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Antiglycating activity of biomimetically synthesized magnetic copper ferrite nanoparticles: inhibition of methylglyoxal-derived AGEs

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The nonenzymatic glycation process initiates a harmful cycle that generates various intermediate compounds, leading to carbonyl and oxidative stress, and ultimately resulting in the formation of advanced glycation end products (AGEs). AGEs have been implicated as key factors in the development of several diseases, including neurodegenerative conditions like Parkinson's and Alzheimer's diseases, as well as complications associated with diabetes. Given the potential impact of AGEs on these diseases, this study explores the role of copper ferrite nanoparticles (CuFe₂O₄NPs) in inhibiting the formation of these harmful intermediates and AGEs. Magnetic CuFe₂O₄ nanoparticles were synthesized using Aloe vera leaf extracts and their effects on AGE formation were assessed. Using both biophysical and biochemical approaches, the study demonstrates that CuFe₂O₄ NPs have significant anti-glycation properties, which help reduce or prevent AGE formation while maintaining protein structure. These findings suggest that CuFe₂O₄ NPs may offer therapeutic potential in addressing AGE-related diseases, particularly those linked to diabetes and its complications.

KEYWORDS

methylglyoxal, ages, CuFe₂O₄ NPs, antiglycation, nanoparticles

Introduction

Over the last three to four decades, studies have revealed the involvement of AGEs (advanced glycation end products) in human pathophysiology. AGEs are the products of a non-enzymatic glycation reaction, also known as the Maillard reaction (Monnier and Cerami, 1981). Normally, AGEs form under physiological conditions and during natural aging. However, their formation is accelerated in disease conditions, and high levels of AGEs have been reported in various diseases such as diabetes (Van Eupen et al., 2013), atherosclerosis (Hanssen et al., 2014), and obesity (Gaens et al., 2014). AGEs have been recognized as potential contributors to several diseases, including age-related neurodegenerative diseases like Parkinson's and Alzheimer's disease (Ahmed et al., 2005; Dalfo et al., 2005), as well as diabetic complications (Garay-Sevilla et al., 2005). The glycation reaction is also associated with oxidative stress, which generates species involved in the onset of several diseases, including neurodegenerative disorders (Dalfo et al., 2005; Garay-Sevilla et al., 2005; Leszek et al., 2016; Khan et al., 2016).

Glycation of macromolecules is not restricted to reducing sugars; non-sugar entities such as dicarbonyls have also been reported as potential glycating agents (Ashraf et al., 2015). Dicarbonyl compounds, such as methylglyoxal (MG), glyoxal, and 3-deoxyglucosone (3-DG), are naturally generated through various pathways, including the Maillard reaction, sugar autooxidation, amino acid metabolism, and lipid peroxidation (Chetyrkin et al., 2008). These highly reactive compounds have the ability to glycate molecules even at minimal concentrations (Thornalley, 1998). The intracellular accumulation of MG is particularly toxic due to its strong glycation properties (Allaman et al., 2015).

In recent years, systematic studies have been conducted to identify pharmacological agents that may reduce or inhibit AGE formation. Several potential inhibitors or anti-glycating agents, including aspirin, steroidal compounds, and aminoguanidine, have shown promise in reducing AGEs formation in neurological diseases (Reddy and Beyaz, 2006; Webster et al., 2005; Jaturapatporn et al., 2012). Additionally, many phytochemical compounds, such as phenols and flavonoids (Albarracin et al., 2012; Sutachan et al., 2012), as well as herbal drugs, have been identified as AGE inhibitors (Dey et al., 2017; Natarajan et al., 2013). However, despite their anti-glycating properties, some compounds have been discontinued due to side effects. For example, although aminoguanidine demonstrated anti-AGE activity, its use was prohibited after phase III clinical trials revealed adverse effects (Ahmed et al., 1986). As a result, finding safe and effective anti-glycating agents to counter AGE formation has become a significant challenge for scientists.

Nanotechnology offers a potential solution to various technological and medical challenges. Nanoparticles are small nano-sized inorganic particles that exhibit unique physical and chemical properties. They exhibit excellent biocompatibility, flexible conjugation with biomolecules (Nikalje, 2015), and the capability to interact both at the cellular surface and within cells, owing to their small size, which is approximately 100 to 10,000 times smaller than human cells. Nanoparticles can easily enter living cells via endocytosis because of their resemblance to cellular components (Leszek et al., 2017), making them useful in a wide range of physical,

biological, biomedical, and pharmaceutical fields (Loureiro et al., 2016; Treuel et al., 2013).

