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POTENCIAL USO DE NANOPARTÍCULAS DE ÓXIDO DE COBRE EM PRODUTOS DE APLICAÇÃO TÓPICA CUTÂNEA: AVALIAÇÕES INICIAIS

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Ciências da Saúde, Universidade de Brasília, como requisito parcial à obtenção do título de Mestre em Ciências Farmacêuticas.

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RESUMO

Com a propagação de doenças infecciosas e o surgimento de bactérias super-resistentes a antibióticos convencionais, as nanopartículas, especialmente as de óxido de cobre, têm se revelado como uma promissora alternativa antimicrobiana, para isso o primeiro passo é avaliar o comportamento desse nanomaterial na pele visando uma aplicação tópica. O trabalho trata de avaliações para um possível desenvolvimento de produto antisséptico: (i) avaliar a atividade antimicrobiana e (ii) desenvolver e validar de um método bioanalítico capaz de quantificar o Cobre (Cu) na pele a partir de um possível novo produto. Para isso, nanopartículas de óxido de cobre (CuO-NP) foram sintetizadas e a concentração mínima inibitória (MIC) dessas partículas foi avaliada frente à *Staphylococcus aureus* ATCC 29213. Para a quantificação de Cu na pele, foi desenvolvido e validado um método por Espectrometria de Absorção Atômica por Chama (EAA). Dados de microscopia eletrônica de transmissão revelaram partículas obtidas em bastão com comprimento e largura de 30.8 ± 0.7 e 6.1 ± 0.1 nm, respectivamente. O MIC contra *S. aureus* foi de 424 µg/mL. Outros métodos, como espectroscopia de UV-visível e fotoluminescência, podem confirmar um fenômeno de agregação. Para desenvolvimento do método bioanalítico foi proposto um método de digestão da pele utilizando os ácidos H_2O_2, HNO_3, H_2SO_4 e HCl. O método desenvolvido e validado revelou ser seletivo, com linearidade r^2: 0.993 e limites de detecção (0.1709 µg/ml) e quantificação (0.5178 µg/ml), além de ter sido avaliado quanto aos parâmetros de robustez, precisão e exatidão com taxas de recuperação de Cu acima de 88.0 ± 3.2 µg/ml. Foi possível verificar, após estudo de permeação *in vitro* utilizando pele de orelha de suíno que as CuO-NP não penetraram a pele intacta. Desta forma, a ação superficial de possíveis produtos antissépticos pode ser esperado a partir de novos ensaios. De qualquer forma, representam apenas avaliações iniciais e mais estudos são necessários neste sentido.

Palavras-chave: atividade antibacteriana; dose resposta bifásica; nanopartículas de óxido de cobre; método bioanalítico; EAA

ABSTRACT

With the spread of infectious diseases and the emergence of bacteria that are super resistant to conventional antibiotics, nanoparticles, especially those of copper oxide, have proved to be a promising antimicrobial alternative, for which the first step is to evaluate the behavior of this nanomaterial on the skin for topical application. The work deals with evaluations for the possible development of an antiseptic product: (i) to evaluate the antimicrobial activity and (ii) to develop and validate a bioanalytical method capable of quantifying Copper (Cu) in the skin from a possible new product. For this, copper oxide nanoparticles (CuO-NP) were synthesized and these particles minimum inhibitory concentration (MIC) was evaluated against *Staphylococcus aureus* ATCC 29213. For the quantification of Cu in the skin, Spectrometry Flame Atomic Absorption (EAA) developed and validated a method. Transmission electron microscopy data revealed particles obtained from sticks with length and width of 30.8 ± 0.7 and 6.1 ± 0.1 nm, respectively. The MIC against *S. aureus* was 424 µg/mL. Other methods such as UV-visible and photoluminescence spectroscopies could confirm an aggregation phenomenon. For the development of the bioanalytical method, a skin digestion method using H_2O_2, HNO_3, H_2SO_4, and HCl acids was proposed. The developed and validated method proved to be selective, with linearity r^2: 0.993 and limits of detection (0.1709 µg/ml) and quantification (0.5178 µg/ml), in addition to having been evaluated for parameters of robustness, precision, and accuracy with rates of Cu recovery above 88.0 ± 3.2 µg/ml. After an *in vitro* permeation study using swine ear skin, it was possible to verify that the CuO-NP did not penetrate the intact skin. Thus, the surface action of possible antiseptic products can be expected from new tests. In any case, they only represent initial assessments and further studies are needed in this regard.

