter, with direct effects on cellular uptake, stability, and tissue distribution [21–24]. Thus, we used dynamic light scattering (DLS) to determine the size range, size distribution (PDI), and surface charge of LPHNPs [15]. Based on DLS, the modified solvent evaporation method yielded a relatively monodispersed LPHNPs and BNPs with a size range of 150–300 nm and narrow size distribution (PDI between 0.14–0.22). As summarized in **Table 1**, and illustrated in **Fig 3A**, the incorporation of cationic or zwitterionic lipids in the LPHNP formulation, resulting in a significant reduction in size ($P<0.05$) compared to those of non-lipid layered formulations (BNPs). For instance, cationic (CA) or zwitterionic (ZA) LPHNPs were 203 ± 6.6 and 191 ± 5.4 nm, respectively, which were smaller than the non-lipid layered BNP formulation (NA), i.e., 226 ± 9.6 nm. Consistent with previous reports from our group and other research groups, incorporation of either cationic or zwitterionic lipids significantly reduce the size of formed NPs, which could be explained by the fact that the processing of those NPs in a single step was stabilized by the function of the lipids with an emulsifying agent, possibly reducing the coalescence of particles [14, 18].

We utilized a double emulsion solvent evaporation technique and the encapsulation of drug (Doxycycline or Vancomycin), drastically affect the size of these NPs, as indicated in **Table 1**. For instance, NA, CA and ZA were smaller than NB, NC, CB, CC, ZC. Additionally, BNP non-lipid layered formulations encapsulating Doxycycline (NB) or Vancomycin (NC) were larger than Cationic and Zwitterionic LPHNPs encapsulating Doxy (CB and ZB) or Vanco (CC and ZC).

Surface charge is another critical factor; it dictates the NP interactions with cells, penetration, and plays an essential role in colloidal stability [23, 25]. Thus, we investigated the surface charge (Zeta potential) of cationic (C) and zwitterionic (Z) LPHNPs, and non-lipid layered BNPs (N) using the Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) [15, 18]. As expected, the inclusion of either Cationic or Zwitterionic lipids into the LPHNP formulations resulted in a differently charged surface, as shown in **Table 1 and Fig 3B**.

Our results confirmed that the surface charge of BNPs is not affected by the incorporation of an antibiotic, i.e., Doxy (NB) or Vanco (NCB). In contrast, the inclusion of either cationic (C) or zwitterionic (Z) lipids into the LPHNP formulation resulted in a charge reduction from -29 ± 2.1 mV towards positive or neutral values [17].

Table 1 Details of formulation and characterization of non-lipid layered BNPs and cationic or zwitterionic LPHNPs loaded with Doxy or Vancomycin

Formulation -group	Variations	Compositions	Z-Ave size	PDI	Charge	EE%
non-lipid layered NPs	NA	Bare PLGA NPs (Without drug)	226±9.6	0.191±0.023	-26±2.6	-
	NB	Bare PLGA NPs with Doxycycline	257±8.4	0.213±0.024	-27±3.7	63
	NC	Bare PLGA NPs With Vancomycin	249±5.4	0.19±0.023	-29±2.1	57
cationic LPHNPs	CA	Cationic LPHNPs (Without drug)	203 ±6.6	0.178±0.017	+39±1.3	-
	CB	Cationic LPHNPs with Doxycycline	231±7.4	0.22±0.018	+38±3.4	71
	CC	Cationic LPHNPs With Vancomycin	223 ±6.2	0.2±0.02	+40±2.3	64
zwitterionic LPHNPs	ZA	Zwitterionic LPHNP (Without drug)	191 ±5.4	0.147±0.017	-7±3.5	-
	ZB	Zwitterionic LPHNPs With Doxycycline	222 ±4.7	0.22±0.012	-8±2.8	79
	ZC	Zwitterionic LPHNPs With Vancomycin	214 ±3.3	0.19±0.017	-8±1.2	76

As summarized in **Table 1**, the addition of cationic lipid to the NP formulations (CA, CB, and CC) resulted in positively charged surfaces and the inclusion of a zwitterionic lipid to the NP formulations (ZA, ZB, and ZC) resulted in charge reductions from that of the native surface of BNPs (-29 ± 2.1 mV).

