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Research Article

Enhancement of a Modified Nano Restorative Mixture in Relation to Antibacterial Activity, MTT, and Spectroscopic Properties

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Abstract

Background: Evidence-based medical studies support the effectiveness of silver diamine fluoride (SDF) in removing or even suppressing caries lesions, considering it a non-invasive treatment modality in dental health care. ***Objective***: Investigate the impact of zinc nanoparticles on the antibacterial activity, cytotoxicity, and optical properties of modified SDF. ***Methods***: Mix zinc nanoparticles with SDF filling material in three variable ratios to create four experimental groups. The microbial sensitivity test for these groups was performed by measuring the diameters of the inhibition zones on the Muller-Hinton agar medium. Cytotoxicity assessment using the MTT test, by ISO-10993-5 guidelines, examined the cellular viability. A spectrophotometer analysis was used to evaluate the absorption in wavelengths ranging from 190 to 780 nm. ***Results***: Revealed a dose-dependent efficacy of zinc nanoparticles with a better antibacterial effect than SDF alone; the cell bioavailability of zinc experimental groups remains within the acceptable measurements. Spectrophotometry was not correlated with adding zinc nanoparticles, especially within the visible light range. ***Conclusions***: Mixtures of zinc nanoparticles with SDF were found to have more effective activity against the three human pathogens. At the same time, their cytotoxic pictures are acceptable but without a remarkable color change and aesthetic enhancement.

Keywords: Caries, *Enterococcus faecalis*, *Lactobacillus*, Silver diamine fluoride, *Streptococcus*, Zinc nanoparticles.

تحسين مادة ترميمية مختلطة نانوية معدلة فيما يتعلق بالخواص المضادة للبكتيريا، وخواص اختبار MTT والخصائص الطيفية

الخلاصة

الخلفية: تدعم الدراسات الطبية القائمة على الأدلة فعالية فلوريد ثنائي أمين الفضه (SDF) في إزالة أو تثبيط الآفات النخرية، مما يجعله خيارًا علاجيًا غير جراحي في مجال رعاية صحة الفم والأسنان. **الهدف:** دراسة تأثير جسيمات الزنك النانوية على النشاط المضاد للبكتيريا، والسمية الخلوية، والخصائص البصرية لمادة SDF المعدلة. **الطرائق:** تم خلط جسيمات الزنك النانوية مع مادة فلورايد ثنائي أمين الفضه بنسب مختلفة لتكوين أربع مجموعات تجريبية. تم إجراء اختبار الحساسية الميكروبية لهذه المجموعات من خلال قياس أقطار مناطق التثبيط على وسط مولر-هينتون، وتم تحليل البيانات إحصائيًا باستخدام اختبار(t-test) ، واعتُبر الفرق ذو دلالة إحصائية عندما كانت قيمة (p) أقل من 0.05. تم تقييم السمية الخلوية باستخدام اختبار MTT وفقًا لإرشادات ISO-10993-5 لفحص حيوية الخلايا. كما تم استخدام جهاز المطياف الضوئي لتحليل الامتصاص عند أطوال موجية تتراوح بين 190 إلى 780 نانومتر. **النتائج:** أظهرت جسيمات الزنك النانوية فعالية تعتمد على الجرعة، حيث كان لها تأثير مضاد للبكتيريا أقوى مقارنة بـ فلورايد ثنائي امين الفضه وحده، كما بقيت حيوية الخلايا ضمن المستويات المقبولة في المجموعات التجريبية التي تحتوي على الزنك. ولم يظهر التحليل الطيفي تغيرات واضحة مرتبطة بإضافة جسيمات الزنك، خاصة ضمن نطاق الضوء المرئي. **الاستنتاجات:** تبين أن خلط جسيمات الزنك النانوية مع فلورايد ثنائي أمين الفضه يُعزز من فعاليتها ضد ثلاثة أنواع من مسببات الأمراض البشرية، مع المحافظة على مستوى مقبول من السمية الخلوية، ودون حدوث تغير لوني ملحوظ أو تحسين جمالي واضح.

