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Biocidal Potency of Polymers with Bulky Cations

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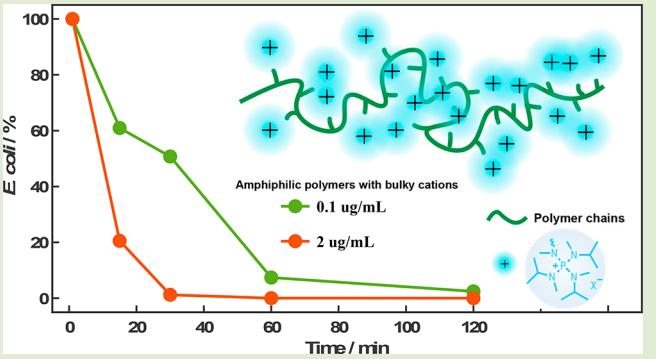
ABSTRACT: The performance of antimicrobial polymers depends sensitively on the type of cationic species, charge density, and spatial arrangement of cations. Here we report antimicrobial polymers bearing unusually bulky tetraaminophosphonium groups as the source of highly delocalized cationic charge. The bulky cations drastically enhanced the biocidal activity of amphiphilic polymers, leading to remarkably potent activity in the submicromolar range. The cationic polynorbornenes with pendent tetraaminophosphonium groups killed over 98% *E. coli* at a concentration of 0.1 μg/mL and caused a 4-log reduction of *E. coli* within 2 h at a concentration of 2 μg/mL, showing very rapid and potent bactericidal activity. The polymers are also highly hemolytic at similar concentrations, indicating a biocidal activity profile. Polymers of a similar chemical structure but with more flexible backbones were made to examine the effects of the flexibility of polymer chains on their activity, which turned out to be marginal. We also explore variants with different spacer arm groups separating the cations from the backbone main chain. The antibacterial activity was comparably potent in all cases, but the polymers with shorter spacer arm groups showed more rapid bactericidal kinetics. Interestingly, pronounced counterion effects were observed. Tightly bound PF_6^- counteranions showed poor activity at high concentrations due to gross aggregate formation and precipitation from the assay media, whereas loosely bound Cl^- counterions resulted in very potent activity that monotonically increased with increasing concentration. In this paper, we reveal that bulky phosphonium cations are associated with markedly enhanced biocidal activity, which provides an innovative strategy to develop more effective self-disinfecting materials.



Synthetic polymers bearing cationic and hydrophobic groups have long been investigated as antibacterial materials due to their eminently tailorable structure and composition, facile and cost-effective synthesis, and fine-tuned control over antibacterial potency as well as toxicity to mammalian cells. It is widely accepted that a major key determinant of antibacterial polymer activity involves striking a careful balance between cationic charge and hydrophobicity (i.e., "amphiphilic balance").[1−6] Cationic charge serves as the driving force of electrostatic attraction to biomembranes whereas the hydrophobic moieties are prone to insertion into the hydrophobic core of the lipid bilayers.[3,4,7−9] Due to the preponderance of anionic phospholipids on the outer leaflet of bacterial cell membranes, most antibacterial polymers are designed with multivalent cationic charges, which are crucial for the electrostatic attraction to the bacterial cell envelope.[9−11] A rich diversity of chemical structures have been used as the source of positive charge, including ammonium,[5,12−19] pyridinium,[20−22] imidazolium,[20,23,24] thiazolium,[25,26] triazolium,[25] phosphonium,[14,20,27] sulfonium,[19,28] and guanidium.[4,8,19,29−31] As synthetic mimics of Host Defense Peptides (HDPs) that contain lysine residues, polymers containing primary amines as the source of cationic charge have exhibited rapid and potent broad-spectrum activity with minimal toxicity to human cells,[3,19,32−34] but many amphiphilic polymers with other cationic residues also showed potent antibacterial efficacy. Strategies on improving antibacterial activities by focusing on cationic groups have been investigated from different angles, such as changing the ratio of different cationic groups,[35] and increasing the density of protonated amino groups.[34,36] Antibacterial phosphonium polymers have been extensively investigated; molecular designs have encompassed a variety of different backbone structures,[14,20,27] molecular weights,[37] functional groups conjugated to phosphonium,[27,38,39] and lengths of spacer groups within the backbone.[40]