The morphologies of antibiotic-encapsulated LPHNPs and BNPs were examined to ensure that they were hybrid nanoparticles with lipid and polymeric cores, rather than a random composition of liposomes and uncovered polymeric NPs [13, 15, 16]. As shown in **Fig 2C, D** and supplementary image (SI) **SI1** (FESEM), the Vanco or Doxy-encapsulated BNPs and LPHNPs exhibited a nano-spherical shape, with no distinct morphological differences among the LPHNP groups. Additionally, EF-TEM images (**Fig 2D**) showed the core-shell hybrid structure of LPHNPs and confirmed the presence of the lipid layer on the PLGANP surfaces. A Cryo-TEM analysis was performed to inspect further the LPHNP structures identified by FE-SEM and EF-TEM [26]. Both cationic and zwitterionic lipid layered LPHNPs exhibited a perfectly spherical shape (**Fig 2**), and the apparent particle sizes corresponded well to the NP sizes calculated by DLS (**Table 1**). Highly electron-dense structures on the LPHNPs, which were easily melted upon electron beam exposure, and extended and loose structures were present in the dispersed phase, as reported by Colombo *et al.*²⁵ To obtain direct evidence for lipid self-assembly on the PLGA NP core, we incorporated fluorescent NBD-PC lipids into cationic or zwitterionic lipids. As shown in **Fig 2**, the CLSM image showed green fluorescence indicating a perfect nano-sphere shell layer, demonstrating that the simple and efficient process led to the formation of lipid layered hybrid nanoparticles [27]. The stability of antibiotic-encapsulated LPHNPs and BNPs was examined to ensure that the formulations are appropriate for prolonged storage and commercial feasibility. The z-average size was used to study NP stability by DLS at specific intervals. As shown in supplementary **SI6** all the LPHNP formulations stored at 4°C remained stable during the entire observation period of 2 weeks, maintaining a slightly higher z-average than that on the first day of production and showing no visible signs of instability, such as sedimentation or aggregation

The drug entrapment efficiencies for Vanco- or Doxy-encapsulated BNPs and LPHNPs was shown in Fig summarized in **Table 1**. The DLS results showed that antibiotic encapsulation in LPHNPs and BNPs resulted in a moderate size increase. The encapsulation efficiencies of Doxy-BNPs (NB) and Cationic or Zwitterionic lipid-layered LPHNPs (CB, and ZB) were 63%, 71%, and 79%, respectively. We also observed that the Vanco encapsulation efficiencies of BNPs (NC) and Cationic or Zwitterionic lipid layered LPHNPs (CC and ZC) were 57%, 64%, and 76%, respectively. The drug incorporation efficiency of hydrophilic antibiotics in non-lipid layered formulations (BNPs) was lower than that in lipid layered formulations.

***In vitro* drug release from LPHNPs and BNPs**

The cumulative antibiotic release from Doxy- or Vanco-encapsulated BNPs and cationic or zwitterionic lipid-layered LPHNPs were assessed for 120 h. As shown in **Fig 3**, the cationic (C) or zwitterionic (Z) lipid layered LPHNPs displayed the sustained release of Doxy or Vanco with minimal burst release. In contrast, non-lipid layered BNPs (N) showed an initial burst release, followed by sustained drug release [28, 29]. For instance, the antibiotic release rates of Doxy-BNPs (NB) at 12, 24, and 48 h were 65%, 69%, and 75%, respectively, while Doxy release rates from cationic (CB) and zwitterionic (ZB) lipid-layered LPHNPs at 24, 48, and 72 h were 38%, 54%, and 66% and 32%, 47%, and 61%, respectively. Similarly, the cationic (CB) and zwitterionic (ZB) lipid-layered LPHNPs encapsulating Vanco had a slower release rate than the non-lipid layered BNP-Vanco formulation (ZC). As shown in **Fig 3**, the amounts of Vanco released from BNPs