Keywords: antibacterial activity; biphasic dose response; Copper oxide nanoparticles; bioanalytical method; FAAS

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INTRODUÇÃO

A pele danificada, quando exposta, perde sua função de barreira, o que a torna suscetível e vulnerável a infecções bacterianas prolongando, dessa forma, o tempo de cicatrização da ferida. Esse ambiente leva ao estabelecimento de feridas crônicas e não cicatrizadas, além disso, o aparecimento de um ambiente úmido nesses locais tem demonstrado relevância, pois propicia o crescimento de microrganismos oportunistas (ZHANG et al., 2022). Para isso, o uso de antimicrobianos está em destaque.

As bactérias apresentam peculiaridades estruturais, todavia ambos os tipos, gram positivas e negativas tem uma superfície carregada negativamente (SLAVIN et al., 2017). As gram positivas como *Staphylococcus aureus* possuem uma espessa camada de peptidoglicano formado por cadeias lineares e ácidos teicóicos carregados negativamente que se estendem da parede celular à superfície, enquanto as gram negativas revelam estrutura um pouco mais complexa, possuindo além de uma camada mais fina de peptidoglicano, uma membrana externa fosfolipídica com lipopolissacarídeos que contribuem no aumento da carga superficial negativa (SÁNCHEZ-LÓPEZ et al., 2020)

Os antibióticos convencionais têm sido amplamente utilizados para o tratamento de feridas superficiais a fim de controlar as infecções bacterianas. O uso crescente desses fármacos tem efeito no surgimento de resistência a antibióticos, o que facilita a seleção de cepas multirresistentes (LIAO et al., 2019).

Nesse cenário, surgem as nanopartículas a base de metais, as quais têm chamado atenção devido às suas propriedades antimicrobianas, óticas e catalíticas (ROY et al., 2016). Essas nanopartículas inorgânicas têm se popularizado e representam uma solução promissora ao uso demasiado de antibióticos tradicionais (SLAVIN et al., 2017).

Nesse contexto, as nanopartículas óxido metálicas derivadas de cobre, têm se revelado alternativas auxiliares efetivas no processo de assepsia de feridas devido à atividade antimicrobiana. Ademais, com a presença de doenças infecciosas atuando como ameaça à saúde global e a emergente seleção de cepas nas últimas décadas, tratamentos baseados na tecnologia de nanopartículas com atividade antimicrobiana é uma promissora alternativa na busca pelo controle da resistência bacteriana (ERMINI; VOLIANI, 2021; ZANONI et al., 2019).

As nanopartículas à base de metais apresentam mecanismos de toxicidade bacteriana inespecíficos, pois não se ligam a um receptor específico, ampliando, por conseguinte, o espectro de atividade antibacteriano (SÁNCHEZ-LÓPEZ et al., 2020). Estudos indicam que determinadas características desses materiais, como a carga superficial, fazem com que as

nanopartículas sejam mais ou menos internalizadas por microrganismos (ERMINI; VOLIANI, 2021; PARSAI; KUMAR, 2019). Enquanto que o cobre pode causar danos a diversas funções celulares e tornar-se um inibidor microbiano eficaz diante de sua toxicidade. Em geral, o cobre é capaz de gerar espécies reativas de oxigênio (MA et al., 2022; FU et al., 2014), além de estar envolvido na imunidade inata durante a fagocitose bacteriana (MA et al., 2022; DUPONT et al., 2011).

Diante disso, é necessário o estudo da aplicação destas nanopartículas em matrizes biológicas como a pele, unhas e cabelo. Vale destacar, dentre elas, a pele suína tendo em vista as vantagens apresentadas como baixo custo, fácil transporte e armazenamento, além da semelhança à pele humana (FORERO-MENDIETA et al., 2022). Essa matriz revela uma grande área de exposição intencional e não intencional a produtos químicos na forma de partículas, cosméticos e produtos de manuseio (ZANONI et al., 2019), por isso a aplicação de CuO-NP e sua consequente quantificação na pele é tão relevante.

Tendo em vista a atual abordagem de administração tópica de nanopartículas com atividade antimicrobiana na pele, faz-se necessário o desenvolvimento de um método para quantificar CuO-NP, e dessa forma, conhecer a quantidade permeada tendo em vista a vantagem desse tipo de administração.

