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Biomedical Application Study of Nano-Hydroxyapatite: Anti-Biofilm, Antioxidant, and Anti-Hemolytic Performance

Ali Abbas Jawad *, Suzan Saadi Hussain, Osama A. Dakhil

Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq

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ABSTRACT

Hydroxyapatite is the perfect biomaterial for Biomedical Application due to its hemocompatibility and biocompatibility, which have been seriously studied by several researchers. The present research focuses on the anti-biofilm, anti-oxidant, and anti-hemolytic properties of hydroxyapatite (HAp) nanoparticles produced using Senna italica in an environmentally friendly manner. This comes after it proved effective as an antibacterial agent against S.aureus, P.aeruginosa and E.cloacae bacteria isolated from postoperative endophthalmitis in a previous study. HAp-NPs exhibits anti-biofilm activity, resulting in the conversion of S.aureus, P.aeruginosa and E.cloacae from strong biofilm-forming bacteria to weak biofilms in vitro. The analysis of antioxidant properties revealed that the HAp-NPs demonstrated significantly higher radical scavenging activity (up to 89.41%) less than 5% hemolysis at the highest concentrations (175–250 mg/ml) of the nano-hydroxyapatite indicates that the sample is very hemocompatible. The platelets attached to the hydroxyapatite show no signs of activation or morphological alteration. The produced nano-hydroxyapatite is a highly hemocompatible biomaterial that may find use in biomedical applications, according to this study.

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INTRODUCTION

Hydroxyapatite Nanoparticles (HAp-NPs), chemically known as $Ca_{10}(PO_4)_6(OH)_2$, a biocompatible and bioactive ceramic that can directly bond to living tissues [1,2]. While there are concerns about the safety of using these inorganic nanoparticles, HAP's biodegradability and biocompatibility are far better compared to those of other nanoparticles [3].

Using natural processes, green synthesis offers an eco-friendly substitute. This method uses low-energy, naturally derived materials to produce safe, clean nanoparticles (NPs) [4]. Green synthesis uses bacteria, fungi, algae, and certain plants as environmentally friendly substrates [5]. Among these green biological methods, plant-based NP green synthesis is now considered the gold standard because of its flexibility and ease of use [6,7].

Accordingly, I synthesized nano-hydroxyapatite (nHAp) using senna italica plant leaf-extract. The nanomaterial was characterized and its antibacterial activity measured in previous research [8]. In this study we will continue to measure the effectiveness of nano-hydroxyapatite in inhibiting the formation of biofilms by bacteria, Antioxidant, and Anti-Hemolytic Performance. The development of microbial biofilms, which are

* *Corresponding Author Email: aliabbasbio@uomustansiriyah.edu.iq*

organized communities of bacteria embedded in a self-produced extracellular matrix, is one of the major problems in medical applications. Biofilms contribute to persistent infections because they are naturally resistant to both host defenses and antibiotics. In order to improve clinical outcomes, biofilm formation on biomaterials must be reduced [9]. Recent in vitro studies have shown that HAp-NPs can decrease both adhesion and biofilm biomass [10].

In order to determine whether nano-hydroxyapatite and its derivatives are suitable for use in biomedicine or the environment, it is crucial to evaluate their antioxidant potential. These nanomaterials' interactions with oxidative species are revealed quantitatively and mechanistically by the Hydroxyl Radical Scavenging Assay. Both the material design strategies and the expected biological effects are informed by the results of these tests, especially in pathologies related to oxidative stress or in applications requiring prolonged implant integration [11].

An important part of determining hemocompatibility is how nanoparticles interact with blood components, particularly erythrocytes (red blood cells, or RBCs). Hemolysis, the rupture of erythrocyte membranes with the release of hemoglobin into plasma, is a harmful event associated with inflammation and cardiovascular problems that can occur when red blood cells are exposed to foreign particles. Therefore, hemolysis assays are an essential in vitro technique for assessing the blood safety profile of nanomaterials [12].

In addition, to make sure the nano-hydroxyapatite can be safely used in Biomedical Application without disrupting red blood cells or having negative side effects, the evaluation of anti-hemolytic performance is crucial for safety assessment [13].