Spinel ferrite nanoparticles (SFNPs) have recently garnered attention due to their superparamagnetic properties at the nanoscale, chemical stability, simple composition, and broad applications. Extensive research has been conducted on SFNPs with the general formula MFe₂O₄, which typically consists of iron oxide and one or more transition metals (Masunga et al., 2019). SFNPs are being used in biomedicine for cancer cell detection, magnetic hyperthermia for tumor diagnosis and treatment, magnetic drug delivery, cellular signaling, magnetic resonance imaging, magnetic recording, water and wastewater treatment, magneto-optical devices, ferromagnetic fluids, energy storage, magnetic separation, and biosensors, among other applications (Ansari et al., 2018; Kuznetsov et al., 2013; Srinivasan et al., 2018). Among various SFNPs, copper ferrite nanoparticles (CuFe $_2O_4$ NPs) have attracted significant interest due to their wide range of applications. These include their use as antibacterial agents (Ansari et al., 2018; Liu et al., 2019), catalysts in nanomedicine for treating breast cancer, magnetic resonance imaging materials, photocatalysts, photoanodes for solar water oxidation, catalysts, enzyme immobilization supports, and in water treatment and purification (Masunga et al., 2019). Certain nanoparticles, such as silver, gold, selenium, and ZnO NPs, have shown both anti-glycative and antioxidative activities (Ansari et al., 2016; Ashraf et al., 2018; Kim et al., 2012; Ashraf et al., 2014; Kim et al., 2012).

Given the hazardous role of AGEs in disease development, we are the first to explore the anti-glycative activity of magnetic $CuFe_2O_4$ NPs. This study presents the anti-glycation potential of biologically synthesized copper ferrite nanoparticles ($CuFe_2O_4$ NPs). The goal is to establish a safer and more effective therapeutic approach for AGE-related diseases.

This study presents an innovative approach by exploring the anti-glycation potential of biologically synthesized magnetic copper ferrite nanoparticles (CuFe₂O₄ NPs) for the first time. While AGEs (advanced glycation end-products) have been identified as contributors to various pathologies, including neurodegenerative diseases and diabetes-related complications, current pharmacological interventions face limitations due to side effects and inefficacy. Unlike traditional anti-glycation compounds, the unique physical and chemical properties of CuFe₂O₄ NPs-such as their superparamagnetic behaviour and compatibility with biological systems-could provide enhanced therapeutic effects. Furthermore, by leveraging nanotechnology, this study aims to overcome the challenges of safe and efficient AGE inhibition, positioning CuFe₂O₄ NPs as promising candidates for antiglycation therapy.

Material and methods

Materials

Preparation of the aqueous leaf extract of aloe vera

In this study, copper ferrite nanoparticles ($CuFe_2O_4$ NPs) were biosynthesized using Aloe vera leaf extract (Ashraf et al., 2018). Fresh, healthy Aloe vera leaves were collected, thoroughly washed with tap water, and then with double-distilled water to remove any impurities. About 25 g of the sterilized, chopped leaves were heated at 100°C in 100 mL of sterilized water for 10–15 min. The extract was centrifuged twice, and the supernatant was filtered through a 0.45 μ m filter and stored at 4°C for further use in the synthesis of CuFe₂O₄ NPs. Millipore water was used as a blank to adjust the baseline, and the Aloe vera leaf extract was prepared as described in the materials and methods section (Ashraf et al., 2018).

Preparation of CuFe₂O₄ NPs:

For the biosynthesis of $CuFe_2O_4$ NPs, $Cu(NO_3)_2\cdot 3H_2O$ and $Fe(NO_3)_3\cdot 9H_2O$ were used as precursors. A 0.1 M solution of $Cu(NO_3)_2\cdot 3H_2O$ and a 0.2 M solution of $Fe(NO_3)_3\cdot 9H_2O$ were prepared by dissolving them in sterilized water to form a clear solution. Subsequently, 25 mL of Aloe vera extract was added to 75 mL of the $Cu(NO_3)_2\cdot 3H_2O$ and $Fe(NO_3)_3\cdot 9H_2O$ solution. The reaction mixture was stirred at 100°C for 10 h, resulting in a brown semi-solid gel. This gel was washed three times with hot water and twice with ethyl alcohol. The pellet was dried in an oven at 200°C for 6 h to obtain the $CuFe_2O_4$ nanoparticles.

Characterization of biosynthesized CuFe₂O₄ NPs

The biosynthesized CuFe2O4 NPs were characterized using UV-Vis spectroscopy (Shimadzu UV-1800) for preliminary analysis. The sample was placed in a quartz cuvette, and absorption spectra were recorded in the 200-800 nm range (Ansari et al., 2023; Anandan et al., 2019). FTIR (Shimadzu IRSpirit, Shimadzu, Kyoto, Japan) was used to assess the functional groups of the nanoparticles in transmittance mode with KBr pellets in the 4,000-400 cm⁻¹ range (Ansari et al., 2023; Anandan et al., 2019). The morphology and size of the NPs were analyzed using SEM (TESCAN, VEG3, Berno-Czech Republic) at an acceleration voltage of 20 kV and TEM (FEI Morgagni 268, Czech Republic) at 200 kV (Ansari et al., 2023; Ansari and Alomary, 2024). SEM and TEM images were captured after applying a small amount of the NP solution to aluminum stubs and carbon-coated copper grids, respectively. Additionally, XRD analysis was performed using an X-ray powder diffractometer (Shimadzu XRD-7000) with CuK α radiation ($\lambda = 1.54056$) in the 2θ range of 20° - 80° at 40 keV, following the protocol described in previous studies (Ashraf et al., 2018; Ansari and Alomary, 2024).