Diante do exposto, o trabalho teve como objetivo avaliar a atividade antibacteriana frente à *Staphylococcus aureus*, além de validar um método de quantificação de nanopartículas de óxido de cobre e analisar o perfil de permeação cutânea do cobre na pele suína.

OBJETIVO GERAL

- Avaliar a atividade antibacteriana da nanopartícula de óxido de cobre frente a cepas ATCC 20213 de *Staphylococcus aureus* e validar um método bioanalítico capaz de quantificar o cobre da pele a partir de um possível novo produto.

OBJETIVOS ESPECÍFICOS

- Avaliar a concentração inibitória mínima das nanopartículas de óxido de cobre em ensaios *in vitro* na presença de *S. aureus*;
- Adaptar um método de digestão para a pele suína;
- Validar um método bioanalítico capaz de quantificar o cobre na pele.

REFERENCIAL TEÓRICO

Aplicação das nanopartículas

As nanopartículas têm aplicações em diversos campos, incluindo fabricação de produtos químicos e aplicações biológicas, o que tem aumentado o interesse e o uso destes materiais no mercado (ZANONI et al., 2019; GAWANDE et al., 2016). Enquanto que nanopartículas à base de cobre têm aplicações como aditivos em produtos para pele (RAMTEKE et al., 2020), além de atuarem como revestimento contra a proliferação microbiana na prática odontológica (POGGIO et al., 2020).

Antes que houvesse algum conhecimento sobre germes e vírus, o poder desinfetante do cobre já era observado, pois os soldados egípcios e os babilônios faziam uso de aparas de metal das espadas, tendo o cobre como constituinte, em feridas abertas para diminuir a infecção (POGGIO et al., 2020). Atualmente, o cobre foi reconhecido pela Agência de Proteção Ambiental Americana (EPA) como melhor metal antimicrobiano, o que levou à aprovação de registros de ligas de cobre como materiais antimicrobianos com benefícios à saúde pública devido à facilidade de acesso e baixo custo a esse material assim como sua biocompatibilidade (ERMINI; VOLIANI, 2021; POGGIO et al., 2020).

Técnicas de preparo das nanopartículas de cobre

No que tange ao preparo de nanopartículas de cobre, diversas vias têm sido estudadas buscando a obtenção de melhores sínteses metálicas envolvendo polímeros, dentre eles a polivinilpirrolidona e quitosana, assim como surfactantes com a função de estabilizar e formar revestimentos na superfície das nanopartículas (USMAN et al., 2013). Para síntese verde, alguns autores têm explorado o envolvimento de extratos de plantas a fim de estabilizar as nanopartículas (SHAMELI et al., 2012).

Posto isto, é válido ressaltar que diversas técnicas podem ser utilizadas para preparar nanopartículas de cobre tais como: radiação induzida, redução térmica, síntese de vapor de metal, técnicas de microemulsão e irradiação a laser(USMAN et al., 2013).

Estes processos dão origem à materiais de diferentes formas, dentre eles bastonetes curtos, esferas, hastes e bastão, os quais demonstram diversos pontos críticos que podem ser alterados como tamanho, forma, química e área de superfície, os quais têm influência na resposta biológica das nanopartículas (MISRA et al., 2014).

Destaque das nanopartículas de cobre

Historicamente, os materiais à base de cobre apresentavam vantagens no uso devido à sua atividade bactericida observada desde as civilizações antigas como a antiga persa e,

atualmente, o cobre tem sido explorado como agente de purificação de água e inativação de bactérias e outros microrganismos (AKHAVAN; GHADERI, 2010).

Certas bactérias do gênero *Staphylococcus*, notadamente a espécie *S. aureus* ocupa um dos primeiros lugares entre os agentes causadores de infecções nosocomiais (OTTO, 2009). Dessa forma, é válido ressaltar que os antibióticos ajudaram a combater notórios microrganismos, entretanto nas últimas décadas, têm ocorrido a seleção de cepas resistentes aos tratamentos preconizados (RAMTEKE et al., 2020). Inclusive as organizações internacionais da saúde alertam exatamente para o crescimento exponencial de surgimento de dessas cepas resistentes causados pelo uso indiscriminado de antimicrobianos (ERMINI; VOLIANI, 2021; AKHAVAN; GHADERI, 2010).