were 57%, 70%, and 76% at 12, 24, and 48 h, respectively, while the amounts of Vanco released from cationic (CC) and zwitterionic (ZC) lipid-layered LPHNPs at same time points were 37%, 53%, and 68% and 32%, 42%, and 60%, respectively. Furthermore, all LPHNP formulations exhibited minimal burst release with the sustained antibiotic release, unlike the non-lipid layered BNPs. This difference could likely be explained by the lipid on the NP core, which could slow the drug release kinetics by acting as a molecular fence between the polymeric matrix and aqueous phase [16, 17].

Antibacterial activity against

Staphylococcus aureus or *Mycobacterium smegmatis*

S. aureus and *M. smegmatis* were used to investigate the bactericidal activity of free antibiotics and Doxy- or Vanco-encapsulated cationic (C) or zwitterionic (Z) lipid-layered LPHNPs and BNPs (F1) by broth-microdilution assay. Antibiotic-loaded cationic (C) or zwitterionic (Z) LPHNP and BNP (N) formulations were directly incubated with a suspension of *M. segmatitis* or *S. aureus* at 37°C [2]. The MIC values for all of the Doxy- or Vanco-loaded cationic (C) or zwitterionic (Z) LPNHP and BNP (N) formulations are presented in **Table 2**. All the tested Doxy- or Vanco-loaded cationic (F2) or zwitterionic (F3) LPHNP and BNP (F1) formulations inhibited the growth of *M. segmatitis* or *S. aureus*, as presented in **Table 2**. The MIC of free Doxy was 4 µg/mL; however, cationic LPHNP-Doxy (F2B) showed a MIC of 4 µg/mL, indicating stronger inhibitory activity against *M. smegmatis* indicating that the formulations exhibited higher MIC than the free antibiotic. *S. aureus* and *M. smegmatis* were used to investigate the bactericidal activity of free antibiotics and Doxy- or Vanco-encapsulated cationic (C) or zwitterionic (Z) lipid-layered LPHNPs and BNPs (F1) by broth-microdilution assay. Antibiotic-loaded cationic (C) or zwitterionic (Z) LPHNP and BNP (N) formulations were directly incubated with a suspension of *M. segmatitis* or *S. aureus* at 37°C [2].

Table 2 Minimum inhibitory concentration assay of non-lipid layered BNPs and cationic or zwitterionic LPHNPs loaded with Doxy or Vancomycin with controls.

Compounds	MIC (µg.mL-1)
Doxy	4
F1B	16
F2B	4
F3B	16
Vanco	4
F1C	16
F2C	2
F3C	32

The MIC values for all of the Doxy- or Vanco-loaded cationic (C) or zwitterionic (Z) LPHNP and BNP (N) formulations are presented in **Table 2**. All the tested Doxy- or Vanco-loaded cationic (F2) or zwitterionic (F3) LPHNP and BNP (F1) formulations inhibited the growth of *M. segmatitis* or *S. aureus*, as presented in **Table 2**. The MIC of free Doxy was 4 µg/mL; however, cationic LPHNP-Doxy (F2B) showed a MIC of 4 µg/mL, indicating stronger inhibitory activity against *M. smegmatis*, indicating that the formulations exhibited higher MIC than the free antibiotics.

In addition, our results showed that the synergism between the antibiotics and cationic-LPHNPs enhanced the antibacterial activity against *S. aureus* and *M. smegmatis*. Our plausible explanation for the enhanced in vitro antibacterial activity is the combinatorial antibacterial effect of the antibiotic and physiochemical properties, such as the size and charge, of the lipid layer LPHNPs [30]. We demonstrated that the cationic charge of DOTAP promoted stronger adhesion to the negatively charged bacteria, such as *S. aureus* and *M. smegmatis* (-42.9 ± 5.9 mV) [30, 31].