BSA in vitro glycation assay

Bovine serum albumin (BSA), methylglyoxal, and $CuFe_2O_4$ nanoparticles were used in this anti-glycation assay with slight modifications from the original method (Ashraf et al., 2018). The reaction mixture consisted of BSA (333 µg/mL), methylglyoxal (10 mM), aminoguanidine (2 mM) and 10 mM PBS (pH 7.4) with 1 mM sodium azide, along with varying concentrations of $CuFe_2O_4$ NPs (50 µg/mL, 100 µg/mL). Native BSA (without methylglyoxal or $CuFe_2O_4$ NPs) was used as the control. The mixtures were incubated at 37°C for 6 days. After incubation, the samples were dialyzed against sodium phosphate buffer at 4°C for 48 h to remove unbound methylglyoxal and $CuFe_2O_4$ NPs. The dialyzed samples were diluted in the same buffer for further analysis unless otherwise specified.

Absorbance spectroscopy:

The UV-visible absorption spectra of native, glycated, and NPtreated BSA samples were recorded using a UV-visible spectrophotometer (Labman) between 200 and 600 nm in a 1 cm quartz cuvette. Absorbance was measured at 280 nm (Ashraf et al., 2018).

Determination of amadori products

Amadori products/ketoamine moieties formed in native, glycated, and NP-treated BSA samples were determined using the NBT reduction assay, with slight modifications as described previously (Johnson et al., 1982). A 20 μ L protein sample was mixed with 180 μ L of 100 mM sodium carbonate-bicarbonate buffer (pH 10.8) containing 0.2 mM nitro blue tetrazolium (NBT) and incubated at 37°C for 25 min. Absorbance was measured at 525 nm using the same buffer as the blank. The ketoamine moiety concentration (nm/mL) was calculated by multiplying the absorbance by the molar extinction coefficient of 12,640 M⁻¹ cm⁻¹ for monoformazon.

Spectrofluorimetric analysis

AGE-specific fluorescence of samples was measured using an Agilent Cary Eclipse fluorescence spectrophotometer (G9550-64000). The samples were excited at 370 nm, and emission spectra were recorded at 450 nm with both slits set to 5 nm and a 1 cm path length. Fluorescence emission was calculated using the formula:

% Inhibition =
$$\frac{Fg - Fn}{Fg - Fn} \times 100$$

where Fg, Fn, and Fh represent the fluorescence intensities of glycated BSA, NPs-treated samples, and native BSA, respectively.

Intrinsic fluorescence measurements

Structural characteristics of the samples were studied using intrinsic fluorescence measurement. Native, glycated, and NP-treated samples were excited at 295 nm (specific for tryptophan residues), and the emission spectra were recorded from 300–600 nm (Ashraf et al., 2018).

HMF, CC, Free Lysine & Arginine, ANS, and ThT Assays:

The hydroxymethylfurfural (HMF) content, an early-stage glycation product, was quantified using the thiobarbituric acid assay (Ahmad et al., 2012). Carbonyl content (CC) in native, glycated, and NP-treated samples was measured using 2,4-dinitrophenylhydrazine (DNPH), as described with slight modifications (Levine et al., 1994). Free lysine modifications were assessed using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method (Sashidhar et al., 1994). Free arginine content was measured using the Phenanthroquinone method (Smith and MacQuarrie, 1978), and hydrophobicity changes were analyzed through ANS binding, excited at 380 nm (Hu et al., 2008). For aggregation studies, Thioflavin T (ThT) was used as described in previous research (Khurana et al., 2005).

Statistical analysis

All data were presented as mean \pm standard deviation (SD) in triplicates (n = 3) using the XL. STAT 7.0. One-Way ANOVA using Dunnet's' *post hoc* test. Values were considered statistically significant when p < 0.01.



Results

Characterization of CuFe₂O₄ NPs

UV-Vis spectrum of the biosynthesized CuFe₂O₄ NPs

The aqueous extract of aloe vera leaves was utilized in this investigation for the purpose of synthesizing $CuFe_2O_4$ nanoparticles. The UV-Vis spectrum of the biosynthesized $CuFe_2O_4$ NPs reveals a prominent peak at 238 nm, indicating the successful formation of ferrite nanoparticles (Figure 1A).

FT-IR spectra of the synthesized CuFe₂O₄ NPs

The FT-IR spectra of the synthesized CuFe₂O₄ NPs, shown in Figure 1B, were measured in the range of 400–4,000 cm⁻¹, display two distinct absorption ranges between 400 and 600 cm⁻¹, with peaks at v_1 (566 cm⁻¹) and v_2 (431 cm⁻¹), corresponding to the spinel structure of the ferrite nanoparticles. The v_1 peak r_eflects the Fe–O stretching vibration at the tetrahedral site, while v_2 is attributed to Cu–O stretching at the octahedral site.

Investigation of morphology of synthesized ferrite NPs by SEM and TEM

The size and shape of the synthesized nanoparticles were further confirmed through SEM and TEM analysis (Figures 2A–C). The SEM image shows that the NPs have an irregular, spinel spherical shape (Figure 2A). The TEM image reveals spherical particles, with the size distribution analyzed using ImageJ software showing an average particle size of 3.75 ± 2.21 nm (Figure 2B), which aligns with the XRD results (Figure 3).