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INTRODUCTION

Children under the age of six are at risk for early childhood caries (ECC), a particularly aggressive kind of dental disease, owing to a significant negative influence on their quality of life [1]. Since ECC affects about half of preschool-aged children worldwide, it is acknowledged as a serious public health concern [2]. Even though there are therapies for ECC, difficulties are still present, particularly in underprivileged areas, so novel, noninvasive treatment methods are necessary [3]. Silver diamine fluoride (SDF), a clear liquid that combines the antibacterial properties of silver and the remineralizing properties of fluoride, is a promising therapeutic agent for treating caries lesions in young children and individuals with special care needs. Numerous in vitro investigations have demonstrated its efficacy in decreasing particular cariogenic bacteria as well as its capacity to remineralize enamel and dentin [4-6]. It is currently known that the fluoride component strengthens the tooth structure and reduces its solubility when it is

attacked by the acid byproducts of bacterial metabolism [7]. However, SDF may also interfere with the biofilm, killing bacteria that cause the local environmental imbalance that demineralizes dental tissues [8]. By altering the bacterial effects on the tissue and promoting remineralization, SDF thus becomes one of the therapies accessible to fight caries. The first SDF product, Saforide (Bee Brand Medico Dental Co., Ltd., Osaka, Japan), was approved in 1970 after doctors Nishino and Yamaga in Japan used ammoniacal silver fluoride to treat dental caries in 1969 [9] and later developed a way to combine the effects of fluoride and silver. According to both, fluoride entered up to 50–100 μm of sound enamel, and silver penetrated up to 20 μm of sound enamel, reaching the pulp chamber in the dentin [10]. Because this chemical discolors the decalcified soft dentin black, they advised that its usage be restricted to posterior teeth and provided detailed instructions. Metal nanoparticles known as zinc nanoparticles (Zn NPs) have novel prospects for biological uses ranging from diagnosis to therapy. These nanoparticles have a wide range of applications, including the effects of their mechanical, regenerative, and antimicrobial qualities. Larger surface area, regulated manufacturing, and the ability to change desired physical and chemical properties are all made possible by the nanoscale, making them interact with biomolecules in novel ways. Additionally, they contain a larger proportion of surface atoms, which increases surface reactivity and optimizes their capabilities [11,12]. Every tissue in the human body contains zinc, which is also present in over 200 different kinds of enzymes [13] and is a necessary mineral for normal skeletal growth [14,15]. Additionally, it is involved in many physiological and metabolic processes and plays a crucial role in the immune and nervous systems [16]. As a result, its wide range of applications in dentistry are attributed to its special optical, magnetic, morphological, electrical, catalytic, mechanical, and photochemical properties, all of which are easily adjustable to meet specific needs, such as changing the size, doping with additional compounds, or modifying the synthesis conditions. Because zinc nanoparticles have proved to be promising candidates in the biomedical field due to their biocompatibility, low toxicity to humans, and cost-effectiveness due to their increased specific surface area and enhanced particle surface activity, the desired characteristics improve as the particle size decreases [17,18]. Therefore, the purpose of this study is to use spectrophotometric analysis to evaluate the cytotoxicity and antibacterial effectiveness of adding zinc nanoparticles to SDF as well as the reduction of SDF-induced dentin discoloration.

METHODS

Preparation of zinc nanoparticles-containing SDF

The basic materials used are silver diamine fluoride (SDF) liquid (India: Kids-e-dental LLP) and zinc nanoparticles (Zn Nps) with high purity (99.9%, 35-45 nm) supplied from US Research Nanomaterials, Inc., 3302 Twig Leaf Lane, Houston, TX 77084, USA. The Nano groups are group II (SDF+3% Zn Nps), group III (SDF+4% Zn Nps), group IV (SDF+5% Zn Nps), and group I SDF control. All the mixing was performed in a sterile condition to exclude contamination. The exact amount of Zn NPs was weighed, and then a very thorough titration with SDF was performed to prepare the required accurate mix percentage.

