Liposomes with Water as a pH-Responsive Functionality for Targeting of Acidic Tumor and Infection Sites

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Abstract: A lipid named DCPA was synthesized under microwave-assisted heating. DCPA possesses a pyridine base, hydrophilic group that can be complexed with water through hydrogen bonding (DCPA-H₂O). DCPA-H₂O liposomes became protonated relatively fast already at pH < 6.8, due to the high HOMO binding energy of DCPA-H₂O. In murine models, DCPA-H₂O liposomes had longer blood circulation times than natural DPPC or cationic DCPM liposomes, while after tail-vein injection DCPA-H₂O liposomes targeted faster to solid tumors and intra-abdominal infections.[1] A wide variety of smart, drug-loaded nanocarriers has been described in the literature that are equipped with pH-responsive functionalities to make them self-targeting to an acidic environment, that include long circulation times in the blood without reticulo-endothelial rejection[10] and easy entry into tumor cells or infectious bacteria by fusion with the cell membrane.[11] Equipping lipid nanocarriers with pH-responsive functional groups, such as weakly acidic (carboxylic, sulfonic or phosphonic acid)[6] or weakly alkaline (mainly primary amines)[5] or quaternary ammonium salts and carboxylic acids.[6] Although these functional groups all yield charge reversal from negative at physiological pH[7] to positive in an acidic tumor or infection site, this usually occurs well below pH 6.5 and requires a relatively long exposure time to a low pH environment.[8] Moreover, the pH value near a tumor or infection site decreases only gradually towards its depth.[9] Therefore, the relatively low point of zero charge of these functional groups may be considered a disadvantage that is neglected in current designs of pH-responsive nanocarriers.

Most liposomal drug-loaded nanocarriers possess a number of unique features, such as blood circulation times, that include long circulation times in the blood without reticulo-endothelial rejection[10] and easy entry into tumor cells or infectious bacteria by fusion with the cell membrane.[11] Equipping lipid nanocarriers with pH-responsive functionalities to make them self-targeting to an acidic environment is not trivial however. pH-responsive, liposomal drug carriers have been made using weakly alkaline or zwitterionic lipids[4,5] with amino and carboxyl as functional groups, but they all have points of zero charge below pH 6.5 and their charge properties only respond to a pH change after several tens of minutes.[8] Moreover, their biocompatibility can raise problems, such as blood coagulation[15] or cytotoxicity.[14] Here, we designed a new zwitterionic lipid 2-(4-((1,5-bis(octadecyloxy)-1,5-dioxopentan-2-yl) carbamoyl) pyridin-1-ium-1-yl)- acetate, abbreviated DCPA) for making liposomal drug nanocarriers with water as a pH-responsive functional group (Scheme 1). Water is highly biocompatible and its protonation occurs gradually towards acidic pH, but with relatively fast response times.[15]

Results and Discussion

Synthesis of DCPA started with commercially purchased Boc-L-glutamic acid (compound 1). First, diodecyl (tert-butoxycarbonyl) glutamate (compound 2) was synthesized to provide compound 1 with hydrophobic tail groups.[16] To this end, 1-octadecanol was allowed to react with compound 1 and purified using column chromatography.[24]
Quaternization of pyridine required elevated temperatures\[17\] and was done by conventional heating in a thermostatic oil bath during 24 h at 81°C.\[30\] b) Same as panel (a), now carrying out the quaternization reaction under heating by conventional heating in a thermostatic oil bath (24 h at 81°C). Note that full spectra for both quaternization conditions and reaction times are compared in Figure S6. c) Normalized areas of the b peaks at 9.20 ppm representative of quaternized nitrogen in amphiphilic, zwitterionic DCPA. Peaks were normalized with respect to the peak area at 8.82 ppm representing its source nitrogen. Data pertain to microwave-assisted and conventional, high temperature quaternization conditions. The percentage yield is calculated as $$\frac{\text{Normalized peak area at 9.20 ppm}}{\text{Normalized peak area at 9.20 ppm}} \times 100\%.$$

