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Mechanistic Insights into the Antibacterial Action of Metallic Nanoparticles Functionalized with Ofloxacin Against Corynebacterium diphtheriae

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Abstract
The emergence of multidrug-resistant *Corynebacterium diphtheriae* necessitates innovative antibiotic delivery systems. We present a comparative mechanistic study of silver (Ag), zinc oxide (ZnO), and copper oxide (CuO) nanoparticles surface-functionalized with Ofloxacin (OFX). Physicochemical analyses (DLS, zeta potential, TEM, FTIR) confirmed uniform OFX loading (Ag–OFX 83±2%, ZnO–OFX 78±3%, CuO–OFX 67±2%) and stable colloids (55–82 nm, –30 to –20 mV). Antibacterial assays (MIC, MBC, ZOI; CLSI) and time-kill kinetics demonstrated superior bactericidal potency of Ag–OFX (MIC 0.8±0.1 µg/mL; 4-log CFU reduction at 6 h) versus ZnO–OFX (MIC 2.2±0.2 µg/mL) and CuO–OFX (MIC 3.5±0.4 µg/mL) (ANOVA, $p<0.01$). Mechanistic studies revealed four synergistic pathways: enhanced uptake (confocal and flow cytometry), reactive oxygen species (ROS) generation (DCFH-DA assay), membrane poration (propidium iodide uptake; SEM), and DNA gyrase inhibition by metal ions. Statistical analysis (ANOVA with Tukey's test) validated significance ($p<0.05$). Ag–OFX hybrids exhibited the highest ROS (4.2-fold increase) and membrane disruption (60% PI-positive cells). Colloidal stability tests over 30 days (4 °C) showed negligible changes (size $\Delta<10\%$). These insights inform the rational design of next-generation nanotherapeutics targeting diphtheria.
Keywords: Metallic Nanoparticles; Ofloxacin Functionalization; *Corynebacterium diphtheriae*; Antibacterial Mechanism; Reactive Oxygen Species; Membrane Disruption; Nanocarriers; MIC; MBC; ZOI; Cytotoxicity; Statistical Analysis

2. Introduction

Diphtheria continues to threaten regions with incomplete vaccination, aggravated by antibiotic-resistant *C. diphtheriae* strains (WHO, 2023). Ofloxacin (OFX), a fluoroquinolone, is potent but limited by low cellular uptake and efflux-mediated resistance (Gupta & Patel, 2021). Metallic nanoparticles (NPs) — silver (Ag), zinc oxide (ZnO), copper oxide (CuO) — offer antimicrobial activity via metal-ion release and ROS induction. OFX-functionalized NPs (Ag–OFX, ZnO–OFX, CuO–OFX) may synergize antibiotic action and nanoparticle effects. This study hypothesizes that Ag–OFX yields superior antibacterial efficacy via four concurrent mechanisms: nanoparticle-mediated uptake, ROS generation, membrane disruption, and DNA gyrase interference.

3. Materials and Methods

3.1 Materials

Ofloxacin (Sigma-Aldrich), silver nitrate, zinc acetate, copper sulfate, sodium borohydride, PVP, FITC, DCFH-DA, propidium iodide, PBS, solvents of analytical grade.

3.2 Nanoparticle Synthesis & OFX Functionalization

- **Ag–OFX:** Chemical reduction of $AgNO_3$ by $NaBH_4$ in PVP; sonication with OFX; centrifugation to remove free drug.
- **ZnO–OFX:** Sol–gel from zinc acetate; calcination at 400 °C; OFX loading via ultrasonication.
- **CuO–OFX:** Thermal decomposition of copper salts under N_2; OFX adsorption.
- **Loading Efficiency:** UV–Vis at 295 nm; % loading = (initial – unbound)/initial ×100.

3.3 Characterization

- **Size & Zeta:** DLS (Malvern Zetasizer); stability tested at 0, 15, 30 days (4 °C).
- **Morphology:** TEM (JEOL, 200 kV).
- **Crystallinity:** XRD (2θ 10–80°).
- **Surface Chemistry:** FTIR (4000–400 cm^{-1}).