In this work, we examine polynorbornenes bearing unusually bulky tetraaminophosphonium groups as a novel source of cationic charge. These materials were originally developed by Noonan and co-workers to act as anion exchange membranes with superior transport properties,[41] but incidentally also

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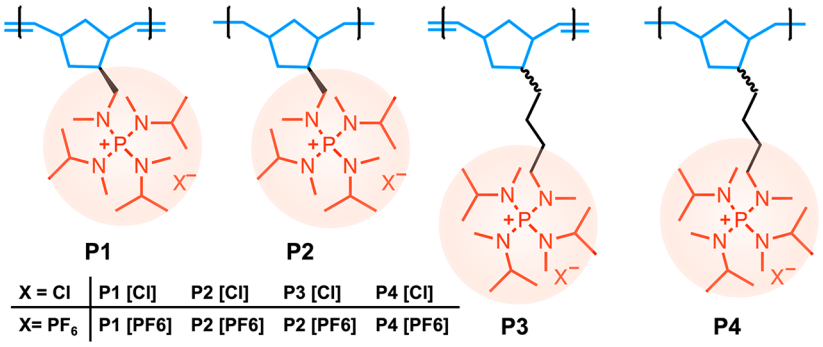
Scheme 1. Chemical Structures of P1, P2, P3, and P4; Degree of Polymerization (DP) = 25



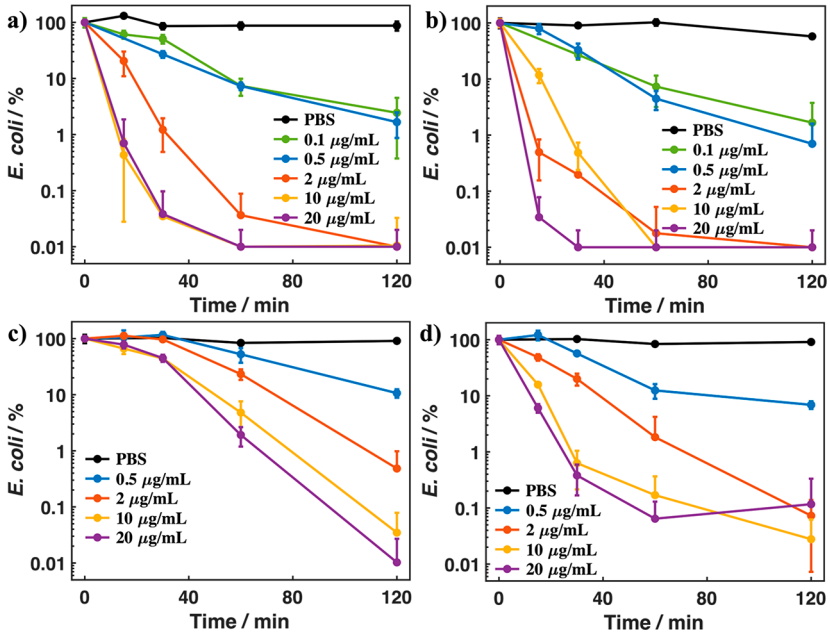


Figure 1. Bactericidal kinetics of (a) **P1** [Cl], (b) **P2** [Cl], (c) **P3** [Cl], and (d) **P4** [Cl] against *E. coli* in PBS at different concentrations.

attracted the attention of researchers in the antibacterial polymer field because the highly delocalized cationic charges appear primed for multivalent interactions with anionic phospholipid headgroups. Although trialkyl- and triaryl-phosphoniums have been reported in antimicrobial macromolecules previously,[14,20,27] this work showed for the first time that triaminoalkyl-phosphonium moieties as the source of cationic charge in antimicrobial polymers. Relative to alkyl and phenyl counterparts, these amino substituents enable spreading of the positive charge over larger volumes, which we hypothesize is related to their enhanced antibacterial activity. We present here a total of four structural variants: polynorbornenes with bulky cations tethered to the backbone via short (methylene) or longer (butylene) side chain linkers. We also studied each of these types before and after hydrogenation of the backbone double bonds, to assess the role of backbone flexibility. Additionally, we also probe the effects of the counterion type.