Additionally, our experimental observations strongly suggested a synergistic antibacterial effect of the cationic surface of LPHNPs (C) and encapsulated antibiotics (Doxy or Vanco). However, synergistic effects were not observed for the zwitterionic or anionic surfaces of LPHNPs (Z) or BNPs (N) and encapsulated antibiotics.

Cytotoxicity.

Cationic lipids exhibit moderate to high cytotoxicity. Our experimental results confirmed similar cytotoxicity for cationic LPHNPs and BNP-Doxy or Vanco formulations, as shown in supplementary **S15**. Notably, at a low NP concentration (10 µg/mL), all LPHNPs and BNPs exhibited low cytotoxicity (cell viability >80%). However, when the NP concentration increased, cell viability decreased. Zwitterionic LPHNPs did not affect cell viability, even at a higher NP concentration (60 µg), which can likely be explained by the biomimetic effect of PC lipids

Surface charge of LPHNPs influence the macrophages uptake.

After red fluorescent dye (DiD) was encapsulated within LPHNPs and BNPs, particle uptake (LPHNPs and BNPs) by J774.1 cells were quantified by flow cytometry and further visualized by CLSM [2, 18]. After 24 h of incubation, the BNPs and cationic-LPHNPs were more efficiently taken up by J774.1 cell than the zwitterionic LPHNPs (**Fig 4**). The intracellular distribution of fluorescently labeled NPs was further analyzed by CLSM to confirm that NPs were present in the cells rather than absorbed into the cell membrane [32]. The **Fig5** shows that the cationic LPHNPs (C) surfaces were more rapidly recognized and processed by phagocytes than the zwitterionic surfaces (Z). Furthermore, Cationic LPHNPs (C) show higher uptake nearly 93% followed by BNPs were 80% respectively. Interestingly, Zwitterionic LPHNPs were taken by macrophages less efficiently (49%) as compared to Cationic and non-lipid layered NPs.

These results depict that macrophage recognition, uptake efficiency, and subsequent intracellular trafficking of tested nanocarriers is positively correlated with the surface properties.

Intracellular antibacterial activity of LPHNPs and BNP

The intracellular antimicrobial activity of cationic or zwitterionic LPHNPs and non-lipid layered BNPs were tested in macrophages *in vitro*. Free Doxy or Vanco treatment decreased the intracellular bacterial load (2-log-CFU compared with the initial infection) for *M. smegmatis* and *S. aureus* (Fig 5).

Treatment with cationic LPHNP–Doxy (CB) and LPHNP–Vanco (CC) resulted in a reduction of 4 or 3 log CFU compared with the untreated infected cells as shown in (Fig 6) and (Fig 7). Similarly, treatment with non-lipid layered BNPs encapsulating Doxy (NB) and Vanco (NC) resulted in bacterial reductions of 3 and 2 log CFU compared with the initial infection. However, zwitterionic LPHNP-Doxy (CB) and LPHNP-Vanco (CC) showed moderate reductions of intracellular antibacterial activity (2 and 1 log CFU) as compared to the initial infection. To confirm the intracellular antibacterial activity confocal microscopy was performed using LIVE/DEAD BacLight bacterial viability assays to distinguish between dead (labeled in red) and live bacteria (labeled in green) within macrophages [2] after treatment (SI 3 and 4).

Fig 5. illustrates the intracellular antibacterial activity obtained after different NPs formulations and free antibiotics. Intracellular *M. smegmatis* and *S. aureus* were alive in macrophages after treatment with a free antibiotic (Doxy or Vanco), which demonstrated that the limited entry of free antibiotics inside the macrophages. On the contrary, the treatment with either Cationic LPHNPs loaded with Doxy or Vanco resulted in a bacterial CFU reduction compared to the free drug in bacterial counts, which represented a reduction of 80% intracellular bacteria replication compared to the untreated infected cells. Treatment with BNP-antibiotic (Doxy or Vanco) effectively killed most intracellular *M. smegmatis* or *S. aureus* and treatment with zwitterionic LPHNP-antibiotic formulations were moderately effective in the eradication of intracellular pathogens. These results indicated that the surface charge of nanocarriers plays a crucial role in NP uptake, thereby influencing the intracellular antibacterial efficacy.