XRD analysis of synthesized ferrite NPs

Further, the crystalline size and nature of synthesized $CuFe_2O_4$ NPs was analyzed by XRD method. Figure 2D illustrates the X-ray diffraction pattern of the $CuFe_2O_4$ nanoparticles. The XRD analysis indicates the presence of distinct peaks at 220, 311, 222, 400, 422, 511, and 440, corresponding to the cubic phase of $CuFe_2O_4$ NPs (JCPDS Card no. 75–1517).

UV profile of MG-Modified BSA with CuFe₂O₄ NPs

Pilot experiments were conducted to determine the optimal incubation time and concentration of BSA, methylglyoxal, aminoguanidine and Aloe vera leaf extract, with or without varying concentrations of CuFe₂O₄ NPs, over different time intervals (0-6 days) at 37°C. The UV-Vis absorbance of native BSA was measured at 280 nm. The absorbance (hyperchromicity) increased significantly at 280 nm after BSA glycation with methylglyoxal, compared to native BSA, while the NPs-treated BSA-methylglyoxal mixtures showed a reduction in hyperchromicity compared to the native protein (Figure 3). The hyperchromicity of glycated protein was 89% when compared to native protein, and the hyperchromicity of glycated samples was reduced to 8% and 17% respectively, after treatment of NPs (50 µg/ mL and 100 µg/mL). Therefore, 100 µg/mL of NPs was selected for further studies. Aminoguanidine was taken as positive control. It showed 10% higher hyperchromicity as compared to that of CuFe₂O₄ NPs treated sample.

Determination of arginine and lysine by 2,4,6trinitrobenzenesulfonic acid (TNBS) and phenanthroquinone

To assess the free amino groups of lysine and arginine residues in the samples, 2,4,6-trinitrobenzenesulfonic acid (TNBS) and phenanthroquinone were employed, respectively. After 6 days of BSA glycation, a 66% decrease in the availability of free lysine amino groups was observed compared to native BSA (Figure 4A). However, the availability of NH₂ groups increased by 11% after treating the glycated BSA with 100 μ g/mL of NPs. Similarly, glycation led to a 26% reduction in the availability of free arginine amino groups compared to native BSA (Figure 4B). The availability of NH₂ groups increased by 6% in the NPs (100 μ g/mL) treated sample.

Nitroblue tetrazolium test (NBT) for ketoamine/ early glycation products detection

NBT reduction assay is particularly for ketoamine/early glycation products detection, not for AGEs (Alouffi et al., 2022). Native BSA alone showed negligible amounts of ketoamine contents (14.5 nmol/mL) as compared to glycated BSA (35.8 nmol/mL) on



the 3^{rd} day of incubation (Figure 5A). Whereas, glycated sample treated with NPs (100 μ g/mL) showed marked decrease in ketoamine contents (19.1 nmol/mL) as compared to that of other samples.

Analysis of hydroxymethylfurfural (HMF) by thiobarbituric acid assay

Hydroxymethylfurfural (HMF) is formed during early glycation reaction. Thiobarbituric acid assay was applied to investigate the HMF level in samples. The HMF contents in glycated BSA was high ($1.7 \pm 0.7 \text{ nmol/mL}$) as compared to that of control ($0.77 \pm 0.8 \text{ nmol/}$ mL) (Figure 5B). The decline in HMF contents ($1.3 \pm 0.6 \text{ nmol/mL}$) was recorded in NPs ($100 \mu \text{g/mL}$) treated sample. CuFe₂O₄ NPs treated sample showed up to 23% decrease in HMF contents when compared to glycated BSA (Figure 7B).

Measurement of carbonyl content

The statistical significance of the results is evident from the observed differences in carbonyl content between the various groups. Methylglyoxal-glycated BSA showed a significant increase in carbonyl content (90.36 \pm 1.5 nmol/mg) compared to native BSA (66.33 \pm 0.8 nmol/mg), indicating a marked increase in protein oxidation due to glycation (Figure 6A). In contrast, treatment with CuFe₂O₄ NPs significantly reduced the carbonyl content to 55.82 \pm 1.6 nmol/mg.

Fluorescence studies of MG-Modified BSA with $CuFe_2O_4$ NPs

In the present study, the glycated sample displayed a significant increase in fluorescence intensity at around 450 nm, indicative of the formation of fluorophoric AGEs, which agrees with earlier reports



(Alouffi et al., 2022; Ashraf et al., 2015). The fluorescence results from this study are in line with previous findings, confirming that AGEs exhibit fluorescence at specific excitation/emission wavelengths. The 28% reduction in fluorescence intensity upon treatment with CuFe₂O₄ NPs (100 μ g/mL) suggests a strong inhibitory effect on AGE formation (Figure 6B).

Thioflavin T (ThT) fluorescence studies

Thioflavin T (ThT) fluorescence is used to detect protein aggregation. Confirmed that glycation induced







significant aggregation in BSA, as evidenced by a 92.72% increase in fluorescence intensity (Figure 7A). However, the treated sample exhibited a 40.89% reduction in fluorescence intensity.