The polynorbornene **P1** displays pendent tris(isopropyl-(methyl)amino) (dimethylamino)phosphonium group with a hexafluorophosphate (PF_6^-) counterion. After exchanging the counterions to Cl^- this polymer is named **P1** [Cl]. Hydrogenation of **P1** yielded **P2**, which putatively has greater backbone flexibility. Similar chemistry is employed for **P3** and **P4**, but with a longer butylene spacer group between the norbornene and the bulk cation in the side chain. Synthetic procedures of polymers can be found in previous work[41] and are also described in detail in the Supporting Information. Chemical structures of all polymers in this work are shown in Scheme 1.

Each of the polynorbornenes with bulky cations and chloride counterions indeed showed rapid bactericidal activity at low concentration (Figure 1). For example, **P1** [Cl] caused over 98% reduction of viable *E. coli* cells at just 0.1 μg/mL, which is a lower concentration range than almost all known membrane-disrupting antimicrobial agents. Furthermore, killing becomes more rapid and more complete with increasing concentration: at 2 μg/mL, **P1** [Cl] induced a >4-log reduction (>99.99% killing) of *E. coli* within 2 h. At still higher concentration (20 μg/mL), a >4-log reduction was achieved in just 30 min. A comparison of the antibacterial activity between the bulky phosphonium and quaternary ammonium was made by conducting the same bactericidal kinetics assay but with an analogous polynorbornene bearing pendent butylammonium, dubbed **P3-A** [Cl]. This structure is very similar to **P3** [Cl], but with the bulky phosphonium replaced by a more conventional quaternary ammonium salt (QAS). **P3-A** [Cl] showed no killing at a concentration of 0.5 μg/mL and killed

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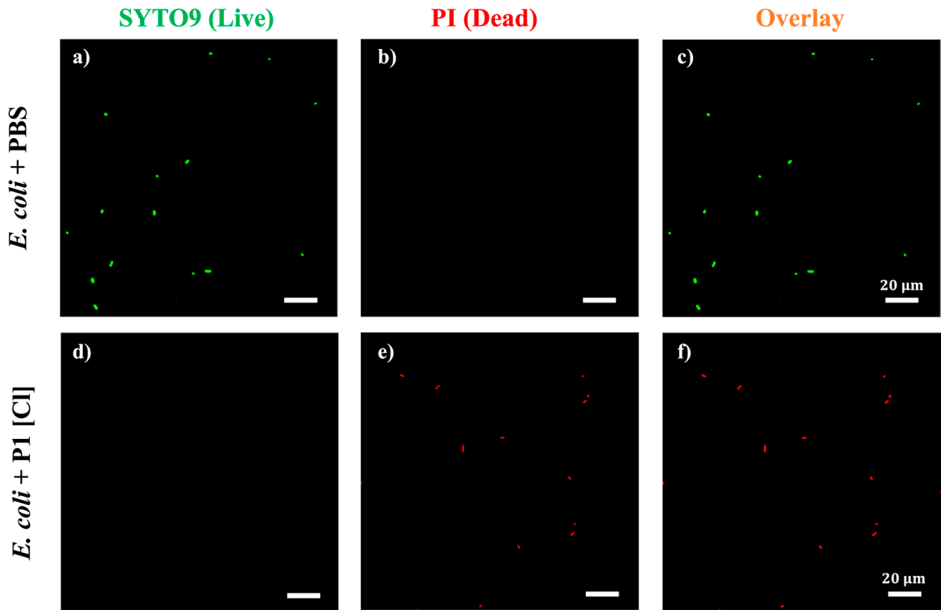

Figure 2. Confocal laser scanning microscopy images of (a–c) *E. coli* in PBS and (d–f) *E. coli* exposed to **P1** [**Cl**] at 2 μg/mL for 2 h.

only 90% *E. coli* at 10 μg/mL in 2 h (Supporting Information, Figure S42), while **P3** [**Cl**] caused 90% and 99.5% reduction of *E. coli* (Figure 1c), respectively. Therefore, we conclude that the larger bulky cation indeed outperformed the conventional QAS units, especially at very low doses.

