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In Situ Synthesis of Catecholamines-Inorganic Nanoflowers and Enhanced Antimicrobial Activity

Abstract

Hybrid nanoflowers synthesized from proteins, enzymes, amino acids and various plant extracts have attracted considerable interest and are used for a variety of purposes. Notably, they exhibit increased stability, as well as enhanced catalytic, antimicrobial and antioxidant activity compared to free molecules. Most synthesis techniques are expected to occur in a solution medium. In this study, we synthesized catecholamine hybrid nanoflowers in situ as a result of a one-day incubation. The in situ catecholamine nanoflower that we synthesized on the surface of a glass exhibits high antimicrobial activity. Thanks to this feature, they have the potential to be used as antimicrobial surface coatings. For surface-bound synthesis, we selected a glass surface as the model surface and catecholamines (dopamine, epinephrine and norepinephrine) as the model organic components. We characterized the in situ catecholamine nanoflower using Scanning Electron Microscopy and contact angle analysis. Additionally, we tested the antimicrobial activities of the in situ catecholamine nanoflower coating against the bacteria *Staphylococcus aureus* and *Escherichia coli* and the fungus *Candida albicans*. The results revealed that the antimicrobial activity of the in situ catecholamine nanoflower coatings had increased significantly. In view of the findings, it is expected that they will have a successful impact as part of antimicrobial surface coatings.

Keywords: Antibacterial coating, catecholamines, nanoflower.

Katekolamin-İnorganik Nano Çiçeklerin Yerinde Sentezi ve Gelişmiş Antimikrobiyal Aktiviteleri

Öz

Proteinler, enzimler, amino asitler ve çeşitli bitki özlerinden sentezlenen hibrit nano çiçekler büyük ilgi görmüş ve çeşitli amaçlarla kullanılmaktadır. Özellikle, serbest moleküllerle karşılaştırıldığında daha yüksek stabiliteye sahip olmanın yanı sıra, katalitik, antimikrobiyal ve antioksidan aktiviteleri de daha yüksektir. Sentez tekniklerinin çoğu bir çözelti ortamında gerçekleştirilir. Bu çalışmada ise, bir günlük inkübasyon sonucunda katekolamin hibrit nano çiçekleri in situ olarak sentezledik. Cam yüzeyinde sentezlediğimiz in situ katekolamin nano çiçekleri yüksek antimikrobiyal aktivite sergilemektedir. Bu özelliği sayesinde, yüzey kaplamaları olarak kullanılma potansiyeline sahiptir. Yüzeye bağlı sentez için, model yüzey olarak cam ve model organik bileşenler olarak katekolaminler (dopamin, epinefrin ve norepinefrin) seçilmiştir. İn situ katekolamin nano çiçekleri taramalı elektron mikroskobu ve temas açısı ölçümü kullanarak karakterize ettik. Ayrıca, in situ katekolamin nano çiçekleri kaplamalarının *Staphylococcus aureus*, *Escherichia coli* ve *Candida albicans*'a karşı antimikrobiyal aktivitesini test ettik. Sonuçlar, in situ katekolamin nano çiçekleri kaplamalarının antibakteriyel aktivitesinin önemli ölçüde arttığını ortaya koydu. Bulgular ışığında, antimikrobiyal yüzey kaplamalarının bir parçası olarak başarılı bir etki yaratmaları beklenmektedir.

Anahtar kelimeler: Antibakteriyel kaplama, katekolaminler, nanoçiçek.

Introduction

Nowadays, various medical devices are implanted in to the body for diagnostic and therapeutic purposes. Unfortunately, serious bacterial infections can occur in the are as where these devices are implanted.[1,2] To reduce the risk of infection, various surface coating techniques have been developed to inhibit bacteria and reduce contamination on medical device surfaces.[3,4] These antibacterial coatings have been shown to be successful in combatting common infection-causing bacteria, such as *Staphylococcus aureus* (*S. aureus*) and, *Escherichia coli* (*E. coli*) and no significant resistance to these surface modifications has been reported.[5] To date, various contact-active antibacterial surface coatings have been developed. But the process of pre-activating commonly preferred metals and ceramics requires numerous lengthy chemical steps.

Recently, polydopamine (PDA) coatings have been created through the auto-oxidation of dopamine, contributing to the antibacterial properties of many materials. The biocompatibility of dopamine-based coatings is another reason for their popularity.[6–9] Inspired by these coatings, this study proposes a new surface coating material. Our team has previously synthesized catecholamine-inorganic nanoflowers (cNF)[10] and demonstrated their antimicrobial activity. R. Zare and colleagues discovered the synthesis of hybrid organic-inorganic nanoflowers and increase in their catalytic activity and stability.[10] In this technique, hybrid nanoflowers were synthesised using protein-based structures as the organic component and copper ions as the inorganic component. It has been demonstrated that the existing protein becomes more stable after incubation in a phosphate buffer for 72 hours. It has also been claimed that this is an effective method of immobilizing enzymes containing protein groups. In studies referencing this work, the organic component has primarily been a protein, an enzyme, an amino acid, or a plant extract.