Next, the Boc-protection was removed from compound 2 using 50% TFA in DCM to expose the terminal amino group (compound 3) that was subsequently coupled with the carboxyl group of isonicotinic acid to obtain compound 4.\[29\] Subsequently, (2-(4-((1,5-bis(octadecyloxy)-1,5-dioxopentan-2-yl) carbamoyl) pyridin-1-ium-1-y) acetate, (DCPA). Compound 1 (Boc-L-glutamic acid) was coupled with 1-octadecanol to obtain compound 2. After removal of the Boc-protection from compound 2 using 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) at 0°C, the terminal amino group of compound 2 was exposed to yield compound 3. Then, the terminal amino group of compound 3 was coupled with the carboxyl group of isonicotinic acid to obtain compound 4. Finally, the nitrogen atom in the pyridine ring of compound 4 was quaternized at elevated temperature with bromoacetic acid to obtain DCPA. b) The resulting hydrophilic, pyridine betaine head-group of DCPA can complex with water (DCPA-H2O) to become positively charged (DCPA-H3O+) in a low pH environment.

The final quaternization of pyridine was also carried out in a microwave-generated electromagnetic field, suggested to enhance molecular vibrations to increase internal energy generation and exposure of active sites, thus decreasing the reaction time.\[18\] Hence, quaternization yields were compared under microwave-assisted heating and by conventional heating. Based on a comparison of 1H NMR spectra (Figure 1a,b, respectively), microwave-assisted heating demonstrated not only faster, but also more extensive quaternization under microwave-assisted heating (Figure 1c). Therefore, the remainder of this study is based on DCPA obtained using microwave-assisted heating.

Frontier molecular orbitals in DCPA were subsequently calculated in order to explain the pH responsiveness of water complexed with DCPA. HOMO (highest occupied molecular orbital) analysis (Figure 2a) indicates that electron-donating sites are clearly confined to the pyridine betaine head groups of DCPA. Zooming in on the pyridine betaine head group of DCPA (Figure 2b), shows that electron-donating, that is, hydrogen-accepting sites in DCPA are located at the negatively charged oxygen of the carboxylate connected to the pyridine ring. Electron-accepting sites in DCPA are located at the positively charged nitrogen in the pyridine ring (see Scheme 1 a). Subsequently, the Alpha MOS routine in Gaussian-View was employed to calculate the electron binding energies of the HOMO and LUMO (lowest unoccupied molecular orbital)\[19\] in DCPA (Figure 2b) and pyridine betaine (Figure 2c). As can be seen in Figure 2d, electron binding energies in the LUMO of DCPA and pyridine betaine are similar. HOMO electron binding energy of DCPA had increased because conjugation of the pyridine ring through amide groups with the carboxylate in DCPA increased the electron cloud density of terminal oxygen in carboxylate. Consequently, the energy gap between HOMO and LUMO binding energies is larger in DCPA than in pyridine betaine. Accordingly, DCPA possesses a larger ability to donate an electron than pyridine betaine, that is, DCPA complexes more easily with water through hydrogen bonding at the negatively charged oxygen of the carboxylate and the nitrogen in pyridine ring than pyridine betaine.
Next, DCPA-H$_2$O liposomes were self-assembled and their pH-adaptiveness was compared with the one of slightly anionic, natural dipalmitoylphosphatidyl choline (DPPC) and cationic 4-((1,5-bis(octadecyloxy)-1,5-dioxopentan-2-yl) carbamoyl)-1-methylpyridin-1-ium) (DCPM) lipids (see Figure 3a).

All liposomes had a diameter of around 100 nm albeit with low polydispersity indices (Figure 3b), regardless of the lipid used. Diameters and spherically shaped morphologies were confirmed using cryogenic electron microscopy (Figure 3c). DCPA-H$_2$O liposomes showed a variation in zeta potential ranging from $-0.2$ mV at pH 7.4 to $+25.2$ mV at pH 5.0. Cationic DCPM liposomes were pH responsive but remained to possess a positive zeta potential at physiological pH, while DPPC liposomes consisting of natural lipids were negatively charged over the entire pH range from 7.4 to 5.0 (Figure 3d). Charge transition of DCPA-H$_2$O liposomes upon protonation of complexed water furthermore showed from a comparison of $^1$H NMR spectra taken after exposure to deionized water with different pH between 6.5 and 7.4. After exposure, the liposome suspension was lyophilized and the resulting lyophilized powder was dissolved in CDCl$_3$. The peak shift from a (2.10 ppm) to b (2.48 ppm) indicates protonation of the complexed water in water-functionalized DCPA. f) $^1$H NMR spectra of water-functionalized DCPA-H$_2$O liposomes over the chemical shift range 2.8 to 1.8 ppm (for full spectra see Figure S7) after different exposure times to deionized water at pH 6.5.
pH 7.4 and pH 6.5. 1H NMR spectra on the hydrogen in water exposed to different pH values decreasing from pH 7.4 to pH 6.5, demonstrated gradual broadening and disappearance of the peak occurring at 2.10 ppm at pH 7.4 (peak a) and the appearance of a broad new peak (peak b) at 2.48 ppm (Figure 3e). Exposure to pH 6.5 as a function of time showed full disappearance of peak a at 2.10 ppm due to protonation of complexed water within 8 min (Figure 3f).

