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Synthesis of Medicinal Leech (Hirudo Verbana) Saliva-Incorporated Hybrid Nanoflowers and Evaluations of Their Biological Applications

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Abstract: In this study, medicinal leech (Hirudo verbana) saliva-incorporated nanoflower (LS-NF) was synthesized and evaluated its peroxidase mimic and antimicrobial activities at the first time. Herein, we reported the formation of medicinal leech saliva (LS)-incorporated organic-inorganic nanoflower (LS-NF) with peroxidase mimic and antimicrobial activities. The medicinal leech saliva was used as an organic component and copper ion (Cu2+) acted as an inorganic part in the synthesis of LS-NF. Leech saliva was obtained from medicinal leeches Hirudo verbana, contains various amino acids, and it can react with Cu2+ ions in phosphate buffer through its accessible amine groups to form flower-shaped structures. These LS-NFs showed quite uniform and monodispersed morphology. They were systematically characterized with SEM, FTIR, and XRD for evaluation of morphology, bond vibrations, and crystal structure, respectively. The LS-NF acted as a Fenton reagent in the presence of hydrogen peroxide (H_2O_2), then we investigated their peroxidase-like and antimicrobial activities through the Fenton reaction. It was concluded that LS-NFs can be used in various scientific and industrial applications, and also as an effective antimicrobial agent.

Keywords: Medicinal leech saliva, Hybrid nanoflower, Fenton reaction, Peroxidase-like activity, Antimicrobial activities

Tibbi Sülük Salyasi İçeren Çiçek Şekilli Hibrit Nanoyapilarin Sentezlenmesi Ve Biyolojik Uygulamalarda Değerlendirilmesi

Özet: Bu çalışmada tıbbi sülük (Hirudo verbana) salyası kullanılarak çiçek şekilli nano yapılar sentezlenmiş ve ilk defa peroksidaz ve antimikrobiyal aktiviteleri değerlendirilmiştir. Araştırmada, tıbbi sülük salyası (H. verbana) kullanarak organik ve inorganik çiçek şekilli nanoyapıların (LS-NF) peroksidaz ve antimikrobiyal etkilere sahip olduğu bildirilmiştir. Çiçek şekilli nano yapıların sentesinde sülük salyası organik bileşen olarak kullanılırken, bakır iyonları (Cu2+) ise inorganik bileşen olarak rol almıştır. Birçok amino asit içeren tıbbi sülük salyası fosfat tampon içindeki Cu2+ iyonlarının amin grupları üzerinden çiçek şekilli nano yapılar oluşturmaktadır. Bu çiçek şekilli nano yapılar oldukça düzgün ve homojen bir dağılım sergiledi. Sentezlenen bu nano yapılar morfolojik, bağ titreşimleri ve kristal yapıları bakımından SEM, FTIR ve XDR analizleri ile sistematik bir şekilde karakterize edildi. Çiçek şekilli nano yapılar (LS-NF) hidrojen peroksit (H_2O_2) varlığında bir Fenton reaktifi olarak davranmış olup, bunlar peroksidaz benzeri ve antibiyotik aktiviteler bakımından incelendi. Çalışmada, LS-NF'lerin etkili bir antimikrobiyal ajan olmalarının yanı sıra birçok bilimsel ve endüstriyel uygulamada kullanılabileceği sonucuna varılmıştır.

Anahtar Kelimeler: Tıbbi sülük salyası, Hibrit nanoçiçek, Fenton reaksiyonu, Peroksidaze benzeri aktivite, Antimikrobiyal aktiviteler

1. Introduction

The discovery of protein-inorganic hybrid nanoflower (NFs) formation with enhanced stability and catalytic activities was reported for the first time by Zare and co-workers (1). The enzyme incorporated-NFs have received great attention from researchers owing to their unique biological activities, and they have been in use for a variety of applications, including biosensor fabrication, catalysis, dye decolorization, and free-radical polymerization (2-11). Wang et al. reported how the allosteric effect and morphology influence enzymatic activities of α-amylase@$CaHPO_4$ NFs. In terms of mechanism, α-amylase was first activated by binding Ca2+ into the allosteric site in inactive α-amylase, then amylase@$CaHPO_4$ NFs showing great activities and stabilities were formed with self-assembly strategy in phosphate buffer (2). Yang et al. demonstrated how to use acetylcholinesterase-based NF as an electrochemical sensor for the detection of dichlorvos via square-wave voltammetry (11). In a further study, Dadi et al. prepared horseradish peroxidase NFs@carbon nanotube (HRP-NF@CNTs) as novel hybrid nanocatalysts with in situ and post-modification procedures. While both HRP-NF@CNT showed an increase in catalytic activities, the HRP-NF@CNT prepared by post-