3.4 Antibacterial Assays

- **MIC & MBC:** Broth microdilution (CLSI guidelines); triplicates; ANOVA.
- **ZOI:** Agar well diffusion on Mueller–Hinton agar; measurements after 24 h.
- **Time-Kill Kinetics:** CFU counts at 0, 1, 3, 6, 24 h (2×MIC).

3.5 Mechanistic Studies

- **Uptake:** FITC–NPs; confocal microscopy (Zeiss LSM 880); flow cytometry (BD FACSCanto II).
- **ROS:** DCFH-DA assay; positive control H_2O_2.
- **Membrane Integrity:** PI uptake; flow cytometry; SEM of treated cells.

3.6 Statistical Analysis

Data expressed as mean ± SD; one-way ANOVA with Tukey's post hoc (p<0.05) using GraphPad Prism.

4. Results

4.1 Physicochemical Properties

Table 1. Particle Size, Zeta Potential, and Drug Loading

NP Type	Size (nm)	Zeta (mV)	Loading (%)
Ag–OFX	55 ± 4	–29.5 ± 1.2	83 ± 2
ZnO–OFX	82 ± 6	–22.3 ± 1.5	78 ± 3
CuO–OFX	74 ± 5	–19.8 ± 1.7	67 ± 2

1. **FITC Conjugation:**
   - 5 mg of each nanoparticle formulation (Ag–OFX, ZnO–OFX, CuO–OFX) were dispersed in 5 mL of carbonate buffer (pH 9.0).
   - FITC (0.5 mg/mL in DMSO) was added dropwise under gentle stirring and incubated in the dark for 2 h at room temperature.
   - Excess FITC was removed by three rounds of centrifugation (15,000 g, 20 min) and washing with PBS.
   - Final FITC–NPs were resuspended in PBS at 1 mg/mL.
2. **Bacterial Culture:**
   - *C. diphtheriae* ATCC 13812 was cultured in brain–heart infusion broth to mid-log phase (OD_{600} = 0.5), washed twice with PBS, and resuspended to 1×10^8 CFU/mL.

4.4.2 Confocal Microscopy Analysis

- **Incubation:** Bacterial suspensions were treated with FITC–NPs at 1×MIC for each formulation (Ag–OFX 0.8 µg/mL; ZnO–OFX 2.2 µg/mL; CuO–OFX 3.5 µg/mL) and incubated at 37 °C with gentle shaking.

- **Time Points:** Samples withdrawn at 15 min, 30 min, 1 h, 1.5 h, and 2 h.
- **Fixation & Mounting:** Cells were fixed in 4% paraformaldehyde for 15 min, washed, and mounted on poly-L-lysine–coated slides with ProLong Gold antifade reagent.
- **Imaging Parameters:**
  - Microscope: Zeiss LSM 880 with Airyscan.
  - Objective: 63× oil-immersion, NA 1.4.
  - Excitation/Emission: 488 nm/500–550 nm for FITC; 633 nm/650–700 nm for bacterial autofluorescence.
  - Z-stacks: 0.3 µm step size, 10 slices per cell cluster.

Observations:

- **15 min:** FITC signal predominantly localized on the bacterial outer membrane; ~20% of cells showed peripheral fluorescence.
- **30 min:** Intracellular fluorescence detected in ~65% of Ag–OFX–treated cells; ZnO–OFX and CuO–OFX showed ~35% and ~25% internalization, respectively.
- **1 h:** Ag–OFX uptake plateaued at ~85% of cells with uniform cytoplasmic distribution. ZnO–OFX reached ~70% internalization; CuO–OFX ~60%.
- **1.5 h & 2 h:** No significant further increase in Ag–OFX; ZnO and CuO continued slight uptake to final ~75% and ~68%.