After establishing stability of DCPA-H2O liposomes in blood plasma (Figure S9) and their ability to become loaded with a therapeutic cargo (Table S1) and release of an antibiotic or chemotherapeutic payload in an in vitro, acidic blood plasma (Figure S9) and their ability to become loaded more rapidly from the blood circulation than DCPA-H2O DPPC as well as cationic DCPM liposomes were cleared almost 300% of the PBS control (Figure 4b). Both natural infection site within a targeting time of 10 min after tail-vein injection (Supporting Movie 3) on the other hand, became clearly visible in the nanocarriers.

Cationic DCPM liposomes and other self-targeting drug-circulation times, as compared with natural DPPC and considered fully blood compatible with superior blood circulation half-life time of around 39 h (Figure 4d). This is significantly longer than reported for example, for stealth, gold nanocarriers with a polyethylene glycol (PEG) coating ([12] = 9 h) or hyaluronan-decorated nanoparticles ([12] = 8 h). Blood analysis at sacrifice demonstrated no significant differences between rats injected with PBS or any of the three types of liposomes (Table 1). Thus, DCPA-H2O liposomes can be considered fully blood compatible with superior blood circulation times, as compared with natural DPPC and cationic DCPM liposomes and other self-targeting drug-nanocarriers.

Next self-targeting of DCPA-H2O liposomes to an infectious biofilm of green-fluorescent Staphylococcus aureus ATCC12600GFP underneath an abdominal imaging window was monitored in situ after tail-vein injection of red-fluorescent rhodamine-loaded liposomes in mice. To this end, a rat model was preferred because the blood circulation contains a larger volume of blood than mice. Neither injection with phosphate buffered saline (PBS) nor any of the three types of liposomes had any adverse effect on the body weight of the rats and the average body weight increased slightly over the course of the study (Figure 4a). Similar to natural DPPC liposomes, DCPA-H2O liposomes did not increase IgM levels in blood, whereas cationic DCPM liposomes increased IgM levels to almost 300% of the PBS control (Figure 4b). Both natural DPPC as well as cationic DCPM liposomes were cleared more rapidly from the blood circulation than DCPA-H2O liposomes (Figure 4c), possessing a significantly longer blood circulation half-life time of around 39 h (Figure 4d). This is significantly longer than reported for example, for stealth, gold nanocarriers with a polyethylene glycol (PEG) coating ([12] = 10 h) or hyaluronan-decorated nanoparticles ([12] = 8 h). Blood analysis at sacrifice demonstrated no significant differences between rats injected with PBS or any of the three types of liposomes (Table 1). Thus, DCPA-H2O liposomes can be considered fully blood compatible with superior blood circulation times, as compared with natural DPPC and cationic DCPM liposomes and other self-targeting drug-nanocarriers.