Diante disso, com o surgimento de potenciais ameaças à saúde, como o relatado e o declínio na descoberta de novos antimicrobianos, surge a necessidade da exploração de abordagens inovadoras (ERMINI; VOLIANI, 2021). Nesse contexto, têm surgido estudos com as nanopartículas à base de metais como o cobre, as quais têm demonstrado comportamentos importantes devido às suas características intrínsecas, revelando resultados promissores de inibição de crescimento de bactérias gram positivas e negativas (ERMINI; VOLIANI, 2021; SÁNCHEZ-LÓPEZ et al., 2020).

Mecanismo de interação do cobre com as bactérias

A literatura relata que o cobre interage com as bactérias de várias maneiras. Estudos revelam a potente ação antimicrobiana das nanopartículas de cobre frente a outros materiais de tamanho comum, entretanto, a razão para tal efeito não está completamente elucidado(MA et al., 2022). Quando comparado a outros materiais moleculares com cobre na sua composição, as nanopartículas contendo este analito podem exercer sua ação por meio de mecanismos exclusivos devido a maior área de superfície e estrutura cristalina diferente (MUTALIK et al., 2022). Em solução, as nanopartículas podem se dissolver mais rapidamente, exercendo maior efeito antimicrobiano através da liberação de maior quantidade de íons metálicos (KARLSSON et al., 2013).

Dentre os diversos relatos na literatura, o processo antimicrobiano dessas nanopartículas pode ser descrito através da produção de espécies reativas de oxigênio, o que resulta em alta toxicidade para as bactérias(ERMINI; VOLIANI, 2021), podem reagir com proteínas e DNA, além de destruir parede e membrana celular (WANG et al., 2013). Ghuglot et al. (2021) afirmam que o cobre em concentração mais baixa, atua como cofator para metaloproteínas e

enzimas, enquanto que em concentrações maiores, têm efeitos tóxicos sobre as células bacterianas e inibe, dessa forma, seu crescimento.

Quantificação do cobre

Dessa forma, é relevante o estudo destas nanopartículas na pele. O uso de matrizes biológicas é importante também para análise de contaminantes tóxicos, uma vez que diferentes estudos analisaram a presença de metais em cabelos e unhas a fim da realização do biomonitoramento de concentração de diferentes metais no corpo humano (ESTEBAN; CASTAÑO, 2009). Tais matrizes biológicas, assim como a pele, permitem a digestão em meio ácido acompanhada de um agente oxidante para degradar a matéria orgânica para serem analisadas por espectrometria de absorção atômica (FORERO-MENDIETA et al., 2022).

Outros oligoelementos como zinco, magnésio e selênio, além de cobre já foram detectados em estudos envolvendo matrizes biológicas a fim de observar a presença destes analitos como contaminantes em crianças por meio da espectroscopia de absorção atômica (LAKSHMI PRIYA; GEETHA, 2011).

Ademais, para quantificação desses materiais, o método de espectrometria de absorção atômica por chama é envolvido, tendo em vista que a análise de traços de metais é difícil devido às concentrações muito baixas e dificuldade de análise em matrizes complexas como visto em estudo proposto por Behbahani et al., (2013), onde foi possível desenvolver um método simples e eficaz capaz de quantificar cobre e prata em solução aquosa em método acoplado a espectrometria de absorção atômica por chama (FAAS). Da mesma forma, Divrikli et al. (2007) desenvolveu um método baseado na concentração de íons metálicos em filtro de membrana de nitrato de celulose para determinação dos analitos por espectrometria de absorção atômica por chama (FAAS) em amostras de águas encanadas.

A análise de água, solo e materiais geológicos também foi possível devido ao desenvolvimento de método partindo da pré-concentração para enriquecimento e separação de metais como o cobre obtendo resultados em nanogramas por litro em espectrometria de absorção atômica em forno de grafite (YANG et al., 2009). Outros analitos de interesse na atualidade também já foram detectados por este método. A prata, outro metal em evidência, atraente devido sua atividade antimicrobiana, ganhou evidência na detecção por espectrometria de absorção atômica por chama em matrizes complexas ao utilizar discos de membrana de octadecil sílica modificados, possibilitando a separação desse metal de interesse na presença de outros íons (ROFOUEI et al., 2009). Finalmente, com o objetivo de detectar nanopartículas de

cobre na pele, Zanoni et al. (2019) realizou estudos de penetração na pele por meio da espectrometria de emissão atômica por plasma acoplado indutivamente (ICP).