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modification exhibited outstanding exhibited greatly enhanced peroxidase activity and stability owing to benefiting from both the HRP-NF and synergistic effect between CNT and Cu3(PO4)2 crystals (6).

In addition to that, the development of dual enzyme combined NFs opened up a new avenue for multiple functions and cascade-type reactions (12-14). For instance; while Gul et al. synthesized dual enzyme-based NFs consisting of HRP and laccase for effective and simultaneous degradation of a cationic dye and an anionic azo dye, Zhu et al. developed a combination of glucose oxidase (GOx) and HRP enzymes in the NFs for quantitative detection of glucose. Simply, glucose is catalyzed by GOx to produce hydrogen peroxide (H_2O_2), then HRP used the H_2O_2 to oxidize a colorless 3,3′,5,5′-Tetramethylbenzidin (TMB) into a blue product (13, 14).

Alternative to enzyme-based NFs, researchers have developed non-enzyme-based NFs and investigate their peroxidase mimic activities in the presence of H_2O_2through mechanism of Fenton reaction (15-22). The NFs can be formed from organic or biological molecules containing functional groups (amine, carboxyl or thiol and etc). For instance, We et al. used natural amino acids (AA) as organic components to form AA-based NFs with an intrinsic peroxidase-mimic activity (22). Aslan et al. reported ethylenediaminetetraacetic acid (EDTA) based NFs and utilized them as novel irrigation solutions for biofilms formed by *Enterococcus faecalis* and *Candida albicans* (19). To extend this concept, whole plant extracts were employed as organic parts for the synthesis of plant extract-based NFs (23-27). These plant extract-based NFs have been used for various scientific and industrial applications, including the fabrication of novel antimicrobial, catalytic, and dye-removal agents. For instance, Demirbas et al. synthesized NFs using umbilical decussate extract and investigated their peroxidase mimic, antimicrobial, and dye degradation properties against guaiacol, bacteria (*Aeromonas hydrophila, Aeromonas sobria, Escherichia coli, Salmonella enterica*, and *Staphylococcus aureus*) and malachite green, respectively (28).

Medicinal leeches, also known as "blood-sucking leeches" (such as *Hirudo verbana*), secrete saliva that occurs naturally in the salivary glands and has anticoagulant properties (29). It has also been reported in the literature that medicinal leeches secrete saliva containing approximately 60 different proteins(30). In this research, we synthesized leech saliva-incorporated nanoflower (LS-NF) and evaluated its peroxidase mimic and antimicrobial activities at the first time. The various characterization methods were used to elucidate the formation of the LS-NFs. All biological activities of the LS-NF were observed by the addition of H_2O_2, which relied on the Fenton reaction mechanism.

2. Materials and Methods

2.1. Synthesis of Leech Saliva-Incorporated Nanoflower

The medicinal leech saliva used in this study was supplied by a local company called Cansuyu Sülük (Kayseri, Türkiye), which produces Hirudo verbana species. The LS-NFs were synthesized by the modification of reported work(22). Briefly, a 120 mM CuSO4 solution was freshly prepared as a stock solution, then a certain volume (the final concentration of Cu2+ is 0.8 Mm) of stock CuSO4 solution was added into 10 mM phosphate buffered saline (PBS) at pH: 7.4. Leech saliva solution (the final concentration is 0.02 mg/mL) was added into above mixture, then resulting mixture was vigorously vortexed for 30 sn to provide homogeneous interaction between saliva molecules and Cu2+ ions. The final mixture was left undisturbed for 3 days of incubation. After incubation, the blue precipitate at the bottom reaction vial can be considered as an indication for the formation of the LS-NFs. The final product, LS-NFs, was washed with deionized water by centrifugation to remove unreacted of metal ions and saliva. The collected LS-NFs were dried in an oven at 60 C. The powder LS-NF was stored for further use.