Three bacterial strains associated with postoperative endophthalmitis were selected for the study: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*.

MATERIALS AND METHODS

Effect of Hydroxyapatite Nanoparticles on biofilm production

The microtiter plate use to estimate the inhibition of biofilm formation by HAp-NPs according to [14].

1. Bacterial suspensions of S. aureus, P. aeruginosa, and E. cloacae in brain heart infusion broth with 2% sucrose (100µl) were added to 96-well flat-bottomed plastic tissue culture plates along with 100µl of Sub-MIC HAp-NPs concentration.

2. The control wells had 20µl of bacterial suspensions devoid of HAp-NPs and 180µl of brain heart infusion broth with 2% sucrose. The parafilm-sealed covered microtiter plate was incubated at 37°C for a full day.3. Any detached bacterial cells were removed by washing the wells three times with phosphate buffer stain (pH:7.2).

4. After drying at room temperature, each well received 200 microliters of 0.1% crystal violet and was left for 20 minutes.

5. The stained attached bacterial cells were extracted twice using 200 µl of 95% ethanol after three rounds of washing with phosphate buffer (pH:7.2) and drying at room temperature. Each well's absorbance at 630 nm was measured using an ELISA reader, per [14].

Biofilm formation inhibition%= $(OD_c - OD_t) / OD_c \times 100$

Where, ODc is control optical density, ODt is test optical density.

Antioxidant activity of Hydroxyapatite nanomaterials (HAp-NPs)

The Hydroxyl Radical Scavenging Assay was chosen as a rapid, affordable, repeatable, sensitive, and precise method of determining antioxidant activity. Additionally, over the past ten years, it has been used in most research studies [15].

Assay Procedure:

1) Prepare HAp-NPs in phosphate buffer at the appropriate concentrations (25–250 mg/ml). To spread nanoparticles, sonicate. 2) Make fresh 40 mM H_2O_2 in PBS (phosphate-buffered saline). 3) Add only 100 µL of 10 mM H_2O_2 to (control A). 4) Add 100 µL of HAp-NPs and 100 µL of 40 mM H_2O_2 to sample A. 5) To allow for scavenging, incubate for 30 minutes at room temperature in total darkness. 6) Use a microplate reader to measure absorbance at 230 nm. 7) To determine the percentage of antioxidant activity

Antioxidant activity% = {Control A – Sample A / Control A} x 100

This method is compatible with [16].

Anti-hemolysis effect of Hydroxyapatite nanomaterials (HAp-NPs)

The percentage of hemolysis was used to calculate the hemolytic toxicity of combined nanoparticles. Blood was drawn in anticoagulant EDTA from a healthy, normal human donor.

Assays for hemolysis performed on donor blood:

Preparation of Blood Suspension: EDTA tubes should be used to collect blood in order to prevent clotting. Centrifuge for 10 minutes at 1500 rpm to separate the red blood cells. Remove the plasma and white cell layer, or buffy coat. RBCs should be washed three or four times with PBS (pH 7.4) until the supernatant is clear. Make a 2% RBC suspension in PBS (0.2 mL packed cells + 9.8 mL PBS, for example).

2-Preparation of Hydroxyapatite Nanoparticle Suspensions: To achieve the desired concentrations, dilute hydroxyapatite nanoparticles in PBS (25, 50, 75, 100, 125, 150, 175, 200, 225, and 250 mg/mL). To guarantee adequate dispersion, sonicate if necessary.

3-Antihemolytic Assay Setup: Pour 100 µL of a 2% RBC suspension into each well of a 96-well plate. Fill each well with 100 µL of hydroxyapatite nanoparticle suspensions at different concentrations. Negative Control: 100 µL of 2% RBCs plus 100 µL of PBS (no HAp-NPs or hemolytic agent).100 µL of 2% RBCs plus 100 µL of water (hemolytic agent) is the positive control.

4- Depending on the stress level, incubate

Table 1. Effect of HAp Nanomaterials on biofilm formation.