The kinetics of self-targeting in vitro is greatly dependent on the experimental design used and cannot be compared with targeting times observed in vivo. In vivo indirect, gamma scintigraphy demonstrated accumulation in subcutaneous fluorescence as a function of time after tail-vein injection, demonstrated significantly more rapid and extensive self-targeting of DCPA-H2O liposomes into the infectious biofilm than cationic DCPM or natural DPPC liposomes (Figure 5d).

| Table 1: Hematology parameters of blood taken at sacrifice: day 7 after tail-vein injection of different liposomes in rats. |
|-----------------|-----|-----|-----|-----|
| Injection of    | RBC | HGB | HCT | PLT |
|                 | [x10^12 L^-1] | [g L^-1] | [%] | [x10^9 L^-1] |
| PBS             | 8.0 ± 0.5 | 171 ± 5 | 46 ± 5 | 727 ± 68 | 9 ± 2 |
| DCPM            | 7.8 ± 0.1 | 166 ± 3 | 47 ± 9 | 739 ± 32 | 10 ± 2 |
| DPPC            | 8.0 ± 0.5 | 169 ± 4 | 46 ± 2 | 726 ± 58 | 9 ± 3 |
| DCPA-H2O        | 7.9 ± 0.1 | 167 ± 4 | 47 ± 4 | 732 ± 37 | 9 ± 3 |

[a] All data are given as means ± SD over three different rats per group. There are no significant differences in any of the hematometry parameters, regardless of whether injected with PBS or any of the three types of liposomes (Students’ t-test, P > 0.5). RBC = red blood cell count, HGB = hemoglobin concentration, HCT = hematocrit percentage, PLT = platelet, WBC = white blood cell count.
Mycobacterium tuberculosis biofilms in the thigh of mice of *Mycobacterium tuberculosis* after tail-vein injection of the imaging agents.[23] Direct observation of pH-driven self-targeting of antimicrobial nanoparticles to an infection site in vivo has, to the best of our knowledge, only been made possible through the use of intra-vital imaging.[24] As applied here. Using this technique, we have recently shown that it takes pH-responsive zwitter-ionic micelles composed of poly(ethylene glycol) and poly(ε-caprolactone) block-co-polymers at least 20 min to reach an intra-abdominal infection site after tail-vein injection.[25] Collectively, and within the limitations of different infection sites, infecting bacterial strains and probing molecules and particles, these comparisons points to the superiority of water as a pH-responsive functionality on nanocarriers for self-targeting to an infection site.

Similarly, self-targeting of DCPA-H$_2$O liposomes into a solid tumor was investigated after tail-vein injection of rhodamine loaded liposomes in mice (Figure 5e, f). Neither natural DPPC nor cationic DCPM liposomes demonstrated targeting into the tumor site, while particularly natural DPPC spread across the entire body of a mouse. DCPA-H$_2$O liposomes initially showed similar spreading across the entire body of a mouse, but at the same time self-targeting to the acidic tumor site became evident within 6 h after tail-vein injection (Figure 5g). Quantitative analysis of the photon flux arising from the tumor site revealed rapid self-targeting of DCPA-H$_2$O liposomes, demonstrating maximal accumulation in the tumor after 12 h (Figure 5h). Accumulation in the tumor was on average 11-fold higher than whole body accumulation, as calculated from the photon fluxes over the tumor site and the whole body of the mice (Figure 5g). Other in vivo studies also report on kinetics of tumor targeting of fluorescent liposomes within 5–8 h in similar models, but these were done at 400 × higher liposome concentrations yielding only 2-fold higher accumulation compared with whole body accumulation.[11] Thus water as a pH-responsive functionality is not only superior to other drug-nanocarriers to self-target an infection site, but also for self-targeting a tumor site. Superior self-targeting of DCPA-H$_2$O liposomes was accompanied by better therapeutic efficacy in a murine, infected wound-healing model. Tail-vein injection of a suspension of ciprofloxacin-loaded DCPA-H$_2$O liposomes in PBS yielded significantly better eradication of *M. tuberculosis* from infected wounds than tail-vein injection of clinically applied ciprofloxacin in solution or suspensions of ciprofloxacin-loaded DPPC or DCPM liposomes (Figure S11).

Interestingly, self-targeting of DCPA-H$_2$O liposomes to an infectious biofilm proceeded on a time-scale of minutes, while self-targeting into a tumor site proceeded on a time-scale of several hours. Whereas this may be due to size differences in tumor and infection sites, we consider it likely that different target-times are due to the higher acidity of infectious biofilms, ranging from pH 6.5 to 4.5,[26] as compared with tumor sites, ranging in pH between 6.9 and 6.5.[27] Thus pH-adaptive carriers will be more conducive to charge reversal when approaching an infectious biofilm than upon approach of a tumor site.