2.2. Catalytic Activity of Leech Saliva- Incorporated Nanoflower

Briefly, LS-NFs (3 mg) were added to the solution containing H_2O_2(22.5 mM), guaiacol (45 mM), and PBS (pH 6.8) (23). The reaction volume was manipulated to adjust the final concentrations of H_2O_2, and guaiacol to 7.5 mM and 15 mM, respectively. The oxidation of guaiacol (2-methoxyphenol) to colored 3,3-dimethoxy-4,4-diphenoquinone was recorded by measuring absorbance values at 470 nm by using a UV-Vis spectrophotometer.

2.3. Antimicrobial Activity of Leech Saliva-Incorporated Nanoflower

In terms of standard microorganisms, Escherichia coli (E. coli) ATCC 35218 (Gram-negative) and Staphylococcus aureus (S. aureus) ATCC 25923 (Gram-positive) used as bacterial strains, and Candida albicans (C. albicans) ATCC 10231 used as a fungus (obtained from the Erciyes University Pharmaceutical Microbiology Laboratory collection). Antimicrobial activities of free leech saliva, LS-NF with H_2O_2, LS-NF, and H_2O_2 were evaluated based on Clinical Laboratory Standards Institute (CLSI) guidelines via broth microdilution method.

In the antibacterial activity procedure, the bacterial cells were cultured in Mueller Hinton broth and incubated at 37 °C for 14 hrs, and their concentration was arranged to 0.5 McFarland. Each antimicrobial agent was mixed with bacterial and fungus strains, and each mixture was incubated at 37 °C for 18–24 hrs for bacteria and 48–72 hrs for fungi. All experiments were carried out in triplicate.

3. Results and Discussions

3.1. Characterization of Leech Saliva-Incorporated Nanoflower

In a typical NF synthesis procedure, Cu2+ ions react with phosphate ions (PO43-) in PBS to form primary copper phosphate complexes (Cu3(PO43-)2). Enzymes or proteins preferentially bind with Cu2+ through their primary amine groups in Cu3(PO43-)2 complexes to create protein-Cu3(PO43-)2 nanocrystals as nuclei. During the incubation process, protein-Cu3(PO43-)2 nanocrystals formed large petals, and each petal was combined with each other to form a flower-shaped structure.

The leech saliva has many polypeptides containing packs of amino acids, so it has various available amine groups to form NFs. The LS-NFs synthesized (in PBS pH 7.4) at room temperature (RT: 25°C) have quite spherical and uniform morphology due to various available amine groups in leech saliva. The LS-NFs are well-monodispersed with diameters of ~3 µm size (Figure 1A).

The boundaries of petals in blooming structures of LS-NF were clearly observed in a magnified SEM image (Figure 1B).

The Cu2+ ions are considered as the cornerstone of the LS-NFs because they form primary nanocrystals Cu3(PO43-)2 to form LS-NFs. However, no flower-shaped structure was formed without Cu2+ ions. When the Cu2+ ions are removed from pre-synthesized LS-NFs, the LS-NFs collapse, and no more flower-shaped morphology is seen. The presence of Cu metal in LS-NF was monitored by Energy dispersive X-ray (EDX) as shown in Figure 1C.

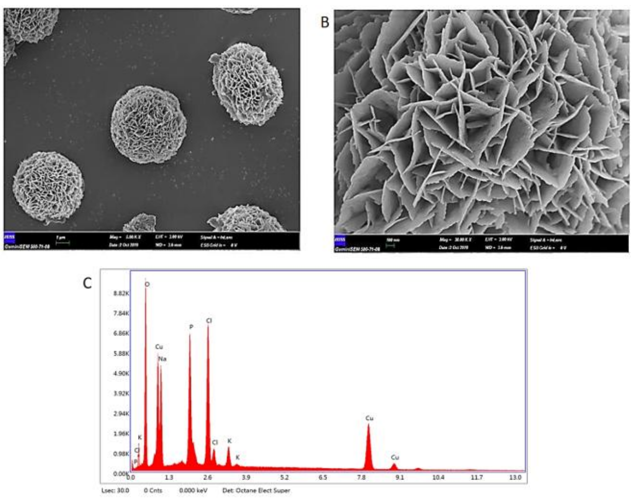

Figure 1. A) SEM image of LS-NFs with 5.00 KX magnification, B) Magnified SEM image of LS-NFs with 30.00 KX magnification. C) The Cu analysis of the LS-NF in the EDX spectrum.