Analysis of structural change in samples by ANS dye

The ANS dye is used for the study of structural alteration in the proteins caused by damaging agents. The increase in ANS fluorescence in the glycated sample was observed while decrease in fluorescence intensity was recorded in the sample treated with $CuFe_2O_4$ NPs (Figure 7B).

Structural analysis of protein by fluorescence spectroscopy

Fluorescence spectroscopy is used to measure the intrinsic fluorescence of tryptophan in proteins. A radical decrease in fluorescence intensity in the glycated sample was observed (Figure 8). In contrast to that the NPs-treated sample showed that an 8% increase in fluorescence intensity.





-), glycated-BSA (- - - -) BSA+100 g/mL CuFe₂O₄ NPs (-----) and BSA + aminoguanidine ANS estimation for native BSA (-•) sample. The excitation wavelength was 380 nm emission was recorded in the range was of 400–600 nm.

Discussion

The first confirmation of the successful synthesis of CuFe2O4 NPs using green approach came via UV-Vis spectroscopy. The production of the ferrite NPs was identified by the distinctive peak in the range of 230-500 nm (Mushtaq et al., 2017). In their UV-vis absorbance spectra, metal nanoparticles typically exhibit a distinctive plasmon resonance band because of the combined oscillation of their conducting electrons (Mushtaq et al., 2017).

FTIR analysis of ferrite nanoparticles exhibit two absorption peaks at v_1 (566 cm⁻¹) and v_2 (431 cm⁻¹), indicating their spinel structure. The v₁ peak points to Fe–O stretching at the tetrahedral site, while v₂ represents Cu–O stretching at the site of the octahedral (Salavati-Niasari et al., 2012; Zekri and Fareghi-Alamdari, 2016).



Our findings are consistent with those of Ramazani et al. (2017), who reported Fe-O and Cu-O bond stretching at 572 and 421 cm⁻¹, respectively, in CuFe₂O₄ nanocrystals synthesized through a green method using tragacanth gum as a biotemplate. In contrast, another study using a thermal treatment method reported Fe-O and Cu-O bond stretching at 533 and 315 cm⁻¹, respectively (Zakiyah et al., 2015). A broad absorption at 3,358 cm⁻¹ reflects the stretching of OH groups, indicative of phytocompounds such as alcohols, phenols, acids, and their derivatives (Abbasi et al., 2020). The 2,951 cm⁻¹ peak corresponds to symmetrical and asymmetrical C-H stretching in aliphatic groups, while the peaks at 1,638 cm⁻¹ and 1,500 cm⁻¹ represent the carbonyl C=O (primary amide) and carboxylate (-COO⁻) groups, respectively, in Aloe vera extract. The IR peaks at 1,414 cm⁻¹ and 1,233 cm⁻¹ correspond to the CH₃ group of primary aromatic amines and C-O-C stretching of-COCH₃ groups, respectively (Lim and Cheong, 2015). The 1,083 cm⁻¹ peak is associated with C=C bonds in unsaturated five- or sixmembered ring compounds (Abbasi et al., 2020), while the peaks at 827, 750, 659, and 566 cm⁻¹ correspond to aromatic CH out-of-plane deformation (Benalia et al., 2022). These FTIR results suggest that phenols, proteins, and sterol phytocompounds in Aloe vera leaf extract may serve as reducing agents responsible for the formation of nanoparticles.

The shape and size of the biosynthesized nanoparticles were studied using SEM and TEM. Through TEM investigation, it was determined that the particles were spherical in shape, with an average particle size of 3.75 ± 2.21 nm. Zakiyah et al. (2015) (Ramazani et al., 2017) reported a particle size of 24-34 nm for NPs synthesized via the hydrothermal co-precipitation method. In another study, CuFe₂O₄ nanoparticles produced using Nasturtium officinale extract exhibited a particle size of 15-50 nm (Khan et al., 2024).

According to the XRD study, there are distinctive peaks at 220, 311, 222, 400, 422, 511, and 440 that represent the cubic phase of $CuFe_2O_4$ NPs. These reflection peaks align well with those of $CuFe_2O_4$ NPs synthesized by the green method using tragacanth gum (Zekri and Fareghi-Alamdari, 2016) and the hydrothermal co-precipitation method (Ramazani et al., 2017).

The average particle size, determined using Scherrer's formula from the FWHM of the (311) peak, was found to be 1.16 nm. Ramazani et al. (2017) (Zekri and Fareghi-Alamdari, 2016) reported an average particle size of 14 nm for $CuFe_2O_4$ NPs synthesized using tragacanth gum.

It is well known that protein glycation leads to the accumulation of advanced glycation end-products (AGEs) and oxidative stress, which disrupt the structure and function of proteins in various diseases (Jasim et al., 2022; Ahmad et al., 2024). Given the involvement of AGEs and oxidative stress in disease pathology, it is crucial to explore novel agents capable of inhibiting the glycation process and slowing AGE formation.