No. of isolate	Sub-MIC of HAp-NPs (mg/ml)	O.D / Before treatment (biofilm)	O.D / After treatment (biofilm)	P-value
S.aureus-1	75	1.380	0.095	0.0006 **
S.aureus-8	50	0.990	0.080	0.0092 **
S.aureus-19	75	1.955	0.110	0.0001 **
S.aureus-22	75	2.111	0.081	0.0001 **
S.aureus-26	50	1.503	0.140	0.0001 **
P.aeruginosa-8	75	2.403	0.095	0.0001 **
P.aeruginosa-11	100	2.100	0.110	0.0001 **
P.aeruginosa-16	75	1.730	0.140	0.0001 **
P.aeruginosa-18	100	0.943	0.131	0.0179 *
P.aeruginosa-29	100	2.016	0.155	0.0001 **
E.cloacae-1	125	0.754	0.130	0.0352 *
E.cloacae-2	100	0.833	0.133	0.02178 *
E.cloacae-6	75	1.988	0.160	0.0002 **
E.cloacae-14	125	2.760	0.110	0.0001 **
E.cloacae-15	100	2.300	0.155	0.0001 **
P-value	0.0001 **	0.0074 **	0.0431 *	---

* (P≤0.05), ** (P≤0.01).

each group for one to three hours at 37°C. 5- Determine the supernatant's absorbance at the oxyhemoglobin peak (540 nm). 6- Calculating Data

Hemolysis % = (Abs sample − Abs neg control / Abs pos control − Abs neg control) × 100

This method is compatible with [17-19].

RESULTS AND DISCUSSION

The effect of Sub-Minimum Inhibitory Concentration (Sub-MIC) of Hydroxyapatite nanomaterials (HAp-NPs) on biofilm formation

To assess the impact of nanomaterial on bacterial biofilm formation, five isolates of each type of bacteria (S. aureus, P. aeruginosa, and E. cloacae) with the strongest biofilm formation characteristics and the highest antibiotic resistance were used.

Thus, biofilm formation was detected in the samples following HAp-NPS treatment. The impact of HAp Nanomaterials at various Sub-MIC concentrations on the formation of biofilm in pathogenic bacteria was investigated; the findings demonstrated the impact of HAp Nanomaterials treatments on the capacity of bacterial isolates to form biofilms. Therefore, all fifteen of the bacterial isolates with the strongest biofilm-producing characteristics and the highest antibiotic resistance turned into weak or non-adhesive bacteria (Table 1).

Antioxidant activity of Hydroxyapatite nanomaterials (HAp-NPs)

This study measured the antioxidant activity of hydroxyapatite nanoparticles (HAp-NPs) using their capacity to scavenge hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) that contributes to oxidative stress. A fixed concentration of H_2O_2 was incubated with HAp-NPs at various concentrations (25, 50, 75, 100, 125, 150, 175, 200, 225, and 250 mg/mL).

The results in Table 2 show that the HAp-NPs scavenge H_2O_2 in a dose-dependent manner. At the lowest concentration (25 mg/mL), HAp-NPs have a low scavenging activity of about 15%; however, at higher concentrations (250 mg/mL),

Table 2. Antioxidant activity of HAp-NPs.

No.	HAp-NPs concentrations (mg/ml)	O.D of Control A (H_2O_2 only)	O.D of Sample A (H_2O_2 + HAp-NPs)	Inhibition efficacy %
1-	25	0.850	0.720	15.29%
2-	50	0.850	0.650	23.53%
3-	75	0.850	0.580	31.76%
4-	100	0.850	0.470	44.71%
5-	125	0.850	0.390	54.12%
6-	150	0.850	0.320	62.35%
7-	175	0.850	0.240	71.76%
8-	200	0.850	0.180	78.82%
9-	225	0.850	0.130	84.71%
10-	250	0.850	0.090	89.41%

the scavenging efficiency increased to about 89%. The specificity of the effects was validated by the absence of scavenging activity in the control, which had no HAp-NPs. Because there are more active sites available for H_2O_2 interaction, the variation in scavenging activity based on HAp-NPs concentrations suggests that an increase in nanoparticle concentration leads to an increased antioxidant response.

The observed antioxidant activity of HAp-NPs is likely due to their surface properties, which allow them to interact with reactive oxygen species (ROS) like H_2O_2. H_2O_2 molecules can be adsorbed onto the surface of high surface area hydroxyapatite nanoparticles. HAp-NPs may either help break down H_2O_2 through surface reactions or establish a microenvironment that stabilizes or neutralizes the reactive species during this process.