The antibacterial activity of the hydrogenated **P2** was comparable to that of the more rigid unsaturated **P1**. Also, **P3** and **P4** showed similar bactericidal activity as well. Taken together, these results suggest that backbone flexibility is seemingly not a predominant activity determinant in this particular class of materials. The side chain spacer groups did have some effect on activity: the polymers with longer butylene tethers (**P3** and **P4**) exhibited somewhat slower bactericidal kinetics relative to the polymers with shorter methylene tethers (**P1** and **P2**), although all four examples have similar end-point MBC values. This suggests that the side chain spacer arms in this study play a minor role in the bactericidal activity, in contrast to prior studies.[42] The counterion effects were rather stark by comparison: polymers showed much weaker activity at high concentration when the anion was hexafluorophosphate (PF_6^-), which is more tightly bound to phosphonium than chloride.[37] In summary, developing bulky cations as the source of positive charge is a promising strategy to design antibacterial polymers with higher antibacterial activity at low dose, and the identity of the counterions was a significant factor in the design.

Despite their differences in bactericidal kinetics, all four polymers (with chloride anions) showed similar Minimum Bactericidal Concentration (MBC) in phosphate buffered saline (PBS) below 5 μg/mL, which is indeed potent activity. We also attempted to measure the minimal inhibitory concentration (MIC) against *E. coli* in nutrient-rich growth media (Mueller Hinton Broth), i.e., the lowest concentration that completely prevents the growth of bacteria in standard incubation conditions (5×10^5 CFU/mL bacteria concentration, MH broth, 37 °C, 18 h). In general, all four polymers, in the chloride series, showed similar MIC values of 12.5 or 6.25 μg/mL, whereas the MBC values were in the range of 2–4 μg/mL. Such low MIC and MBC values indicated potent antibacterial activity in all cases. An important side note is that the MIC assay was done in MH broth, whereas MBC is done in phosphate buffered saline (PBS). Because hydrophobic polymers can aggregate or bind to globular proteins in the nutrient-rich MH media, the reported activity may be less potent than observed in PBS.

We also confirmed that these polycations are broad-spectrum bactericides with potent activity in the low micromolar range against a panel of clinically relevant Gram-positive and Gram-negative microbes: *S. aureus*, *S. epidermidis*, *A. baumannii*, and *P. aeruginosa*. The specific MBC and MIC values of all bacterial strains tested are given in the Supporting Information (Table S1).

As a complementary method to the colony counting assay of bactericidal kinetics, confocal laser scanning microscopy and a commercially available LIVE/DEAD staining kit were used to examine the bacteria cells exposed to polymer. Dilute solutions (2 μg/mL) of each polymer in the [**Cl**] series, were incubated with *E. coli* suspensions separately at 37 °C for 2 h. After the polymers were removed from *E. coli*, the bacteria were stained with the LIVE/DEAD staining kit which consists of SYTO 9 and propidium iodide (PI). SYTO 9 (green channel) stains all cells, while PI (red channel) only stains dead cells when intercalated into DNA. Hence, the presence of red emission indicates membrane permeabilization of cells. The untreated control group showed *E. coli* cells all stained with green by SYTO 9 but without any red staining, indicating all live bacteria. In contrast, *E. coli* treated with **P1** [**Cl**] showed bright red emission, indicative of membrane permeabilization of all cells, while the green emission was too weak to be seen under the same parameters with the control group (Figure 2). Similarly, *E. coli* treated with **P2** [**Cl**], **P3** [**Cl**], and **P4** [**Cl**] also emitted bright red under confocal microscope. (Supporting Information, Figure S47). In all cases, the red-stained dead cells appear to retain the characteristic rod shape of *E. coli*, suggesting that membrane permeabilization and resulting death may not require gross lysis of the whole cells.