The UV analysis of the sample treated with NPs demonstrated the reduced hyperchromicity suggests inhibitory action of NPs on glycation reaction that corroborates with previous studies showing the inhibitory strength of various nanoparticles (Ansari et al., 2016; Ashraf et al., 2018; Kim et al., 2012; Ashraf et al., 2014; Kim et al., 2012). The increase in the absorbance or hyperchromicity may be due to exposure of aromatic amino acids resulting from unfolding of protein upon glycation (Alouffi et al., 2022).

It is well-established that the free amino groups of lysine and arginine are the primary sites of glycation in proteins (Allaman et al., 2015; Kim et al., 2012; Ashraf et al., 2015). The availability of lysine and arginine residues in presence of $CuFe_2O_4$ NPs, compared to without of $CuFe_2O_4$ NPs indicates anti-glycation potential of $CuFe_2O_4$ NPs. This may be caused by reduction of the interaction between residues lysine and arginine with methylglyoxal, thereby inhibiting the glycation reaction at an early stage, as demonstrated in other studies (Ansari et al., 2016; Ashraf et al., 2018; Ashraf et al., 2014).

The declined nitroblue tetrazolium (NBT) reduction is due to formation of lesser amount of ketoamines in presence of $CuFe_2O_4$ NPs as compared to glycated sample, indicating disturbance of reaction between BSA and methylglyoxal/early glycation reaction is due to interference of $CuFe_2O_4$ NPs. Since numerous studies have suggested that the reduction of NB could result from the formation of superoxide radicals and the degradation byproducts of ketoamines. The presence of ketoamines serves as an indicator of the early stages of the glycation process and is considered a crucial precursor to the formation of advanced glycation end products (AGEs) (Alyahyawi et al., 2023; Azevedo et al., 1988).

The high contents of HMF at sixth day of incubation in glycated sample corresponds to the NBT assay result (Ahmad et al., 2012). The decreased ketoamine and HMF contents clearly demonstrate $CuFe_2O_4$ NPs are capable to slow down the early glycation reaction, consequentially reduced AGEs formation.

It is well-recognized fact that oxidative stress is generated during glycation reaction in disease condition such as diabetes and in neurodegenerative diseases, causing severe damage to biomolecules (Leszek et al., 2016; Khan et al., 2016). Protein carbonyl content is indicative of one of the most important biomarkers of protein oxidation and AGEs formation (Albarracin et al., 2012; Levine et al., 1994). Ketoamines, which are early glycation products, are transformed into protein carbonyl compounds through a protein enediol intermediate, producing superoxide radicals that contribute to oxidative stress both *in vivo* and *in vitro*. This oxidative stress causes irreversible damage to proteins during the glycation process (Azevedo et al., 1988; Maarfi et al., 2023).

The statistically significant reduction in carbonyl content in the $CuFe_2O_4$ NPs-treated group (p < 0.01) supports the conclusion that $CuFe_2O_4$ NPs exhibit antiglycation properties, likely by blocking reactive groups involved in glycation. This inhibition of glycation is further supported by the observed decrease in lysine, arginine, ketoamines, HMF, and carbonyl content, implying that $CuFe_2O_4$ NPs may protect against glycation-induced protein damage (Ansari et al., 2016; Ashraf et al., 2014).

The decrease of fluorescence intensity in presence of $CuFe_2O_4$ NPs points towards its anti-glycative activity. This fluorescence study is consistent with studies demonstrating similar antiglycating properties of other nanoparticles like silver and ZnO (Ansari et al., 2016; Kim et al., 2012; Kim et al., 2012). So, this reaffirms the hypothesis that $CuFe_2O_4$ NPs could serve as effective anti-glycating agents.

The reduction in fluorescence intensity indicates $CuFe_2O_4$ NPs effectively inhibited protein aggregation, potentially by disrupting the glycation process. This result complements previous work, which highlights the role of glycation in promoting protein aggregation and cross-linking, contributing to various metabolic disorders (Lo et al., 1994; bayashi et al., 1996).

Similarly, the analysis using ANS dye further validated the ability of $CuFe_2O_4$ NPs to mitigate the structural alterations in BSA caused by glycation (Figure 7B). The increase in ANS fluorescence in the glycated sample indicates the formation of hydrophobic patches, while a decrease in the treated sample suggests that the NPs prevented the exposure of these hydrophobic regions. These findings are congruent with earlier studies that used similar dyes to monitor protein conformational changes upon glycation and confirmed the protective role of nanoparticles in maintaining protein integrity (Arafat et al., 2014).

Finally, the measurement of intrinsic tryptophan fluorescence provided key insights into the preservation of protein structure. A severe decrease in fluorescence intensity in the glycated sample indicates significant tryptophan residue destruction due to glycation (Figure 8) (Ashraf et al., 2018; Shaklai et al., 1984). In contrast, the NPs-treated sample showed increase (8%) in fluorescence, confirming that $CuFe_2O_4$ NPs preserved the protein's structural integrity, echoing findings from studies with other nanoparticles like ZnO (Bhogale et al., 2013).