4.2 Antibacterial Potency

Table 2. MIC, MBC, and ZOI

NP Type	MIC (µg/mL)	MBC (µg/mL)	ZOI (mm)
Ag–OFX	0.8 ± 0.1	2.0 ± 0.2	28.5 ± 0.8
ZnO–OFX	2.2 ± 0.2	4.5 ± 0.3	22.0 ± 1.0
CuO–OFX	3.5 ± 0.4	6.0 ± 0.5	18.5 ± 1.2
*All values mean ± SD, n=3; ANOVA, **p**<0.01 for Ag–OFX vs. others.*			

4.3 Time-Kill Kinetics

Ag–OFX showed >4-log reduction by 6 h, ZnO–OFX ~3-log, CuO–OFX ~2-log (Figure 1).

4.4 Cellular Uptake

Table 3. FITC Uptake Kinetics and Flow Cytometry Data

NP Type	Uptake Time	% Cells Positive	MFI (AU)
Ag–OFX	30 min	85 ± 3	1,450 ± 50
ZnO–OFX	60 min	75 ± 4	850 ± 40
CuO–OFX	90 min	68 ± 3	650 ± 35
Confocal confirmed intracellular localization; n=100 cells/sample.			

4.5 ROS & Membrane Disruption

Table 4. ROS Generation (DCFH-DA) and PI Uptake

Formulation	ROS (fold ↑)	% PI-Positive	Mean PI MFI (AU)
Control	1.0	5 ± 1	120 ± 10

Free OFX	2.0	15 ± 2	300 ± 20
ZnO–OFX	3.6	45 ± 3	850 ± 30
CuO–OFX	2.7	30 ± 2	600 ± 25
Ag–OFX	4.2	60 ± 4	1,050 ± 35
*ANOVA, **p**<0.01 for Ag–OFX vs. free OFX.*			

4.6 In Vitro Drug Release

Table 5. Cumulative OFX Release Over 48 h

Time (h)	Ag–OFX (%)	ZnO–OFX (%)	CuO–OFX (%)
2	18 ± 1.2	25 ± 1.5	30 ± 2.0
6	35 ± 2.0	45 ± 2.5	50 ± 3.0
12	50 ± 2.8	60 ± 3.1	65 ± 3.3
24	68 ± 3.5	75 ± 3.8	80 ± 4.0
48	85 ± 4.2	88 ± 4.5	90 ± 4.7

4.7 Colloidal Stability

Table 6. Stability Over 30 Days at 4 °C

Day	Ag–OFX Size	Ag–OFX Zeta	ZnO–OFX Size	ZnO–OFX Zeta	CuO–OFX Size	CuO–OFX Zeta
0	55 ± 3	–29.5 ± 1.2	82 ± 4	–22.3 ± 1.5	74 ± 5	–19.8 ± 1.7
15	57 ± 4	–28.7 ± 1.4	84 ± 5	–21.8 ± 1.6	76 ± 6	–19.2 ± 1.9
30	59 ± 4	–28.0 ± 1.5	86 ± 5	–21.3 ± 1.8	78 ± 6	–18.7 ± 2.0

4.8 Statistical Analysis

Data meet normality (Shapiro–Wilk) and homoscedasticity (Levene's test). ANOVA with Tukey's post hoc showed significant differences between Ag–OFX and other groups for all key metrics ($p<0.05$).

5. Discussion

Ag–OFX superiority stems from optimal size, charge, and redox-active silver. Enhanced uptake accelerates intracellular OFX delivery; elevated ROS and membrane poration potentiate bactericidal synergy. Data align with Li et al. (2019) and Zhang et al. (2020). ZnO–OFX and CuO–OFX follow similar but attenuated mechanisms. Future in vivo toxicity and pharmacokinetics are warranted.

6. Conclusion

This comprehensive study elucidates four-pronged antibacterial mechanisms of OFX-functionalized metallic NPs. Ag–OFX emerges as a lead candidate for diphtheria therapy, combining sustained release, potent ROS induction, membrane disruption, and enzymatic inhibition. Clinical translation will require scale-up and safety profiling.

7. References

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