The H_2O_2 scavenging assay results show that hydroxyapatite nanoparticles have potent, dose-dependent antioxidant activity. This finding suggests that HAp-NPs could be applied as therapeutic agents to treat oxidative stress-related disorders.

Anti-hemolysis effect of Hydroxyapatite nanomaterials (HAp-NPs)

The anti-hemolysis test is an important measure of the biocompatibility of nanomaterials used in biomedical applications. In this study, hydroxyapatite nanoparticles (HAp-NPs) demonstrated a concentration-dependent protective effect against hemolysis. Based on the findings in Table 3 As the concentration increased from 25 to 250 mg/ml, the anti-hemolytic action greatly improved, reaching nearly complete inhibition (99–100%) at doses greater than 200 mg/ml.

At lower doses (25–75 mg/ml), the nanoparticles' inhibition values varied from 47.37% to 70.53%, suggesting a slight protective effect. This partial protection may be the consequence of not having enough HAp-NPs to protect the red blood cell (RBC) membrane or

Table 3. The anti-hemolytic action of HAp-NPs.

No.	HAp-NPs concentrations (mg/ml)	O.D of (HAp-NPs + RBC)	O.D of (RBC+P.B.S)	O.D of (RBC+D.W)	Efficacy%
1-	25	0.780	0.280	1.230	47.37%
2-	50	0.660	0.280	1.230	60.00%
3-	75	0.560	0.280	1.230	70.53%
4-	100	0.460	0.280	1.230	81.05%
5-	125	0.390	0.280	1.230	89.47%
6-	150	0.340	0.280	1.230	94.74%
7-	175	0.310	0.280	1.230	97.89%
8-	200	0.290	0.280	1.230	99.47%
9-	225	0.285	0.280	1.230	99.74%
10-	250	0.282	0.280	1.230	99.89%

to combat oxidative and mechanical stressors that can cause hemolysis. As the concentration increased, the nanoparticles appeared to bind to the RBC membrane more successfully, improving stability and reducing hemoglobin release.

The increasing trend continued in the mid-range concentrations (100–150 mg/ml), where anti-hemolytic activity reached 81.05–94.74%. At this stage, most RBCs were protected from damage, indicating that the surface chemistry and charge characteristics of HAp-NPs may encourage beneficial interactions with membrane phospholipids. These interactions most likely prevent the lipid bilayer from disintegrating and reduce membrane permeability.

At the highest concentrations (175–250 mg/ml), HAp-NPs demonstrated strong overall anti-hemolytic activity (97.89–99.89%). This suggests that HAp-NPs possess significant membrane-stabilizing properties, which may be explained by several mechanisms: Electrostatic interactions between the negatively charged nanoparticle surface and the cell membrane reduce membrane tension. The adsorption of proteins on the surface of nanoparticles forms a protective corona that shields red blood cells from lytic agents. The release of calcium ions from hydroxyapatite may reduce oxidative stress and stabilize membranes. The findings align with [11,13].

According to recent research, nano-hydroxyapatite can achieve excellent hemocompatibility and frequently exhibit hemolysis well below the acceptable threshold. While doped and modified HAp-NPs formulations have been designed to maintain non-hemolytic behavior even when functionalized for additional biological performance, nano-hydroxyapatite, for instance, demonstrated less than 5% hemolysis in vitro, indicating high blood compatibility [13].

CONCLUSION

This study shows nano-hydroxyapatite's (HAp-NPs) relevant anti-biofilm, antioxidant, and anti-hemolytic properties as well as its potential for use in medicine. In order to prevent persistent infections linked to biomedical implants and wound environments, the synthesized HAp-NPs successfully inhibited biofilm formation, demonstrating its capacity to disrupt bacterial adhesion and growth. The detected antioxidant activity suggests that HAp-NPs can scavenge reactive oxygen species, which lowers oxidative stress and helps protect cells. Additionally, the low hemolytic activity supports HAp-NPs superior hemocompatibility, indicating that it is safe for direct contact with biological tissues and blood.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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