Measurement of antibacterial activity

A Muller-Hinton medium (BD France) was used in the agar diffusion test (ADT) to ascertain the various mix testing groups' inhibitory effectiveness against three types of gram-positive bacteria, including *Streptococcus, Lactobacillus, and Enterococcus faecalis*. In nutrient broth (Biolife USA), each bacterium was grown and incubated at 37ºC for 18-24 hrs. Later on, a volume of 0.1 ml from each bacterial suspension was uniformly spread on the surface of nutrient agar and incubated at 37°C for 24 hours. A single well-isolated colony was then transferred into a test tube containing 5 ml of normal saline to prepare a bacterial suspension with moderate turbidity, visually matched to a standard turbidity equivalent to approximately 1.5×10^8 CFU/ml. A portion of this suspension was gently and evenly swabbed across the surface of Mueller-Hinton agar plates using a sterile cotton swab. The inoculated plates were left undisturbed for 10 minutes to allow absorption. Subsequently, three wells of 5 mm in diameter were aseptically made in each agar plate. After removing the agar plugs, 50 µl of the purified and treated exopolysaccharides (EPS) were introduced into each well using a micropipette. At the same time, distilled water (D.W.) was added to the central well to serve as a control. The plates were then incubated at 37°C for 18 hours, after which the diameters of the inhibition zones around the wells were measured and recorded.

Analysis of cytotoxic activity

Tetrazolium salt reduction has become an accepted and reliable approach for assessing cell proliferation. The compound 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) is a yellow tetrazolium salt that is reduced metabolically by active cells, partially facilitated by cellular dehydrogenase enzymes, leading to the formation of reducing equivalents, which in turn result in the intracellular accumulation of purple formazan crystals. These crystals can then be solubilized and quantitatively measured using spectrophotometry. In this study, the assay kit used included two main components: 25 ml of MTT reagent, stored at 24°C, and a detergent reagent provided in two bottles of 125 ml each, which are stored at room temperature.

Spectrophotometer analysis

A double-beam UV-Vis spectrophotometer (UV-2401PC, Shimadzu Corporation, Kyoto, Japan) equipped with an integrating sphere was utilized. A

standardized mounting system was designed to ensure consistent measurements. The spectrophotometer was connected to a computer, which captured the spectral reflectance curves of the mixtures across the ultraviolet and visible range (190–1100 nm).

Statistical analysis

The SPSS statistical software (SPSS Version 22 for Windows; Chicago, IL, USA) was used to analyze the data. The Kruskal assessed the inhibition zone sizes–Wallis test ($p < 0.05$) in millimeters and showed the differences among the test mixtures (group II: 3% Zn NPs, group III: 4% Zn NPs, group IV: 5% Zn NPs) with the control group I (SDF alone) according to the antibacterial analysis for the three types of bacterial species. Post-hoc Bonferroni pairwise comparisons tests with P-values were corrected for multiple comparisons using the Bonferroni adjustment method.

RESULTS

Table 1 revealed there is a p= 0.01 according to the Kruskal-Wallis Test, indicating a significant difference among the test groups, while using *post-hoc* Bonferroni pairwise comparisons revealed the significance between group I (*Streptococcus* SDF control) and group IV (Streptococcus SDF + 5% zinc Nps 5%) with *a p*-value of 0.01.

Table 1: Mean range of inhibition zones diameters (mm) of mixed SDF groups compared to SDF alone against *Streptococcus* species (n=3 for each group)

Streptococcus/SDF + zinc nanoparticles				
Group	Kruskal-Wallis Test		*Post hoc* Bonferroni test	
	Median (Range)	*p*-value	Sample 1-Sample 2	*p*-value
Streptococcus/SDF+zinc Nps (3%)	15(1)	0.01	*Streptococcus*/SDF+ zinc Nps (3%) – *Streptococcus*/SDF control	1.0
Streptococcus/SDF +zinc Nps (4%)	22(1)		*Streptococcus*/SDF+ zinc Nps (3%) – *Streptococcus*/SDF + zinc Nps (4%)	1.0
Streptococcus/SDF+zinc Nps (5%)	24(1)		*Streptococcus*/SDF + zinc Nps (3%) – *Streptococcus*/SDF + zinc Nps (5%)	0.2
Streptococcus/SDF control	13(1)		*Streptococcus*/SDF control – *Streptococcus*/SDF + zinc Nps (4%)	1.0
			Streptococcus/SDF control – *Streptococcus*/SDF + zinc Nps (5%)	0.01
			Streptococcus/SDF + zinc Nps (4%) __ *Streptococcus*/SDF + zinc Nps (5%)	1.0

Values were expressed as mean±SE.