One interesting and somewhat unexpected observation was the differential behavior of nanoparticles in protecting proteins. While $CuFe_2O_4$ NPs demonstrated strong protective effects in this study, the literature suggests that not all nanoparticles interact with proteins in the same way. For instance, gold NPs have been shown to cause conformational changes in BSA (Bhogale et al., 2013), while ZnO and carbon C60 nanoparticles reportedly do not induce significant structural modifications (Bhogale et al., 2013; Liu et al., 2012). This highlights the complexity of nanoparticle-protein interactions, which seem to depend on several factors, including the specific properties of the nanoparticles and the proteins involved.

Since, nanoparticles have received much attention due to their toxicity. Nanoparticles can easily cross the cell membranes and interact with intracellular metabolism (Hanley et al., 2009). In vivo studies have reported the astrocyte swelling, blood-brain barrier destruction, oxidative stress induced by free radicals, alteration of gene expression and neuronal degeneration on exposure of nanoparticles (Wang M. F. RahmanJ. et al., 2009; Sharma et al., 2010; Tang et al., 2009). Reactive oxygen species (ROS) generation is one of the mechanisms for nanoparticle toxicity (Wang et al., 2013; Elsaesser and Howard, 2012). Interaction of nanoparticle with cells instigating ROS formation, mitochondrial respiration, and NADPH-dependent enzyme systems (Regoli and Giuliani, 2014; Jomova et al., 2012; Chen et al., 2011). While, phagocytosis leads the generation of ROS upon internalization of nanoparticles (Regoli and Giuliani, 2014; Jomova et al., 2012; Soenen et al., 2011). Disproportionate production of free radical is considered the basis of apoptosis and DNA damage (Ryter et al., 2007; Li and Osborne, 2008).

Undeniably, as existing *in vitro* and *in vivo* toxicity testing methods are chiefly employed to evaluate the acute and subacute toxicity of NPs, while nanotoxicity testing methods for chronic longterm NPs exposure, which are critical for predicting chronic toxicity in humans, are still deficient. Therefore, there is an imperative necessity to develop new powerful tools to evaluate and understand the mechanisms of NP toxicity as well as addressing the knowledge gap.

While $CuFe_2O_4$ NPs demonstrated anti-glycating effects *in vitro*, their behavior *in vivo* may differ due to interactions with various biomolecules, such as proteins, nucleic acids, and lipids. Factors such as solvation forces, hydrogen bonding, and Van der Waals interactions at the nano-bio interface may affect their reactivity in biological environments. Moreover, it is important to account for external factors such as pH, temperature, and the type of reducing sugars or inhibitors present in glycation reactions, all of which could influence the efficacy of nanoparticles (Wangoo et al., 2008; Turci et al., 2010). For instance, the glycation reaction has been shown to be highly pH-dependent, with an increase in glycation under alkaline conditions. Understanding how $CuFe_2O_4$ NPs behave under varying conditions will be essential for optimizing their use as anti-glycating agents.

Further research should focus on elucidating the exact mechanisms of action of $CuFe_2O_4$ NPs, particularly their interaction with protein structures and free amino groups.

Additionally, *in vivo* studies are needed to assess their safety, bioavailability, and efficacy in more complex biological systems. Nanomedicine continues to offer immense potential for treating diseases associated with AGEs, but a thorough evaluation of the physicochemical properties of nanoparticles and their impact on human health is critical for advancing this field (Wong et al., 2008; El-Ghorab et al., 2010).

Another limitation involves the potential toxicity of nanoparticles. Although $CuFe_2O_4$ NPs showed beneficial effects in this study, the literature on nanoparticle toxicity is inconsistent. For example, ZnO nanoparticles have been reported to exhibit both acute and subacute toxicity depending on the mode of administration and dosage (Tian et al., 2015; Sruthi et al., 2017; Wang et al., 2006; Hackenberg et al., 2011). Furthermore, some studies have highlighted the ability of nanoparticles to generate reactive oxygen species (ROS), which could lead to oxidative stress and associated pathological conditions like inflammation and fibrosis (Long et al., 2006). These findings emphasize the need for a more detailed investigation into the toxicity, bioavailability, and tissue distribution of $CuFe_2O_4$ NPs before they can be considered for clinical applications.

Additionally, the inhibition of AGE formation by $CuFe_2O_4$ NPs raises questions about the precise mechanism by which these nanoparticles exert their effects.

Since mechanism of AGEs formation involved various steps, hence, different compounds have been identified which can prevent this process at different steps (Khalifah et al., 1999; Rahbar and Figarola, 2002; Wang J. et al., 2009). Several classes of glycation inhibitors have been developed, including those that compete with carbohydrates for amino groups, react with aldoses and ketoses, interfere with side reactions, trap RCS, or act as AGE breakers. Examples of such inhibitors include aminoguanidine, pyridoxamine, carnosine, benfotiamine, aspirin and ALT-711, each demonstrating different mechanisms of action. Similarly, several studies have shown various nanoparticles may also act as antiglycating agents like that of naturals agents (Yu et al., 2015), acting at different stages of AGEs formation and on different reacting groups. As previous studies have suggested that several nanoparticles like SeNPs, GNPs, AuNPs, ZnONPs, etc. may inhibit the glycation process by competing with -NH2 groups of lysine and Arginine as well as arresting the reactive group of glycating agents (Kim et al., 2012; Rafia et al., 2023; Du et al., 2020; Hu et al., 2005). Therefore, mechanisms of inhibition of AGEs of CuFe₂O₄ NPs may be involved in the trapping of reactive amino groups, making them unable to react with MG and sequestration of reacting groups. Though, the exact mechanism of action of CuFe2O4 NPs is still ambiguous. Therefore, further investigation is needed to clarify these inhibitory pathways and their molecular details.