Interação dos nanosistemas inorgânicos com a pele

Uma vez que o estudo e o uso de nanopartículas metálicas têm se intensificado, os humanos ficaram mais propensos e expostos ocupacionalmente através de produtos de consumo ou no meio ambiente, todavia ainda existem lacunas a serem solucionadas pela comunidade científica (ZANONI et al., 2019). O impacto das nanopartículas óxido metálicas na saúde humana têm sido estudadas devido às preocupações de segurança para os humanos, animais, bem como para a biota aquática (KARLSSON et al., 2008).

Um dos mecanismos mais abordados por trás dos efeitos à saúde induzido por estes materiais é a capacidade da formação de estresse oxidativo causado por espécies reativas de oxigênio (DONALDSON et al., 2001). Entretanto, nenhum estudo se concentrou em investigar e comparar uma variedade de nanopartículas em relação ao dano ao DNA e marcadores de estresse oxidativo em humanos, apesar de serem descritas como potentes causadoras de dano ao material genético das bactérias (KARLSSON et al., 2008).

Este trabalho foi dividido em duas partes. O primeiro capítulo teve o objetivo de confirmar a inibição do crescimento de *S. aureus* tratados com CuO-NP. Este ensaio de viabilidade microbiana *in vitro* foi conduzido com concentrações de CuO-NP espalhadas na faixa de 0,4 a 848,0 μg/ml. Os achados revelaram um comportamento pouco debatido na literatura acerca da bidose resposta representada por duas fases específicas e confirmadas por técnicas de espectroscopia como UV-visível e fotoluminescência.

Dessa forma, o segundo capítulo teve como objetivo desenvolver e validar um método bioanalítico simples e eficiente de quantificação de cobre a fim de realizar ensaios de permeação visando a aplicação do material em formulações tópicas. O método proposto foi testado quanto à robustez para recuperar e quantificar este metal das camadas da pele em concentrações compatíveis com ensaios de permeação, além da determinação dos limites de detecção e quantificação, o que ajuda a fornecer subsídios para realização de ensaios cinéticos.

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Colloidal copper oxide nanoparticles leading to a biphasic dose-response in growth inhibition of *Staphylococcus aureus*

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Aim: The dose response in growth inhibition of *Staphylococcus aureus* treated with colloidal copper oxide nanoparticles (CuO-NP) was evaluated. **Methods:** An *in vitro* microbial viability assay was conducted with CuO-NP concentrations spreading over the 0.4–848.0 μg⁄ml range. The dose–response curve was modeled with a double Hill equation. UV-Visible absorption and photoluminescence spectroscopies allowed tracking concentration-dependent modifications in CuO-NP. **Results:** Two specific phases separated by the critical concentration of 26.5 μg⁄mL were observed in the dose–response curve, with each exhibiting proper IC_{50} parameters, Hill coefficients, and relative amplitudes. Spectroscopy techniques reveal the occurrence of a concentration-triggered aggregation of CuO-NP starting from this critical concentration. **Conclusion:** The findings demonstrate a dose-related change in *S. aureus* sensitivity to CuO-NP, which probably arises from the aggregation of this agent.

Plain language summary: Antibacterial agents are often used to stop the growth of bacteria such as *Staphylococcus aureus (S. aureus)*. Copper oxide nanoparticles (CuO-NP) stand as a promising candidate for this purpose. Generally, the agent´s effectiveness is inspected following a dose-response curve in which de agent´s antibacterial response is plotted against its dose (concentration). In this work, employing an extended mathematical interpretation we were capable of discerning experimentally the existence of two stages of dose-response (biphasic dose-response) in the treatment of *S. aureus* with CuO-NP. These results in combination with insights from spectroscopic techniques lead to the understanding that the biphasic behavior arises from the aggregation of CuO-NP at high concentrations. Therefore, according to the adopted concentration to treat *S. aureus*, the agent can behave as a dispersed nanoparticle or as an aggregated nanoparticle. In summary, understanding whether antibacterial agents transform as a function of concentration is important in determining their practical applications.