In Table 2, the results show significant differences among groups (p= 0.02), and the differences were substantial statistically between (*Lactobacillus* SDF + 3% zinc NPs) Group II and (*Lactobacillus* SDF + 5% zinc NPs) Group IV with *a p*-value of 0.05.

Table 2: Mean range of inhibition zones diameters (mm) of mixed SDF groups compared to SDF alone against *Lactobacillus* (n=3 for each group)

Lactobacillus \SDF + Zinc nanoparticles				
Group	Kruskal-Wallis Test		*Post hoc* Bonferroni test	
	Median (Range)	*p*- value	Sample 1 – Sample 2	*p*-value
Lactobacillus/SDF+ zinc Nps (3%)	21 (1)	0.02	*Lactobacillus*/SDF+ zinc Nps (3%) _ *Lactobacillus*/SDF control	1.0
Lactobacillus\ SDF+ zinc Nps (4%)	24 (2)		*Lactobacillus*/SDF+ zinc Nps (3%) _ *Lactobacillus*/SDF+ zinc Nps (4%)	0.6
Lactobacillus\ SDF+ zinc Nps (5%)	28 (1)		*Lactobacillus*/SDF+ zinc Nps (3%) - *Lactobacillus*/ SDF+ zinc Nps (5%)	0.05
Lactobacillus\ SDF control	22 (2)		*Lactobacillus*/SDF control – *Lactobacillus*/SDF+ zinc Nps (4%)	0.8
			Lactobacillus/SDF control- *Lactobacillus*/SDF+ zinc Nps (5%)	0.07
			Lactobacillus/ SDF+ zinc Nps (4%) - *Lactobacillus* SDF+ zinc Nps (5%)	1.0

Finally, Table 3 also shows a significant difference between groups (p= 0.03) and a significant difference between (*Enterococcus* \ SDF control) group I and (Enterococcus \ SDF + 5% zinc Nps) group IV, with *a p*-value of 0.02. Figure 1 shows the three bacterial species in relation to the four testing groups according to the inhibition zones (mm). In Figure 2 and Table 4, the results demonstrated the relationship between the yellow tetrazolium MTT dose and the mean values of cell viability according to group IV; as we increase the dose, there is acceptable cell proliferation until the dose of 50 µg/ml, where a drop exists and continues even though there is little elevation at the dose of 1000 µg/ml. The predicted IC50 value was 28.14 µg/ml. In Figure 3 and Table 5, a comparison of the results demonstrated a dose-dependent viability percentage, where predictable proliferation properties continue until the dose of 25 µg/ml. Then, a steady drop was reported as we increased the dose of MTT. The predictable IC50 value was 17.79 µg/ml. A spectrophotometer is an advanced digital device capable of accurately and consistently measuring the complex color of tooth structure in numerical terms [19].

Table 3: Mean range of inhibition zones diameters (mm) of mixed SDF groups compared to SDF alone against *Enterococcus* (n=3 for each group)

Enterococcus/SDF+zinc nanoparticles				
Group	Kruskal-Wallis Test		*Post hoc* Bonferroni test	
	Median (Range)	*p*-value	Sample 1 – Sample 2	*p*- value
Enterococcus/SDF+ zinc Nps (3%)	22 (2)	0.03	*Enterococcus*/SDF+zinc Nps (3%) – *Enterococcus*/SDF control	1.0
Enterococcus/SDF+ zinc Nps (4%)	22 (3)		*Enterococcus*/SDF+ zinc Nps (3%) *Enterococcus*/SDF+ zinc Nps (4%)	1.0
Enterococcus/SDF+ zinc Nps (5%)	25 (1)		*Enterococcus*/SDF+ zinc Nps (3%) – *Enterococcus*/SDF+ zinc Nps (5%)	0.2
Enterococcus\ SDF control	20 (1)		*Enterococcus*/SDF control – *Enterococcus*/SDF+ zinc (4%)	1.0
			Enterococcus/SDF control – *Enterococcus*/SDF+ zinc Nps (5%)	0.02
			Enterococcus/SDF+ zinc Nps (4%) – *Enterococcus*\ SDF+ zinc Nps (5%)	0.8

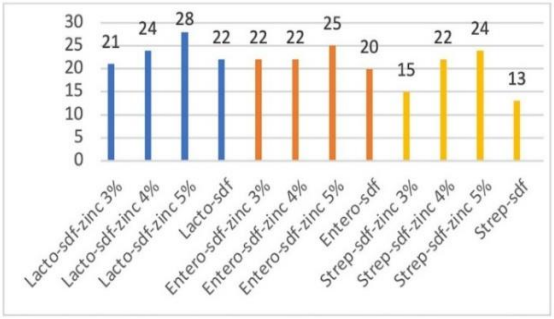

Figure 1: Shows the three bacterial species in relation to the four testing groups according to the inhibition zones (mm).