In our previous study, we were the first to use catecholamines as the organic component in synthesizing cNF. In this study, we demonstrated that cNF structures synthesized on the surface can be used as effective coating materials, considering the effectiveness of dopamine-based coating materials. We synthesized these structures on glass surfaces using dopamine, epinephrine, and norepinephrine molecules as the organic components and copper ions as the inorganic component. We characterized in situ dopamine-inorganic nanoflowers (dNF-Is), epinephrine-inorganic nanoflowers (epNF-Is), and norepinephrine-inorganic nanoflowers (neNF-Is) with Scanning Electron Microscopy (SEM) and contact angle measurement. Additionally, we tested their antimicrobial activities against *S. aureus*, *E. coli* and *Candida albicans* (*C. albicans*).

Materials and Methods

Study Design and Study Period

This study was designed as an experimental study and was conducted between May 2024 and May 2025.

Materials

Dopamine, epinephrine, norepinephrine, Copper (II) sulfatepentahydrate, ($CuSO_4 \cdot 5H_2O$), contents of PBS (NaCl, KCl, Na_2HPO_4, KH_2PO_4, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$) were purchased from Sigma-Aldrich. *E. coli* ATCC 35218, *S. aureus* ATCC 25923 and *C. albicans* ATCC 10231 were obtained from Erciyes University Pharmaceutical Microbiology Laboratory collection. Mueller Hinton Broth (MHB), Triptic soy agar, agar were obtained from Thermo Scientific Oxoid, RPMI 1640 medium was purchased from Gibco.

Synthesis of In Situ Catecholamine-Inorganic Nanoflowers

For the synthesis of cNF-Is, stock solutions of organic components (dopamine, epinephrine, and norepinephrine) were prepared at a concentration of 1 mg/mL in deionized water. A 120 mM stock $CuSO_4$ solution was prepared as the inorganic component. A 10 mM Phosphate Buffer Solution (PBS) was prepared and adjusted to pH 7.4. Catecholamines at 0.02 mg/mLand Cu^{2+} at 0.8 mM were added to the PBS buffer. A homogeneous mixture is critical for the formation of uniform structures. Therefore, it was thoroughly vortexed. The vortexed solution was added to the glass surface in a glass beaker and left to incubate for one day. At the end of incubation, the glass surface was removed from the solution medium and washed with distilled water.[10,11]

Characterization of In Situ Catecholamine-Inorganic Nanoflowers

The SEM system can be used to examine materials in three dimensions using high-energy electrons. SEM imaging is crucial for proving the formation of the targeted flower-like morphology. The cNF-Is, which

grow by adhering to the surface, were scraped off the surface and dripped on to a copper grid, then dried. The structures adhering to the copper grid were imaged using SEM.[10-12]
As a separate analysis, contact angle measurement was performed to prove the wettability property of cNF-Is coatings. The tendency of water drop lets to pass through the gaps between nano structures on the cNF-Is-coated surface is also directly related to bacterial adhesion. Contact angle measurements were performed at 20°C, with the left, right, and average contact angles of water drop lets dropped on to the surface measured and photographed every 0.5 seconds for 15 seconds.[9,13]

Antimicrobial Activity of In Situ Catecholamine-Inorganic Nanoflowers

neNF-Is was selected as a model nanoflower for antimicrobial experiments due to its significantly lower contact angle compared to other cNF-Is. Gram-negative *E. coli* ATCC 35218, Gram-positive *S. aureus* ATCC 25923, and the fungus *C. albicans* ATCC 10231 were used to demonstrate antimicrobial activity. The liquid microdilution method protocol out lined in the Clinical and Laboratory Standards Institute (CLSI) guidelines was followed.[14-16]

Bacterial and fungal cells were cultured on Mueller Hinton agar and RPMI agar respectively, and adjusted to 0.5 McFarland in saline solution. 0.1 mL of MHB medium was added to the 96-well plates for bacterial cells, and 0.1 mL of RPMI 1640 medium was added for fungal cells. neNF-Is was sonicated for 30 minutes to separate them from the surface. Then, 0.01 mL of the microorganism suspensions were added to wells. In the final stage bacterial and fungal cell solutions were separately mixed with $CuSO_4$, free norepinephrine, and neNF-Is, and each sample was incubated in an incubator (37 °C) for one day for bacterial and two or three days for fungal cells. The plate was evaluated spectrophotometrically using an Azure Ao (Biosystem, France) at wavelengths of 590 nm for bacteria and 600 nm for fungi. The experiments were repeated three times, and the percentage inhibition values were calculated.[17] The percentage inhibition values were calculated using the formula in Equation 1.[18]