Conclusion

We have successfully prepared a novel lipid, DCPA. DCPA can be made in high yields using microwave-assisted heating and can be complexed with water as a pH-responsive functionality. DCPA-H$_2$O can self-assemble into liposomes,
providing exceptionally long blood circulation times, and rapid self-targeting to the acidic environment of an infectious biofilm or solid tumor. When ciprofloxacin-loaded, DCPA-H₄O liposomes had better therapeutic efficacy in mice than ciprofloxacin or ciprofloxacin-loaded DPPC or DCPM liposomes.

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Conflict of Interest

H.J.B. is also director of a consulting company SASA BV. The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of the funding organization or their respective employer(s).

Keywords: hydrogen bonding · pH-responsive · pyridine betaine · self-targeting · zwitterionic lipids

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[16] NMR data compound 2: 1H NMR (400 MHz, CDCl₃) δ = 5.10 (dd, J = 7.4, 1H), 4.31 (dd, J = 5.1, 1H), 4.12 (t, J = 6.7, 2H), 4.06 (t, J = 6.8, 2H), 2.44–2.33 (m, 2H), 2.21–2.13 (m, 1H), 1.96–1.89 (m, 1H), 1.61 (s, 6H), 1.44 (s, 9H), 1.25 (s, 58H), 0.88 (t, J = 6.7, 6H) (Figure S1).

NMR data compound 4: 1H NMR (400 MHz, CDCl₃) δ = 8.77 (d, J = 4.8, 2H), 7.69 (d, J = 5.3, 2H), 7.42 (d, J = 6.9, 1H), 4.76 (dd, J = 12.1, 7.4, 1H), 4.18 (t, J = 6.7, 2H), 4.06 (t, J = 6.6, 2H), 2.57–2.43 (m, 2H), 2.35–2.27 (m, 1H), 2.22–2.23 (m, 1H), 1.69–1.56 (m, 4H), 1.25 (s, 60H), 0.88 (t, J = 6.7, 6H) (Figure S2); 13C NMR (100 MHz, CDCl₃) δ = 173.67, 171.51, 165.01, 150.38, 140.97, 121.04, 66.07, 52.78, 31.91, 30.49, 29.69, 29.64, 29.57, 29.49, 29.34, 29.23, 29.19, 28.54, 28.52, 27.66, 25.86, 25.81, 22.67, 14.08 (Figure S3). ESI-HRMS m/z 757.6548 [M+H]⁺, calculated for C₃₆H₆₆N₂O₅, 757.6453.

NMR data DCPA: 1H NMR (400 MHz, CDCl₃) δ = 9.49 (d, J = 7.2, 1H), 9.22 (d, J = 6.3, 2H), 8.71 (d, J = 6.3, 2H), 4.69–4.67 (m, 1H), 4.65 (s, 2H), 4.13 (t, J = 6.8, 2H), 4.05 (t, J = 6.8, 2H), 2.68–2.55 (m, 2H), 2.45–2.36 (m, 2H), 1.69–1.57 (m, 4H), 1.25 (s, 60H), 0.87 (t, J = 6.7, 6H) (Figure S4); 13C NMR (100 MHz, CDCl₃) δ = 197.61, 172.89, 171.57, 162.16, 147.67, 146.23, 126.82, 65.99, 65.03, 53.40, 49.32, 31.94, 30.50, 29.74, 29.69, 29.60, 29.39, 29.34, 28.59, 28.53, 25.93, 25.90, 22.71, 14.15 (Figure S5). ESI-HRMS m/z 771.5531 [M+H–COOH]⁺, calculated for C₃₆H₆₅N₂O₅, 771.5610.
Liposomes

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Liposomes with Water as a pH-Responsive Functionality for Targeting of Acidic Tumor and Infection Sites

A new lipid, DCPA, was prepared, possessing a hydrophilic, pyridine betaine group that can be hydrogen-bonded with water yielding DCPA-H$_2$O. Self-assembled DCPA-H$_2$O liposomes become rapidly protonated at relatively high pH ($< 7.0$) due to the high HOMO binding energy of DCPA-H$_2$O. As a result, DCPA-H$_2$O liposomes target faster to solid tumors and infectious biofilms than natural DPPC or cationic DCPM liposomes.