Discussion

Developing correlations between nanoparticle physicochemical characteristics and its uptake, intracellular trafficking mechanisms in macrophages would provide a basis to overcome the intracellular antibiotic delivery and targeting, and facilitate the design of new, more effective and safer nanomaterial platforms. However intracellular infections remain a challenging task for physicians to eradicate, mainly due to the poor intracellular penetration of most of the commonly used antibiotics. Bacteria use macrophages as a ‘Trojan horse’ to induce a secondary site of infection thereby causing persistent or recurrent infections. Therefore, we performed a representative study, to investigate the effect of cationic or Zwitterionic LPHNPs, when compare to non-lipid layered BNPs. The encapsulation of Doxy or Vanco within the polymeric matrix protected the drug from degradation. Doxycycline is a broad-spectrum tetracycline-based antibiotic, which exerts bactericidal action by inhibiting protein synthesis [29, 33]. Vancomycin is the most commonly used antibiotic for *S. aureus* infections; it inhibits the biosynthesis of

peptidoglycan and the assembly of NAM–NAG–polypeptide in the peptidoglycan chain [5, 28]. Overall, our experimental data indicated that the encapsulation of Doxy or Vanco into the LPHNPs or BNPs significantly reduced the antibacterial efficacy, but may have prolonged activity by extending the time of action of a single dose as compared to that for free antibiotics [29, 33] [5, 34]. This improved antibiotic entrapment ability of LPHNPs can be explained by the ability of lipids to act as a molecular fence, which prevents drug leakage in the external phase during the solvent evaporation process, consistent with the results of Zhang *et al.* (2008) [16]. The cationic surface of LPHNPs promoted the internalization rate, increased the macrophage uptake of NPs, and enhanced the bacterial killing efficiency, as demonstrated by using an *in vitro* model of intracellular bacterial infections. These results are critical for building the knowledge base required to design efficient and specific LPHNP-based nano-antibiotics.

Conclusion

In summary, a comprehensive investigation of the effects of surface charge on the macrophage uptake and intracellular bacterial killing efficiency of LPHNPs loaded with Doxy or Vanco on *M. smegmatis*- or *S. aureus*-infected macrophages provided a basis for optimizing their use in biomedical applications.

Supporting Information

Description of details for demonstrating formulation, characterization and Schematic outline of the preparation method for materials; additional confocal microscopy images, cell viability test, electron microscopy, DLS size distribution.

Materials And Methods

Bacterial strain and macrophages

The bacteria and macrophages were obtained from our laboratory resources. The bacterial cultures were methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC BAA 1683) and *Mycobacterium smegmatis* (ATCC 607) [5, 33]. The mouse leukemic monocyte macrophage cells RAW 264.7 (ATCC TIB-71) and macrophage cells J774A.1 (ATCC TIB-67) were used for all cell culture experiments [2, 34].

Chemicals

The antibiotics (Vancomycin and doxycycline), PLGA (50:50), and polyvinyl alcohol (PVA; molecular weight 13–23 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The lipids 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine *N*-[methoxy-(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG), 1, 2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC), and fluorescent lipid 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The dye 1, 10-dioctadecyl-3, 3', 3'', 3'''-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) was purchased from Invitrogen (Eugene, OR, USA). Nutrient Broth,

Mueller-Hinton Broth (MHB), and Mueller-Hinton Agar (MHA) were obtained from Biolab (Midrand, South Africa). Middlebrook 7H9 broth medium (Becton Dickinson GmbH, Heidelberg, Germany). The LIVE/DEAD BacLight Bacterial Viability Kit was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals and reagents were purchased from Sigma-Aldrich Co. unless otherwise stated.