Inhibition (%) =[$(OD_{positivecontrol}-OD_{sample})$ / $(OD_{positivecontrol}-OD_{negativecontrol})$] x 100 Eq 1

Statistical Analysis

The results were analyzed using GraphPad Prism software, version 8.0.1 (GraphPad Software, San Diego, CA, USA), and commercial license. The experimental results were obtained from at least three replicates. A priori power analysis was not conducted; sample size (n = 3) was determined based on conventions in similar published studies.[10,11,17] A one-way ANOVA test (Tukey's multiple comparison test) was performed to analyse the differences between the groups. A *p*-value of less than 0.01 was considered significant.

Results

The first stage of synthesizing nanoflowers is nucleation, where primary crystals form between the amine groups in catecholamines and the Cu^{2+} ions that make up the inorganic part. In the second stage, known as the growth stage, catecholamine-$Cu_3(PO_4)_2$ leaves form, resembling leaves growing from a nucleus. In the final maturation stage, these leaves come together to form a complex, flower-like structure. As this anisotropic structure grows, all possible complexations in the reaction environment are completed. During the washing stage, structures that did not participate in the reaction are removed.

We synthesised cNF-Is on a glass surface using dopamine, epinephrine, norepinephrine (all at 0.02 mg/ml) and Cu^{2+} (0.8 mM). Figure 1 shows SEM images of the cNF-Is synthesised on the surface. Figures 1A and 1B belong to the dNF-Is, while Figures 1C and 1D belong to the epNF-Is. The nanoflower structures that grow by adhering to the glass surface resemble those that grow after 72 hours of incubation, despite only being incubated for 24 hours. It can be seen that many petal scome together to form a flower-like structure.