The characteristic stretching and bending vibrations of leech saliva in LS-NFs were analyzed by FTIR analysis, as shown on the FTIR spectrum in Figure 2A.

The amine groups (-NH2) gave the bending and stretching vibrations were recorded at 1622 cm−1. Additionally, the peak that appeared at 1139 cm−1 was attributed to the stretching of the C-N bond in NH2. The stretching vibration of hydroxyl bond (-OH) at 3289 cm−1. The characteristic stretching vibration peaks of PO43− (P-O and P=O vibrations in Cu3(PO4)2 primary crystals were seen at 1097 cm−1 and 557 cm−1. All characteristic stretching and bending peaks can be indications of LS-NF formation.

In addition to that, the crystal structure of LS-NF was elucidated by XRD analysis, as given in Figure 2B.

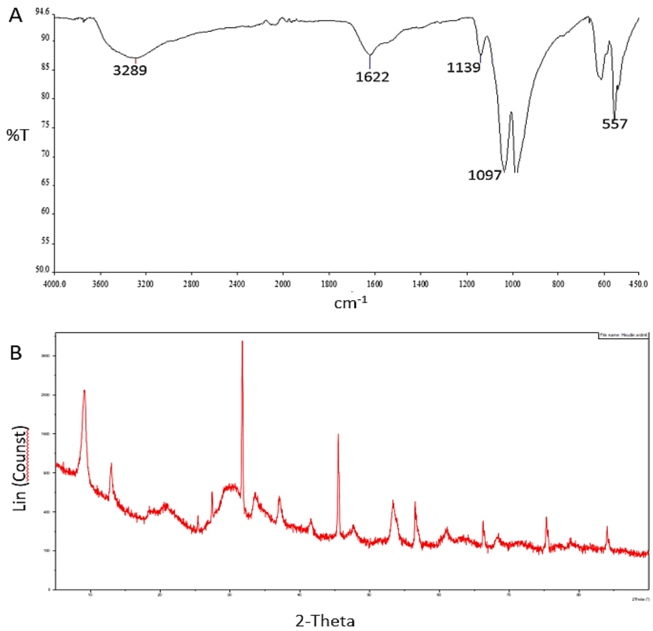

Figure 2. A) FTIR spectrum of LS-NF and B) XRD patterns of LS-NF.

Based on the XRD spectrum, almost all diffraction peaks of Cu3(PO4)2 in the LS-NFs were consistent with the standard of the JCPDS card (00-022-0548). The distinct and clear diffraction peaks in the LS-NFs were observed in XRD spectrum. The diffraction peaks positions at around ~8.5o, ~13o, ~21o, ~27o, ~32o, ~33o, ~45o, ~48.0o, ~53o, ~57o and ~68o in the 2-theta plane. This XRD pattern of LS-NF revealed that its crystal structure is overlapped with the crystal pattern of Cu3(PO4)2·3H2O (JCPDS card (00-022-0548).

3.2. Catalytic Activity of Leech Saliva-Incorporated Nanoflower

Peroxidase-like activities of LS-NF were tested by oxidation of guaiacol. The LS-NF exhibited catalytic activity via a Fenton-like reaction mechanism in the presence of H_2O_2. The Fenton reaction relies on the formation of Cu2+ and Cu1+ ions in the LS-NF shown in equation 1 (Eq. 1). Briefly, Cu2+

ions in Cu3(PO4)2 of the-NF were reduced to Cu1+ by H_2O_2, then Cu1+ reacted with $H_2O_2$to re-create Cu2+ ions and to form reactive hydroxyl radical (ROS). This continuous cascade reaction produces much ROS, which causes oxidation of guaiacol used as a standard substrate.

$$Cu^{2+} + H_2O_2 \text{ ----> } Cu^{1+} + HOO^{\cdot} + H^{+}$$
$$Cu^{1+} + H_2O_2 \text{ ----> } Cu^{2+} + {}^{\cdot}OH + OH^{-} \quad \text{(Eq. 1)}$$

The peroxidase-mimic activity of the LS-NF used as a Fenton reagent (20µg/ml) was examined towards guaiacol. The LS-NF rapidly and efficiently oxidized guaiacol to convert into an oxidized product called " 3,3-dimethoxy-4,4-diphenoquinone". The formation of the product was monitored by measurement of its absorbance peak at 470 nm via Uv-Vis spectrophotometer (Figure 3).