Preparation of antibiotic loaded LPHNPs and BNPs

The antibiotic (Doxy or Vanco)-loaded LPHNPs were prepared as described previously, with slight changes in the modified emulsion solvent-evaporation method [14, 18]. Briefly, the antibiotic (Doxy or Vanco (0.5:1) was dissolved in an aqueous buffer solution. The cationic lipid DOTAP or DOPC (15% w/w to the polymer) and PLGA (3%) were dissolved in dichloromethane. The w/o emulsion was prepared by the addition of antibiotic-containing aqueous buffer to the PLGA solution with sonication (Ultrasonic Probe; Sonics & Materials Inc., Newtown, CT, USA). The resulting w/o emulsion was transferred into a stabilizer (1% w/v PVA) solution and sonicated in an ice bath. The resultant w/o/w emulsion was stirred until the evaporation process was complete. Finally, the antibiotic encapsulated LPHNPs were separated by ultracentrifugation and freeze-dried. Blank, fluorescent (DiD or NBD-PC lipid) nanoparticles were also prepared using the same conditions, with few modifications [17, 18].

Characterization of LPHNPs and BNPs

The particle size (z-average) and size distribution (polydispersity index) of blank, antibiotic-loaded BNPs and LPHNPs were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments), and the surface charges (Zeta potential) of all formulations were determined as described previously. The morphological analysis of all formulations was investigated by field-emission scanning electron microscopy (FESEM; JSM-6700F; JEOL, Tokyo, Japan) at an accelerating voltage of 5 kV [18, 35]. The hybrid nature of LPHNPs was visualized by energy-filtered transmission electron microscopy (EFTEM). EFTEM experiments were performed using a Libra 120 microscope (Carl Zeiss Meditec AG, Jena, Germany), and samples were prepared by depositing 20 μ L of the LPHNPs suspension (0.5 mg/mL) onto a 200-mesh carbon-coated copper grid [14, 18]. The lipid layers on PLGA NPs were analyzed by Cryo-TEM using a Tecnai G2 20 TWIN transmission electron microscope (FEI, Hillsboro, OR, USA). Samples for Cryo-TEM were prepared using a FEI Vitrobot Mark IV, under controlled temperature and humidity conditions within an environmental vitrification system. A small droplet (5 μ L) was deposited onto a Pelco Lacey carbon-film grid and spread carefully. Excess liquid was removed, resulting in the formation of a thin (10–500 nm) sample film [26, 36]. Additionally, the presence of the assembled lipid layer on the PLGA NP core was further confirmed by confocal laser scanning microscopy (CLSM; Carl Zeiss LSM 510 Meta) with fluorescent NBD-PC [17, 18, 27].

Antibiotic(s) encapsulation efficiency and release behavior

The loading efficiency of Doxy or Vanco in BNPs or LPHNPs was quantified by spectrophotometry (UV-160; Shimadzu, Tokyo, Japan) by measuring the amount of non-entrapped Doxy or Vanco in the external aqueous solution [37, 38]. The external aqueous solution was obtained after centrifugation of the

colloidal suspension for 30 min at $18,000 \times g$. *In vitro* drug release was performed for 120 h at 37°C. Antibiotic-encapsulated BNPs and LPHNPs were suspended in 3 mL of PBS with continuous shaking. The eluted Doxy or Vanco was withdrawn at predetermined time points, and an equal volume of fresh buffer was replaced at every sampling point. The samples were analyzed by UV spectrophotometry, as described above.

Determination of minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) of drug-free and Doxy- or Vanco-loaded BNP and LPHNP formulations were determined against *M. smegmatis* or *S. aureus* [5, 33]. Cultures were grown in 10 mL of broth (Middlebrook or MHB) overnight at 37°C with shaking at 200 rpm. These cultures were then washed and diluted to a final concentration of 5×10^5 bacteria/mL, and various concentrations of Doxy- or Vanco-encapsulated NPs or standard concentrations of free antibiotics were added separately (Doxy or Vanco). After 18 h of incubation at 37°C and shaking at 200 rpm, bacterial densities were estimated by diluting and plating on agar followed by incubation overnight at 37°C.