According to experiments results above, all four polymers did not show a wide difference on antibacterial activity, but the

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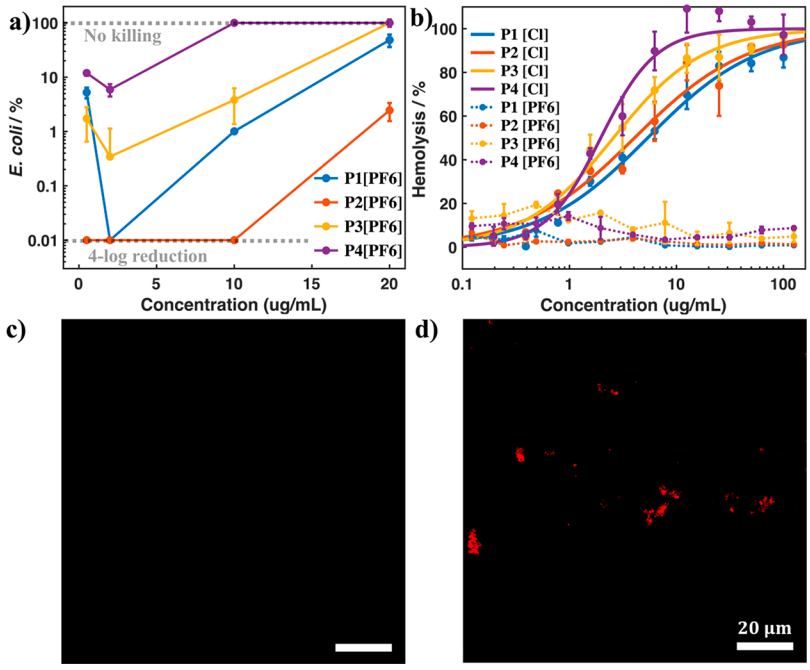

Figure 3. (a) Live *E. coli* percentage after incubation with polymers [**PF6**] at 2 μg/mL in PBS for 2 h. (b) Hemolysis activity of polymers [**Cl**] and polymers [**PF6**] over concentration against sheep RBCs. Confocal laser scanning microscopy images of **P1** [**PF6**]-**RhB** at (c) 2 and (d) 20 μg/mL.

difference of anion species was found to greatly affect their antibacterial and hemolytic activity (Figure 3). We examined the ability of these polycations to lyse red blood cell (RBC) membranes, by established methods. The hemolysis dose–response curves are given in Figure 3b and Hemolytic Concentration (HC_{50}) values are also listed in Table S1. HC_{50} values were calculated by fitting the curves of hemolytic percentage over concentration to the modified Hill equation. The HC_{50} values of the four polymers (1–5 μg/mL) are in the same comparable range as the MIC and MBC, which implies that there is no selectivity for activity against bacteria versus mammalian cells, i.e. the materials are *biocidal* in nature.

All four polymers in this study were first synthesized with PF_6^- as the counterion, which forms tightly associated ion pairs with bulky cations.[37] Relative to the more conventional chloride counterions, the polymers with PF_6^- as the counterion showed poor aqueous solubility and their antibacterial activity depends nonmonotonically on concentration. For **P1**, **P2**, and **P3** [**PF6**], bactericidal activity was observed at concentrations below 10 μg/mL but also anomalous growth was sometimes erratically observed at high concentration. In both the MIC and MBC assays against *E. coli*, turbidity at high concentration was sometimes seen, while optically clear culture medium was observed at lower concentration. (Supporting Information, Figures S11 and S27). Colony counting assay was also conducted to examine the antibacterial activity. *E. coli* was incubated with polymers of increasing concentrations for 2 h. Remaining live cells were quantified by withdrawing bacterial suspensions, streaking on agar, incubating overnight, and colony counting. Weaker killing ability was observed as the concentration of the polymer increased (Figure 3a). In addition, MBC and HC_{50} of Cl^- version of polymers are all lower than that of PF_6^- version against all five bacteria strains tested in this work (Supporting Information, Table S1), showing the difference on bactericidal activity and hemolytic activity by anion species. It is theoretically possible that Cl^- ions in the PBS media would displace some PF_6^- counterions on the polymer chains, but such an exchange effect seems to be marginal, considering the dramatic difference in solubility and activity between polymers with Cl^- and PF_6^- as anions.