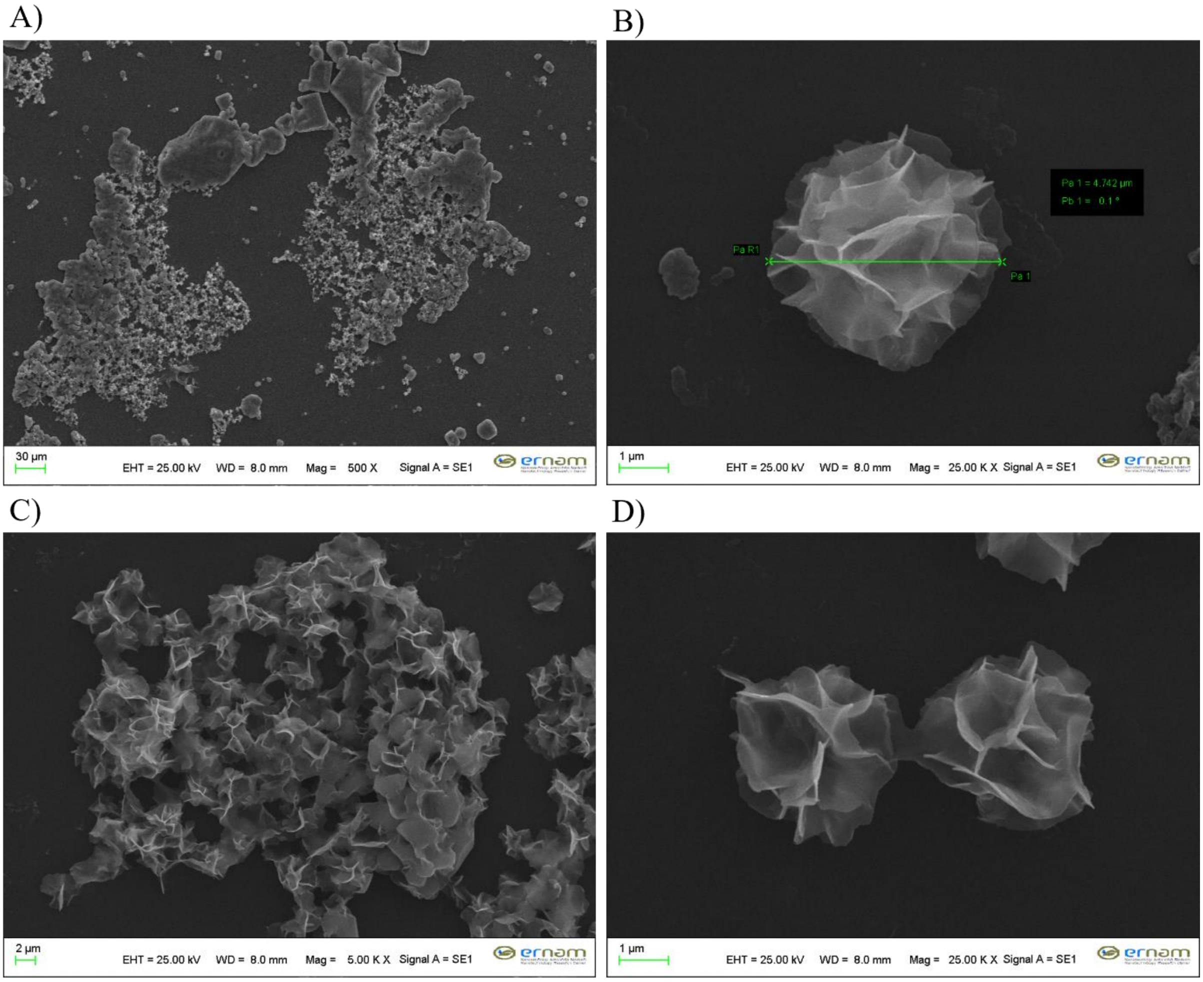

Figure 1. SEM images of cNF-Is. A)dNF-Is (Mag 500X), B) dNF-Is (Mag 25.00 KX), C) epNF-Is (Mag 5.00 KX), D) epNF-Is (Mag 25.00 KX).

Figures 2A and 2B shown eNF-Is structures exhibiting a flower-like morphology. Figures 2C and 2D show an SEM image of $Cu_3(PO_4)_2$ crystals. In this case, only the inorganic component was incubated in the solution medium, with no catecholamines added as the organic component. Figure 2D shows that separate petals have not formed, and the structure has not completed its development.