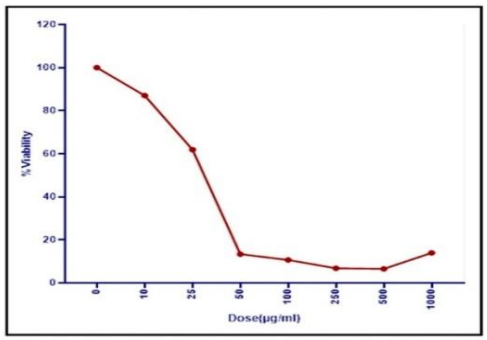

Figure 2: MTT test for the highest additive zinc nanoparticles (5%) mixture, group IV for 48hrs.

Table 4: Percentage cell viability of group IV in relation to doses (mg/ml) (n=3 for each dose)

Dose (µg/ml)	Mean±SD
0	100±4.026
10	78.219±6.971
25	26.939±0.472
50	18.747±5.595
100	12.052±0.937
250	11.658±0.829
500	11.855±0.583
1000	12.524±0.236

Table 5: Percentage cell viability of group I in relation to doses (mg/ml) (n=3 for all doses)

Dose (µg/ml)	Mean±SD
0	100±7.064
10	87.042±2.165
25	61.933±0.503
50	13.326±1.736
100	10.674±0.877
250	6.814±0.353
500	6.512±0.153
1000	13.931±0.655

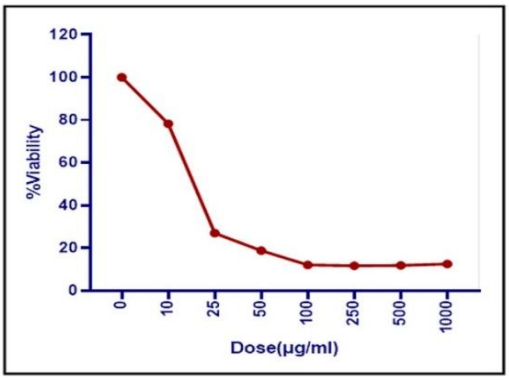

Figure 3: MTT test for the highest additive zinc nanoparticles (5%) mixture, group I for 48hrs

The UV-VIS scanning was recorded in regular patterns with the addition of zinc nanoparticles through the first 48 hours. Table 6 shows the minimum and maximum absorption of visible and ultraviolet light regions.

DISCUSSION

Tooth staining is the main adverse effect of SDF, so the question is whether a new formula of SDF is created by adding an effective supplement to have better clinical behavior or whether it is still the same with fewer side effects. Many in vitro research studies have proposed using the beneficial properties of nanomaterials to replace silver ions linked to ingredients inside SDF [20-23]. The zinc nanoparticles emerged as one of these additive metals, so utilizing its good reputation, they were added into the SDF, and then these three mixtures (groups II, III, and IV) were studied for the antagonist effect against cariogenic germs and the possibility of getting rid of the discoloration effect. The studied data show a highly significant improvement against the bacteria, specifically when increasing the zinc NPs percentage up to 5%, so it is a concentration-dependent value; this result coincides with another modified SDF to improve its antibacterial properties by Bang *et al*. in 2022, copper-doped bio-glass nanoparticles were incorporated with SDF in variable percentages, where the results also showed a cumulative effect of increasing the doses of nanoparticles; increasing the doses of CuBGNPs, the antibacterial impact rises [22].