Cytotoxicity assay

The cytotoxicity of BNPs and LPHNPs on macrophages was evaluated by CCK-8 assays [17, 18]. J774.1 (5×10^3) cells were seeded in 96-well plates and incubated in DMEM containing 10% FBS 24 h before the experiment. Serially diluted BNPs and LPHNPs in serum-free DMEM were added to the cells and incubated for 24 h at 37°C [39, 40]. CCK-8 solution (10 μ L) was added to each well, followed by incubation for 1 h at 37°C. Then, absorbance at 450 nm was measured using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Intracellular uptake of BNPs and LPHNPs by macrophages

The J774.1 (1.8×10^5) cells were seeded prior to 18 h of treatment. The cells were then incubated with fluorescent BNPs and LPHNPs for 24 h at 37°C. After incubation cells were washed and treated with 0.25% trypsin for 10 min, and cells were resuspended directly in PBS for cytometry (FACS). Cell suspensions were analyzed by flow cytometry (Accuri C6; BD Biosciences, Franklin Lakes, NJ, USA) [17-19, 25]. All measurements were performed in triplicate or more. Particle uptake by macrophages was further visualized by CLSM [2, 17, 18, 32]. Briefly, J774.1 cell (8×10^4) was cultured on a cover slip and incubated at 37°C in a 5% CO₂ incubator for 24 h. Then, fluorescent NPs (BNPs and cationic and zwitterionic LPHNPs) were added to the J774.1 cell and incubated for an additional 24 h. Then, these cells were washed twice with PBS and fixed with a paraformaldehyde solution. The fixed J774.1 cell was stained with DAPI and visualized by CLSM (TCS SP5 II; Leica, Heidelberg, Germany).

Intracellular bactericidal efficiency of BNPs and LPHNPs

J774.1 and RAW 264.7 cells (1.5×10^5 cells) were seeded in 24-well plates in DMEM with 10% FBS for 24 h. The cells were infected by *M. smegmatis* or *S. aureus* for 2h in DMEM at a multiplicity of infection

(MOI) of 10 bacteria/cell. After 2 h, gentamicin (200 µg/mL) was added to kill the extracellular bacteria for 2h. After incubation, cells were washed with PBS and free antibiotic(s) or antibiotic (Doxo or Vanco)-loaded BNPs or cationic, zwitterionic LPHNPs were added. After 24 h of incubation, the cells were washed with cold PBS for three times, and the macrophages were treated with ice-cold sterilized MilliQ water to release the intracellular bacteria. The lysates were diluted, plated on agar plates, and cultured overnight at 37°C. Colony-forming units (CFU/mL) were calculated. The medium without the drug was used as a negative control [2, 32]. To visualize the viable and dead bacterial cells within macrophages, the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes) was used. As described above, the J774.1 or RAW 264.7 cells (1.5×10^5 cells) were seeded on a coverslip and the infection was established as described earlier in this section. Macrophages were incubated with a cocktail of the LIVE/DEAD BacLight bacterial viability staining solution for 15 min in the dark in the presence of TritonX. After washing with PBS, cells were visualized under CLSM (TCS SP5 II; Leica) [2].

Statistical analysis

The Bonferroni post-hoc test was used for comparisons between groups after at least three independent sets of experiments performed in triplicate. Differences were considered significant at $P < 0.05$. The Prism software package (version 5.02; GraphPad Software Inc., La Jolla, CA, USA) was used to perform the statistical tests.

Abbreviations

LPHNPs- Lipid–polymer hybrid nanoparticles, BNPs- Bare nanoparticles, Doxy- Doxycycline, Vanco- Vancomycin

Declarations

Availability of Data and Materials

Data and materials are available for any research.

Funding.