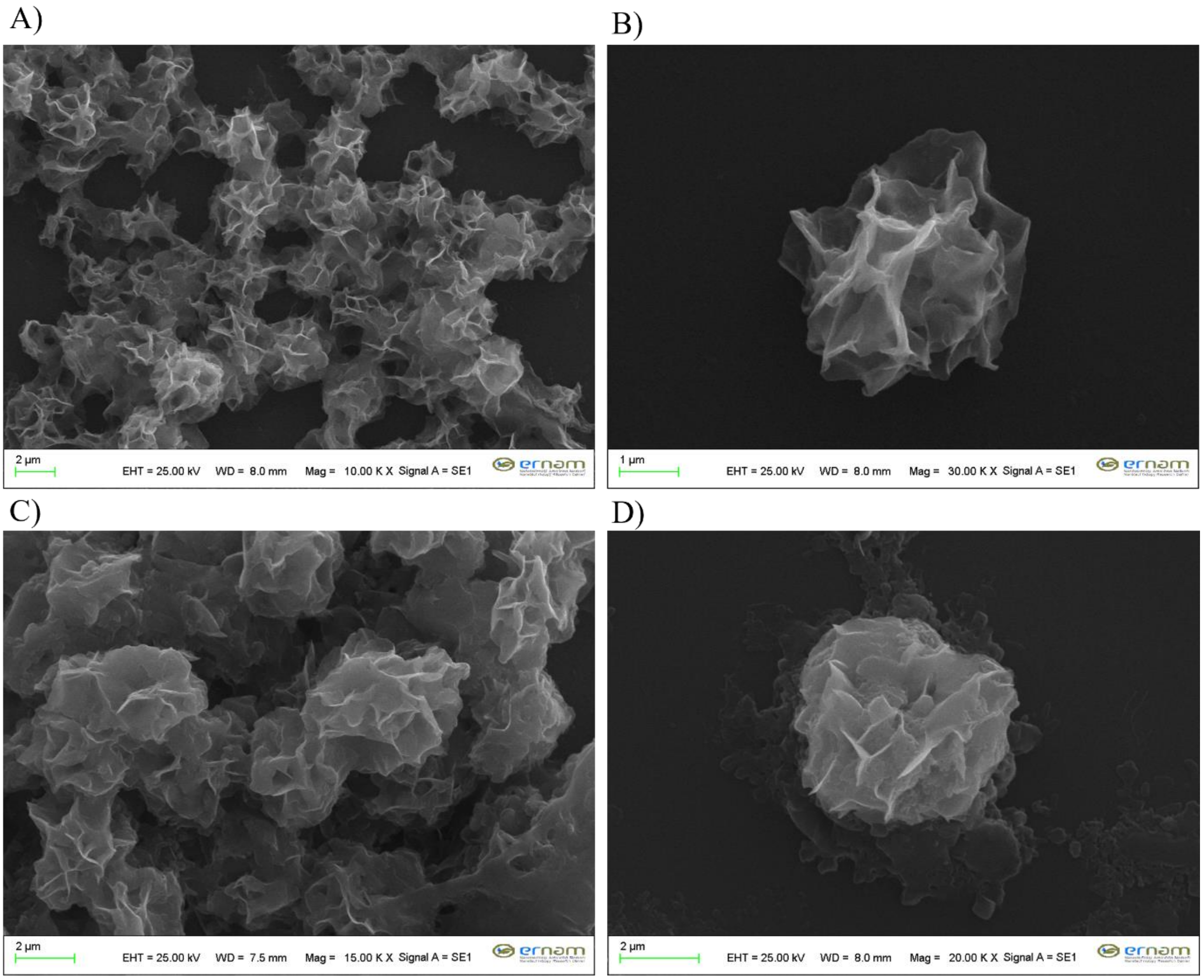

Figure 2. SEM images of cNF-Is and $Cu_3(PO_4)_2$. A) neNF-Is (Mag 10.00 KX), B) neNF-Is (Mag 30.00 KX), C) $Cu_3(PO_4)_2$ (Mag 15.00 KX), D) $Cu_3(PO_4)_2$ (Mag 20.00 KX).

Measuring surface wettability is essential in demonstrating the effectiveness of the cNF-Is coated surface modification process. Figure 3 (A1–A3) shows the contact angles of surfaces coated with dNF-Is. Figure 3A1 shows angles of 55.20°/56.44°, Figure 3A2 shows angles of 42.26°/45.09°, and Figure 3A3 shows angles of 50.14°/47.51°. Figures 3B1, 3B2 and 3B3 show contact angles of 26.23°/25.10°, 26.36°/25.17° and 25.15°/24.14°, respectively. Figure 3C1 shows angles of 22.43°/21.68°, Figure 3C2 shows angles of 26.90°/26.35°, and Figure 3C3 shows angles of 23.74°/23.49°. When the contact angles of dNF-Is, epNF-Is and neNF-Is were compared, the smallest angle was observed for neNF-Is. This indicates that the coating in Figure 3C has a more hydrophilic surface. Additionally, the low apparent contact angles indicate a tendency for water droplets to pass through the gaps between the nanoflowers.