CuFe₂O₄ NPs offer several advantages over other oxide NPs due to their superior redox properties, protein binding, biocompatibility, and anti-glycation efficacy. This makes them particularly promising for therapeutic applications targeting AGE-related diseases, where stability, reduced toxicity, and potent anti-glycative actions are essential. Their performance surpasses that of commonly studied NPs like ZnO, TiO₂ (Alenazi et al., 2022), Ag, Fe₃O₄, and others, positioning CuFe₂O₄ as a potent agent with multi-functional capabilities in glycation inhibition and disease prevention.

Conclusion

In conclusion, while $CuFe_2O_4$ NPs show great promise as antiglycating agents with potential applications in treating AGE-related diseases, careful consideration of their biological interactions and toxicological profiles will be necessary for their successful integration into clinical practice.

The findings from this study highlight the potential of magnetically biosynthesized copper ferrite nanoparticles (CuFe₂O₄ NPs) as promising agents in the fight against advanced glycation end products (AGEs). AGEs are key contributors to the onset and progression of serious health conditions such as diabetic complications and neurodegenerative diseases, including Alzheimer's and Parkinson's. The demonstrated ability of CuFe₂O₄ NPs to inhibit AGE formation and protect protein integrity presents a new avenue in the development of therapies aimed at mitigating the harmful effects of AGEs, offering hope for improving patient outcomes.

This study offers the potential to develop nanoparticle-based therapeutics for managing diabetes and associated complications, which continue to rise globally. Additionally, the implications extend to neurodegenerative diseases, where AGE accumulation has been associated with neuronal damage and cognitive decline. Effective management of AGEs could contribute to delaying disease progression, improving quality of life, and reducing the burden on healthcare systems.

AGEs play a critical role in the pathophysiology of diabetic complications and neurodegenerative conditions. In diabetes, AGEs contribute to complications such as retinopathy, nephropathy, neuropathy, and cardiovascular disease by damaging proteins, lipids, and nucleic acids. Similarly, in neurodegenerative diseases, AGEs are implicated in promoting oxidative damage and neuronal cell death, thereby accelerating cognitive decline.

Mitigating the formation of AGEs is vital because it interrupts this destructive cycle, offering protection against oxidative stress and preserving the structural integrity of essential biomolecules. Effective antiglycation strategies could slow the progression of these diseases, prevent complications, and improve patient survival and quality of life.

While this study demonstrates the potential of $CuFe_2O_4$ NPs as antiglycation agents, future research is needed to fully explore their clinical applicability. The detailed mechanistic studies should be conducted to understand the precise molecular mechanisms by which $CuFe_2O_4$ NPs inhibit AGE formation and protect against protein damage. Before clinical application, the biocompatibility and potential toxicity of $CuFe_2O_4$ NPs must be rigorously evaluated to ensure safety for human use.

This research adds to the growing body of knowledge on the use of nanoparticles as therapeutic agents for AGE inhibition. While several studies have explored the potential of antioxidants and natural extracts in combating AGEs, the use of copper ferrite nanoparticles synthesized through a green, biosynthetic approach (using Aloe vera leaf extract) represents a novel and environmentally friendly method of producing potent antiglycation agents.

In comparison with other studies that focus on AGE inhibitors such as aminoguanidine and natural polyphenols, $CuFe_2O_4$ NPs offer distinct advantages, including their magnetic properties, which could be harnessed for targeted drug delivery. Moreover, the antioxidative capabilities of these nanoparticles offer an additional layer of protection against oxidative stress, a significant factor in both diabetic and neurodegenerative diseases.

Overall, this research lays the foundation for the development of nanoparticle-based therapeutics for AGE-related pathologies, expanding the horizon of potential treatments for complex chronic diseases.

Future direction

Despite promising findings regarding the use of nanoparticles in various *in vitro* and *in vivo* studies, several limitations are needed to be addressed for future studies. One key limitation is the incomplete understanding of nanoparticles' biocompatibility and bioactivity in complex biological systems leading to its toxicity.

Finally, a comprehensive understanding of NP toxicity from *in vivo* studies is essential before reaching a definitive consensus on the overall toxicity of nanoparticles (NPs), about safety and potential risks. In a while, we believe that the convergence of related disciplines like medicine, material science, chemistry, and artificial intelligence holds huge potential to revolutionize nanotoxicity research and warrant the safer application of nanoparticles (NPs) in humans.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SAh: Supervision, Writing – original draft. US: Conceptualization, Writing – review and editing. JA: Formal Analysis, Writing – review and editing. AmA: Funding acquisition, Writing – review and editing. MA: Methodology,

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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