Curves were drawn to reflect the percentage of live *E. coli* as a function of the concentration of polymers. Live bacteria decreased as the polymer concentration increased from 0.5 to 2 μg/mL as expected, but the trend was markedly reversed upon further increase of polymer concentration from 2 to 20 μg/mL, suggesting a loss of activity at higher doses. **P3** [**PF6**] and **P4** [**PF6**] even showed no killing ability at concentrations of 20 μg/mL and above. These polymers are all amphiphilic molecules, so aggregation or poor solubility may explain the phenomenon described above. We attempted to probe aggregation by the pyrene emission method but that effort failed to provide clear evidence of well-defined micelles (Supporting Information, Figure S8). In order to directly assess aggregation of **P1** [**PF6**] at high concentration, we labeled the end group with rhodamine and observed the material under a confocal microscope. Synthesis procedures are detailed in the Supporting Information (Scheme S5). The MBC value of the tagged polymer was similar to the untagged **P1** [**PF6**], suggesting that the effect of the dye labeling did not substantially alter activity (Figure S29). **P1** [**PF6**] with rhodamine attached as the end group (**P1** [**PF6**]-**RhB**) was diluted in PBS to 2 and 20 μg/mL. **P1** [**PF6**]-**RhB** showed weak and uniform fluorescence intensity in PBS at 2 μg/mL (Figure 3c), while it showed micron-sized aggregates at 20 μg/mL (Figure 3d). Thus, nonspecific and gross scale aggregation/precipitation of polymers is the simplest likely explanation for their anomalous loss of antibacterial activity seen at higher concentration. As shown above, such loss of activity at higher concentration is not seen in the case of the same polymers but with chloride anions instead of the PF_6^- anions. According to previous work on counteranion effects, phosphonium and PF_6^- make a tighter ion pair while phosphonium and Cl^- are more loosely bound in aqueous solution.[37] Tighter ion pairing screens the electrostatic

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attraction to negatively charged components of the bacterial membrane and results in weaker antibacterial activity. In contrast, more loosely associated chloride counterions improve the efficiency of electrostatic interaction thus resulted in stronger antibacterial activity. Also, more tight ion pair makes the molecule less water-soluble, so polymers aggregated at high concentration and lost their activity in a nonmonotonic manner.

In conclusion, novel cationic polynorbornenes with bulky phosphonium showed very potent biocidal activity. Here, a phosphonium attached to four amino groups was used as the cationic center in antimicrobial polymers for the first time. This structure provided a new strategy to improve antibacterial activity, with significant killing observed even at concentration below 1 μg/mL. Although they showed high hemolytic toxicity and are thus biocidal agents not suited for medical applications, these materials could be applied as surface coatings to disinfect on contact in future studies. Considering the excellent chemical stability of the tetraaminophosphonium, it is possible that such self-disinfecting surfaces could display improved longevity. Further research into the mechanism of why the bulky cations display such potent antibacterial activity would be interesting. We speculate that spreading out the cationic charge over a larger volume might facilitate multivalent interactions with multiple lipid headgroups, leading to higher killing efficiency at low concentrations relative to smaller ammonium cations. In addition, the activity of these polymers showed little dependence on flexibility of backbones and the spacing between positive charge centers and hydrophobic backbones, but the antibacterial activity was severely impaired by replacing the counteranion with more tightly bounded anions. Such a distinct difference in antibacterial activity by anions could be an interesting entry point to develop polymers that can switch between antibacterial state to nontoxic state.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmacrolett.2c00726.

Materials, instruments, experimental procedures, and biological assay protocols (PDF)

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Author Contributions

CRediT: **Yang Lou** data curation (lead), investigation (lead), methodology (lead), writing-original draft (lead), writing-review & editing (lead); **Jamie C. Gaitor** investigation (equal), methodology (equal), resources (equal); **Megan Treichel** investigation (supporting), methodology (supporting), resources (supporting); **Kevin J.T. Noonan** conceptualization (equal), funding acquisition (equal), investigation (equal), resources (equal), supervision (equal); **Edmund F. Palermo** conceptualization (lead), data curation (supporting), funding acquisition (equal), methodology (supporting), resources (equal), supervision (equal), writing-original draft (supporting), writing-review & editing (supporting).

Notes

The authors declare no competing financial interest.

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