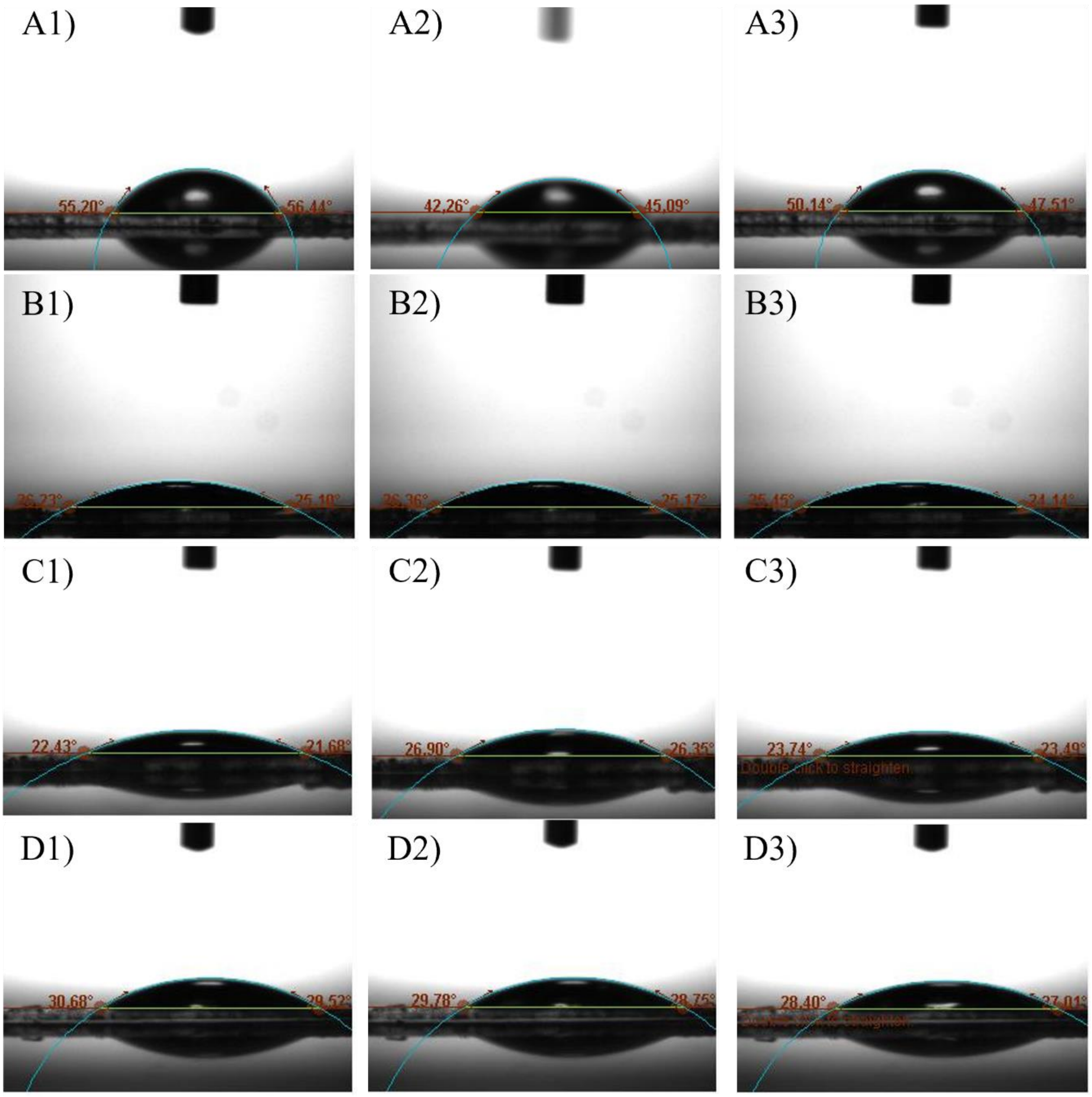

Figure 3. Typical static contact angles of surfaces coated withc NF-Is and $Cu_3(PO_4)_2$. A1-3) dNF-Is, B1-3) epNF-Is, C1-2) neNF-Is, D) $Cu_3(PO_4)_2$-Is.

The antimicrobial activity of cNF-Is synthesised on the surface was tested against model Gram-positive and Gram-negative bacteria, as well as a fungal pathogen. The antimicrobial activity of the neNF-Is structure with the smallest contact angle was investigated. A $CuSO_4$ solution and free norepinephrine were also included in the antimicrobial test in the presence of hydrogen peroxide for comparison purposes. As shown in Figure 4, the $CuSO_4$ solution exhibited 20%, 22% and 15% inhibition (dark blue bar in Figure 4) and free NE exhibited 35%, 38%, and 38% inhibition (light blue bar in Figure 4) against *E. coli*, *S. aureus*, and *C. albicans*, respectively. Finally, the neNF-Is exhibited 90%, 78% and 85% inhibition (green bar in Figure 4) against *E. coli*, *S. aureus* and *C. albicans*, respectively.

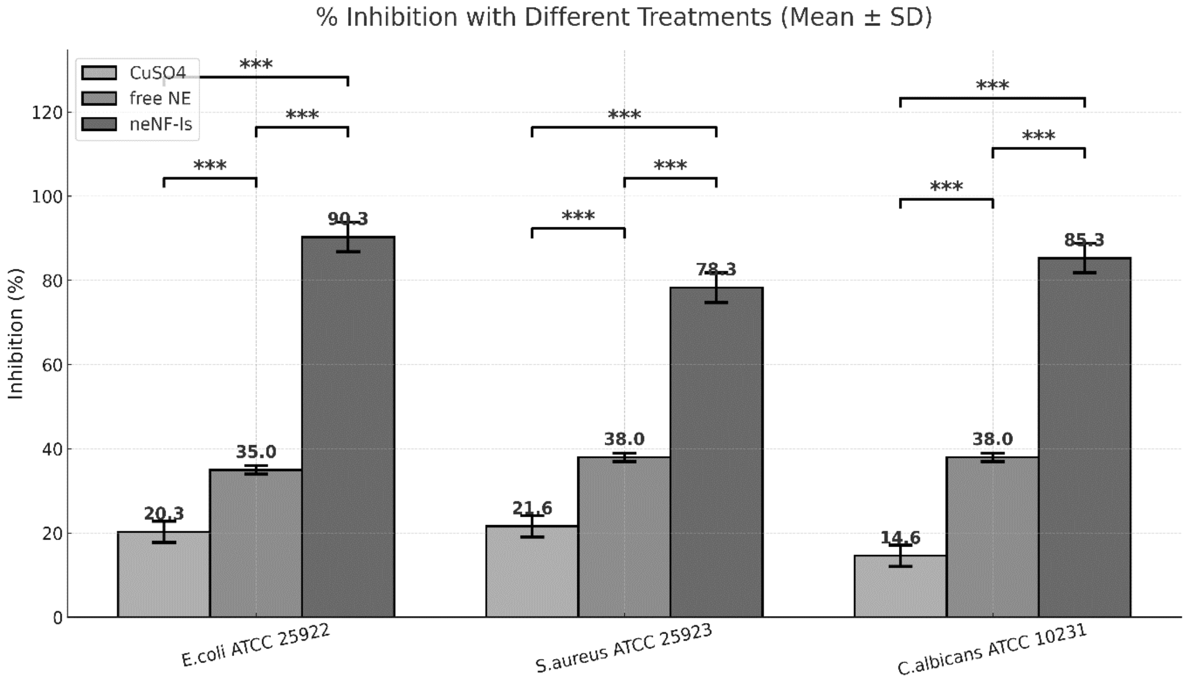

Figure 4. Antimicrobial activity of cNF-Is. SD: standard deviation.