This research was supported by Creative Materials Discovery Program through the National Research Foundation of Korea (NRF) funded by Ministry of Science and ICT(NRF-2018M3D1A1058813), This research was supported by the National Research Foundation of Korea(NRF) funded by Ministry of Science and ICT (NRF-2019R1A2C1007088), and Dongguk University Research Fund of 2018.

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RJCB and HP conceived the idea, performed the experiments, and analyzed the data. RJCB, NT and HP wrote the manuscript. RP, YHC, NT, TM, and SHL edited the manuscript. SHL and HP supervised and funded the project.

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Ethics declaration.

Not applicable.

Consent for publication

We agree to publish this manuscript.

Competing interests

None.

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Figures

Figure 1

Schematic diagram outlines the overall concept of experiment. The cationic and zwitterionic lipids-based surface engineering approach with antibiotics (Doxycycline or Vancomycin) loaded LPHNPs were prepared and examined their surface charge influence on the physiochemical characteristics, antibiotic entrapment, and intracellular release behaviors against *Mycobacterium smegmatis* or *Staphylococcus aureus* infected macrophages in vitro.

Figure 2

Physiochemical characterization of cationic or zwitterionic lipid polymer hybrid and non-lipid layered nanoparticles loaded with either doxycycline or Vancomycin. (A) DLS based Size analysis different Nanoparticles formulations. (B) Surface-charge (ζ -potentials) analysis of different nanoparticles formulations measured by Malvern Zeta sizer. (C) EFTEM images of non-lipid layered, zwitterionic and cationic lipid polymer hybrid nanoparticles. (D) Cryo-electron microscopy analysis of cationic or zwitterionic lipid polymer hybrid nanoparticles, Arrow marks indicates the lamellar assembly lipids on Polymer-NPs (Scale bar:0.2 μ m). (E) CLSM image of fluorescent lipid layered LPHNPs. (F) Encapsulation efficiency of cationic or zwitterionic lipid polymer hybrid and non-lipid layered nanoparticles loaded with either doxycycline or Vancomycin.

Figure 3

In vitro antibiotic cumulative release from (A) doxycycline or (B) Vancomycin loaded non-lipid layered BNPs and cationic or zwitterionic LPHNPs

Figure 4

Effect of surface characteristics of NPs on macrophage cellular uptake and intracellular behaviors. Fluorescent NPs (DiD labelled BNPs and cationic and zwitterionic LPHNPs) were treated with J774.1 cell

(labeled with DAPI) for 24 hours and then the particle uptake efficiency was visualized by (A) CLSM images (Scale bar 50um) and quantified by (B) flow cytometry.

Figure 5

(A) Representative CLSM images of the viable and dead Intracellular *S.aureus* and *M.smegmatitis*. Fig A shows the adhesion of cationic LPHNPs -doxy on the surface of macrophages (dotted white arrow indicating NPs on lipid leaflet of extracellular side) and thick white arrow indicating NPs in intracellular compartment of macrophages. Inlet Fig (2) shows the Live and dead bacteria (post-treatment) with NPs.

Figure 6

Intracellular bactericidal (*M. smegmatitis*) activity of Doxycycline loaded non-lipid layered BNPs and cationic or zwitterionic LPHNPs. (A) Intracellular killing efficiency of doxycycline loaded non-lipid layered BNPs and cationic or zwitterionic LPHNPs was investigated with controls (free drug and DMSO). Representative CLSM images of the viable and dead Intracellular mycobacterium within macrophages after the treatment with free drug, doxycycline loaded non-lipid layered BNPs and cationic or zwitterionic LPHNPs

Figure 7

Intracellular bactericidal (*S. aureus*) activity of Vancomycin loaded non-lipid layered BNPs and cationic or zwitterionic LPHNPs. (A) Intracellular killing efficiency of Vancomycin loaded non-lipid layered BNPs and cationic or zwitterionic LPHNPs was investigated with controls (free drug and DMSO). B-E. Representative CLSM images of the viable and dead Intracellular *S. aureus* within macrophages after the treatment with free drug, doxycycline loaded non-lipid layered BNPs and cationic or zwitterionic LPHNPs

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