Tweetable abstract: Aggregation of copper oxide nanoparticles leads *Staphylococcus aureus* to follow a biphasic dose response in *in vitro* viability assay.

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Keywords: aggregation • antibacterial activity • biphasic dose response • copper oxide nanoparticles • optical spectroscopy • *Staphylococcus aureus*

Staphylococcus aureus is one of the most common infectious bacteria worldwide, responsible for morbidity and mortality of a wide variety of diseases, ranging from moderately severe skin infections to sepsis [1]. Estimates in the literature report that *S. aureus* bacteremia has an incidence rate ranging from 20 to 50 cases per 100,000 per year [2]. Treatment of *S. aureus* infections is complicated by antibiotic resistance. Indeed, resistance to almost all available antibiotics, including methicillin and traditional beta-lactam antibiotics have been reported [3–5]. Currently, vancomycin stands as the last resort for treatment. However, both high and intermediate vancomycin-resistant strains have been reported [6,7], which are an additional public health concern. Novel treatments capable of circumventing bacterial resistance mechanisms are highly needed.

Promising advances in antibacterial activity have been achieved with the use of colloidal systems [8–13]. These systems are interesting for addressing problems related to antibiotic resistance due to their capability of inhibiting microbial growth through a variety of nonspecific and simultaneous effects, meaning that resistance development would require several simultaneous gene mutations [14]. Among the colloidal systems with antibacterial properties, metal oxide nanoparticles (MONPs) stand as tunable agents [8–13]. Specifically, copper oxide nanoparticles (CuO- NPs) have demonstrated excellent antimicrobial properties, and, because of the facility of their synthesis and obtention, they have also been employed in various medical products [14].

In general, the *in vitro* performance of MONPs is associated with their sizes and shapes [15–17], which can be modulated by physicochemical- and thermodynamical parameters such as pH, temperature, and concentration [18– 22]. Since the concentration can affect both the action of MONPs and their overall structure, this kind of system stands as an interesting candidate to exhibit a complex behavior in the treatment of biological models. Therefore, the evaluation of concentration-triggered changes in the *in vitro* activity of MONPs is a critical step aiming at their applications.

Most of the quantitative information of *in vitro* viability assays derives from dose-response curves, which correlate the concentration of the agents under test with parameters such as the minimum inhibitory concentration (IC_{50}), the curve slope coefficient, and the curve's relative amplitude [23–30]. All these features enable characterizing the effectiveness of the agent. Generally, these curves can be suitably modeled assuming a monophasic behavior through the use of the single Hill equation [23–30]. Although this equation is widespread in the literature, its misuse during experimental data interpretation can hinder the achievement of deeper analyzes.

In recent years, it has been demonstrated that some results cannot be properly fitted with the single Hill equation [31,32]. In some cases, the data can comprise multiphasic regimes, which are better fitted with alternative equations such as the double Hill equation. Indeed, important insights can be accessed when different phases are taken into account. As a consequence, besides being useful to revise previously reported single Hill fitted data [31], the multiphasic modeling also brings the opportunity to explore concentration-dependent target effects in cell biology [32].

This work reports a case study in which colloidal rod-shaped CuO-NP exhibits a biphasic dose-response against *Staphylococcus aureus* bacteria, implying two concentration-dependent phases, each characterized by specific IC_{50} parameters, Hill coefficients, and percentage amplitudes. Spectroscopic techniques reveal the occurrence of a concentration-triggered aggregation process in CuO-NP that can be correlated with the *in vitro* results.

Materials & methods

Materials

Copper (II) chloride dihydrate ($CuCl_2.2H_2O$, ≥99.0%) and dialysis tubing cellulose membrane were purchased from Sigma-Aldrich (Steinheim, Germany), sodium hydroxide pellets P.A. (NaOH) was purchased from Vetec (Rio de Janeiro, Brazil), trisodium citrate solution was purchased from Merck KGaA (Darmstadt, Germany), nitric acid (HNO_3, 70%) was purchased from Proquimios (Brazil), Mueller–Hinton broth was purchased from Kasvi (Campinas, Brazil) and resazurin P.A. was purchased from Dinamica (Campinas, Brazil). All reagents were used as received with no additional processing.