Tukey's multiple comparison analyses demonstrated statistically significant differences in inhibition levels among the microorganisms tested (Table 1). Notably, the neNF-Is group exhibited markedly higher inhibitory activity compared with both the $CuSO_4$ and free NE treatments ($p \leq 0.001$). Further more, the difference between the $CuSO_4$ and free NE conditions was also statistically significant in all three microbial species ($p = 0.001$).

Table 1. Statistical analysis results

	Tukey's multiple comparison test	Mean difference	95% confidence interval	*p*-value
S. aureus	$CuSO_4$ vs free NE	16.33	8.06 – 24.61	0.001
	$CuSO_4$ vs neNF-Is	56.67	48.39 – 64.94	<0.001
	free NE vs neNF-Is	40.33	32.06 – 48.61	<0.001
E. coli	$CuSO_4$ vs free NE	14.67	6.39 – 22.94	0.001
	$CuSO_4$ vs neNF-Is	70.00	61.72 – 78.28	<0.001
	free NE vs neNF-Is	55.33	47.06 – 63.61	<0.001
C. albicans	$CuSO_4$ vs free NE	23.33	15.06 – 31.61	0.001
	$CuSO_4$ vs neNF-Is	70.67	62.39 – 78.94	<0.001
	free NE vs neNF-Is	47.33	39.06 – 55.61	<0.001

It is generally thought that the inhibition of pathogenic cells by coatings containing antibacterial materials results from direct damage to cell surfaces.[19,20] To prove this, bare glass surface and cNF-Is coated glass surface were treated with pathogens. The cell surface morphologies of *E. coli*, *S. aureus* and *C. albicans* were then compared. To achieve this, the glass surface were incubated with 3 McFarland pathogen cell suspensions for 5 hours. The cells were then fixed and the slides were dried at room temperature. Figure 5 shows SEM images of cells on bare and coated glass surfaces. Figures 5A1 and 5A2 show images of *E. coli* on bare glass surface and coated glass surfaces, respectively. Figures 5B1 and 5B2 show images of *S. aureus* on bare glass surface and coated glass surfaces, respectively, and Figures 5C1 and 5C2 show images of *C. albicans*.

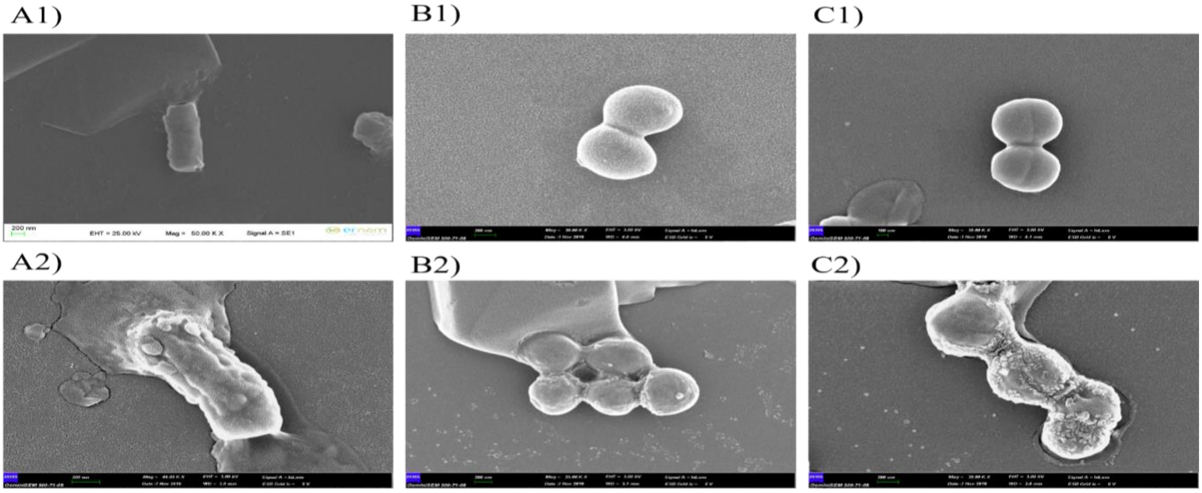

Figure 5. SEM images of the bacterial and fungal pathogens treated with bare (1) and coated (2) glass surface. A) *E. coli*, B) *S. aureus*, C) *C. albicans*.