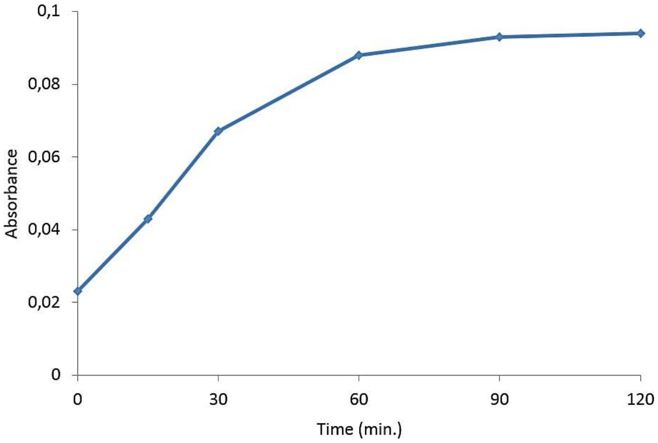

Figure 3. Peroxidase-mimic activity of LS-NF via Fenton reaction.

3.3. Antimicrobial Activity of Leech Saliva-Incorporated Nanoflower

We tested antimicrobial activities of LS-NF with and without H_2O_2, and antimicrobial activities of the free leech saliva and H_2O_2 were used for comparison towards E. coli, S. aureus, and C. albicans (Figure 4).

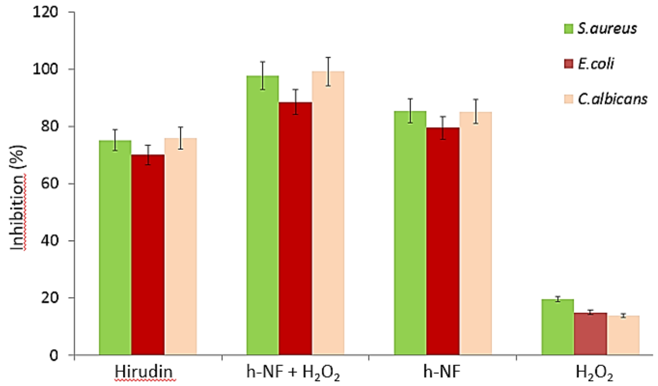

Figure 4 Antimicrobial activities of free leech saliva, LS-NF with H_2O_2, LS-NF, and H_2O_2.

The leech saliva in free from displayed kind of effective antimicrobial activities with ~70% inhibition for all three microorganisms. While almost ~85%, ~97%, and ~99% of E. coli, S. aureus, and C. albicans were respectively killed by the LS-NF in the presence of H_2O_2, only LS-NF without H_2O_2 caused ~78%, ~82%, and ~82% inactivation for E. coli, S. aureus and C. albicans, respectively. In addition to that, the H_2O_2 solution killed around 10% of each bacterial and fungal cell.

We revealed that free leech saliva showed over mild-level antimicrobial activities; however, saliva in NF form exhibited highly effective antimicrobial activities, especially in the presence of H_2O_2, which can be attributed to morphology and ROS production capability of the LS-NFs. The ROS produced by the LS-NF oxidizes the membrane of each cell, and cell membrane rigidity is decomposed, then eventual cell death is observed.

In conclusion, we have examined formation of medicinal leech (Hirudo verbana) saliva-incorporated organic-inorganic nanoflower (LS-NF) with peroxidase mimic and antimicrobial activities through the Fenton reaction. We showed that amino acid rich- leech saliva produced quiet uniform, compact and monodisperse NFs at the first time. The LS-NFs acted as Fenton reagent in the presence of H_2O_2 and generate ROS radicals which are in charge of intrinsic peroxidase-like and antimicrobial activities. We concluded that LS-NFs can be implemented in various scientific and industrial applications, and also as an effective antimicrobial agent.

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Declarations

Conflict of interest

The authors declare no competing interests.

Ethical approval

Not applicable

Consent to participate

Non applicable

Consent to publish

All authors consent to the publication of this manuscript

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