Table 6: Max, and Min absorption values of studied groups corresponding to wave lengths

Wavelength range	SDF only	SDF + 3% Zinc	Plus 4% zinc	Plus 5% zinc
Min in UV	0.037 at 232 nm	0.905 at 190 nm	0.859 at 380 nm	0.272 at 380 nm
Max in UV	3.532 at 323 nm	4.586 at 220 nm	4.643 at 226 nm	4.576 at 212 nm
Min in visible	0.033 at 776 nm	0.299 at 780 nm	0.245 at 780 nm	0.074 at 780 nm
Max in visible	0.039 at 452 nm	1.065 at 382 nm	0.849 at 382 nm	0.267 at 382 nm

Adding nanoparticles to many dental materials has a similar effect by enhancing antibacterial efficiency, as in a study that said Se/ZnO NPs to resin-based composites [24] with higher biocompatibility and mechanical strength. Even root canal materials show the same enhancement of antibacterial properties with lower cytotoxic effects when modified with Nano metal, as when silver-gold nanoparticles have been added to root canal sealants [25]. Many biological characteristics of these nanoparticles are influenced by particle shape, size, concentration, and affinity to agglomerate to unite with other nanoparticles or other materials [26,27]. When zinc nano adhesive was mixed, a significant improvement appeared in the microbial defense mechanism without affecting the bond strength [28-30]. A recent theory, the “Trojan Horse effect," explains these similar results: The acidic lysosomal field leads to the degradation of nanoparticles, which in turn convert basic metals to ionic substances to release toxic, harmful substances, interfering with the cellular ability to make another copy by themselves. Local microenvironment alterations produce reactive oxygen species (ROS) or increase the dissolving effect of these particles; most of these reactions result in an interaction with the -SH group of the cellular enzymes, producing dysfunction of organelles and protein denaturation that destroys DNA, already stopping the replication of DNA and H_2O_2 releasing probability [31]. The investigation of cytotoxicity in the form of an MTT test was another critical issue to be discussed in our study, especially when adding metal nanoparticles with different percentages of up to 5% to be chosen for such investigation. The cell viability values (Tables 4 and 5) showed that both control SDF and mixed 5% Group IV were non-cytotoxic to the fibroblast cell line when a proliferative effect was observed after 48 hours. However, it’s a dose-dependent issue. Truly, there is no previous evidence-based study that discusses the cytotoxicity of pure or additive zinc nanoparticles; others mainly evaluate other zinc derivative compounds or even other zinc applications in dentistry, as in 2021, when Toledano *et al*. incorporated polymeric membranes of implants with zinc and showed high levels of bone healing around them due to optimum regeneration, bone integration, and genesis in animal experiment samples [32]. A low concentration threshold for cytotoxic action is found in polyethylene glycol (PEG) mixed with ZnO NPs as a coating layer [33]. Spectrophotometer analysis showing maximum absorption in UV for all the studied groups was almost close to each other. In contrast, maximum absorption values in the visible region for both groups II and III were mainly close and primarily high, the minimum values in the UV spectrum for control SDF were the lowest and both close and higher for groups II and III, and in the visible spectrum the values of absorption were not much different from the previous one. These groups seemed to have more light absorption in the ultraviolet region than visible and, therefore, were much darker than in VIS light. The addition of Zn NPs had no noticeable effect on the material's light absorption and later dark discoloration, unlike another study where zinc reduced the darkness effect of SDF on dentine in a dose-related impact [34]. Different concentrations of zinc were used to treat the prepared dentin blocks before applying SDF. ImageJ software measured the color changes, showing remarkable color enhancement. Therefore, their results were not confined to ours, which may be due to the addition of zinc directly to the SDF not inducing the desirable effect rather than the application of it separately on dentine before SDF. Further investigation should be done to evaluate the chemical procedure.

Study limitations

This is an *in vitro* study without a clinical application directly to the tooth structure, which reveals the actual effect of a modified combination. So we suggest applying mixed material directly to a dent in blocks prepared previously or applying the zinc nanoparticles to extracted teeth with different percentages before SDF application.

Conclusion

The SDF restorative material is highly antibacterial against one of the most cariogenic and root canal infection bacterial species, and the addition of the zinc nanoparticles increases this property, which makes it a good, promising new modified formulation with an acceptable cell proliferation likelihood, even though it’s a percentage-dependent issue. Unfortunately, adding such antioxidant ions did not reduce the silver oxides and the unesthetic dark discoloration of the SDF as expected.

Conflict of interests

The authors declared no conflict of interest.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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