Discussion

Rapid advances in nanotechnology in recent years have led to the development of various applications for eliminating pathogens that cause infectious diseases. Among these techniques, the production of antimicrobial nanomaterials for use as surface coatings is particularly promising. Many devices have been developed as part of medical innovations and are implanted into the body. Antimicrobial coating materials are critical in preventing potential infections. The continuous disinfection of surfaces using antimicrobial nanomaterials is a significant innovation that prevents microbial contamination without the need for various sterilization steps.[21] In this study, we investigated the antimicrobial efficacy of cNF-Is on surfaces as an alternative coating material. In a previous study, we synthesised catecholamine-inorganic nanoflowers for the first time and systematically investigated their formation mechanism. By examining the reaction parameters, we identified the optimal conditions for producing the desired morphology. Additionally, we demonstrated that catecholamine-inorganic nanoflowers exhibit antimicrobial activity. Su et al. proved that polydopamine synthesised by stirring is an effective surface coating material with strong antimicrobial properties.[9] Inspired by this study, we investigated the potential of cNF-Is as biocompatible coating materials. Figures 1 and 2 show SEM images of dNF-Is, epNF-Is, and neNF-Is. The standard synthesis steps for nanoflowers formed from protein, enzyme and plant extracts include a three days incubation period.[22,23] The nanoflower structures in Figures 1 and 2 adhered to the surface after one day incubation period and quickly attained a flower-like morphology composed of petals.

Figure 3 shows the contact angle measurement results for cNF-Is. Contact angle is a measure of the wetting properties of a liquid on a solid surface. It is also defined as the angle formed where the liquid, gas and solid components meet at the interface. The most common way to measure contact angle is using an optical tensiometer, also called a drop shape analyzer. In this technique, a drop of water is placed on the surface of a solid sample and the contact angle between the drop and the surface is measured without moving the drop.[24] In the analyses conducted, neNF-Is exhibited the lowest contact angle, indicating that surfaces coated with this material interact more with water and bacteria. Coating with titanium oxide nanoflowers has also been shown to be effective in bacterial adhesion due to its high hydrophilic properties.[25]

Figure 4 shows the antimicrobial activity of cNF-Is. For this purpose, neNF-Is, which have the lowest contact angle, free norepinephrine (NE) and $CUSO_4$, were evaluated. neNF-Is showed high antimicrobial activity against both bacterial and fungal pathogens, compared to free NE and $CUSO_4$. Previous studies have demonstrated that the antimicrobial activity of nanoflower structures synthesised from materials such as free proteins, enzymes, and plant-based standard substances increases.[11,26,27]

After we demonstrated the antimicrobial activity of neNF-Is in Figure 4, we obtained SEM images in Figure 5 as a result of treating pathogens with neNF-Is. Cell morphology of pathogens was preserved without damage on the uncoated glass surface, while damage was caused to pathogens on the glass surface coated with neNF-Is. They adhered to the coating material and were damaged. Damage to cell membranes was also observed. These results indicate that the coated surface damages the cell surface of pathogens. The findings are similar to those of other recent studies. The inhibition mechanism of surfaces coated with polydopamine and various antibacterial materials stems from the damage caused to the cell surface.[28,29]

Conclusion

This study has demonstrated the feasibility of synthesizing organic-inorganic nanoflowers from catecholamines, such as dopamine, epinephrine and norepinephrine, on glass surfaces with in a one day incubation period. The ability to synthesize these structures on surfaces suggests their potential use as surface coatings. Contact angles were measured and the antimicrobial activity of neNF-Is with the lowest contact angle against the bacteria *S. aureus* and *E. coli* and the fungus *C. albicans* was investigated. This experiment revealed high antimicrobial efficacy. Additionally, SEM images showed damage to the cell membranes of bacteria and fungi treated with the neNF-Is coated surface, which supports the contact-dependent killing mechanism. Therefore, neNF-Is coatings are an excellent alternative for producing short-term, contact-dependent and biocompatible antimicrobial polymer coatings.

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Declarations:
1. Ethics Committee Approval: This study does not require ethical committee approval. Clinical samples were not used.
2. Informed Consent: Informed consent is not required for this study.
3. Author Contributions: Concept-CCY, IO; Design-CCY, IO; Supervision-CCY, IO; Resources-CCY, NI; Materials-CCY, NI, IO; Data Collection and/or Processing-CCY, NI, IO; Analysis and/or Interpretation-CCY, NI, SK, IO; Literature Search-CCY; Writing Manuscript-CCY, NI, SK, IO; Critical Review-CCY, NI, SK, IO.
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