Synthesis of CuO-NPs

The CuO-NPs colloidal sample was synthesized in two steps following procedures described in the literature [17,33]. First, 75.0 ml of aqueous $CuCl_2.2H_2O$ solution (0.02 mol/l) prepared with ultrapure water (resistivity: 18.0 MOhm/cm) was heated until boiling under magnetic stirring. Next, 0.4 g (0.01 mol) of NaOH was added to the former solution, which was kept under heating and magnetic stirring for an additional 15 min. The resulting black precipitate of CuO-NPs was isolated, washed with ultrapure water and centrifuged three times. In a second step, the washed CuO-NPs were suspended in 50.0 ml trisodium citrate solution at 1% (w/v) and submitted to 10 min of sonication at 125 W in pulsed mode (1 s). Afterward, the sample was purified by dialysis for 48 h using cellulose regenerated bags (molecular weight cutoff = 12,000 g/mol). The concentration of the resulting CuO-NP suspension was obtained by atomic absorption spectroscopy employing a Varian (CA, USA) AA 240FS Fast Sequential flame atomic absorption spectrometer using a flame source derived from air/acetylene mixture. The colloidal sample, originally stored in a HNO_3 (2%) solution, was diluted in ultra-pure water for the measurements

The calibration curve in water was built with certified standard copper solution in a set of concentrations, from 1.5 to 9.0 ppm. All analyses were performed in quintuplicate.

Sample characterization

UV-visible (UV-Vis) absorption spectra were acquired in an Agilent (CA, USA) CARY UV-Vis-NIR spectropho- tometer (1.0-nm wavelength resolution), model 5000, employing a 1.0-mm path length quartz cuvette, whereas steady-state photoluminescence (PL) spectra were measured in a Perkin Elmer (MA, USA) EnSpire plate reader, model Multilabel Reader (1.0 nm wavelength resolution), with the samples being excited at 300 nm. All spectroscopic measurements were conducted at room temperature, employing ultrapure water as solvent. Dynamic light scattering and electrophoretic mobility experiments were conducted using the Zetasizer ZS90 (Malvern Pananalytical, Malvern, UK), with the colloidal system diluted until it reached 12.72 μg/ml to maintain it under the equipment's detection limit. Transmission electronic microscopy (TEM) was performed following this same dilution condition at room temperature in a JEM 1011 (JEOL, MA, USA).

In vitro microbial viability assay

Staphylococcus aureus (ATCC 29213; ATCC, VA, USA) was suspended in Mueller–Hinton broth and the density of the suspension was calibrated to 10^8 UFC/ml following the

procedures described in the literature [34]. The dose– response assay was performed adopting 96-well plates, with each well filled with 200.0 µl of the CuO-NP dispersion, 90 µl of the Mueller–Hinton broth and 10 µl of the inoculum. The concentration of the employed CuO-NP dispersion varied progressively from 0.4 to 848.0 µg/ml. The prepared plates were incubated for 24 h under 37 ºC. Afterward, 50.0 µl of resazurin solution (0.01% w/v) was added to each well, following 2 h of additional incubation in the same temperature condition. Negative and positive controls were set as the bacteria-free well (only broth and CuO-NPs) and the CuO-NP-free well (only broth and bacteria), respectively [35].

Data analysis

All experiments were conducted in triplicate and the quantitative data are shown in the M ± SD format, with M and SD accounting for the mean values and the standard deviations, respectively. For TEM analysis, the width (W) and length (L) of a set of 300 counted CuO-NPs were evaluated employing the ImageJ software. Afterward, the W and L counts were fitted as a normal distribution to inspect the central value and dispersion parameters. The mean value of the experimental dose–response curve was fitted employing the nonlinear equations defined in Equations 1–3. The goodness of the fit was based on the r^2 parameter.

Results

After synthesis, the CuO-NP suspension was properly characterized. TEM confirmed the homogeneous distribution of rod-shaped particles (Figure 1 A & B), which exhibit mean L and W of 30.84 ± 0.75 and 6.09 ± 0.10 nm, respectively (Figure 1C& D).

For a diluted (12.7 µg/ml) CuO-NP sample, the mean hydrodynamic diameter of 80.79 ± 0.81 nm and polydispersity index of 0.29 ± 0.03 were obtained. The electrophoretic mobility of this sample yielded a zeta potential of -12.33 ± 2.00 mV. In Figure 2, the UV-Vis absorption and steady-state PL spectra of CuO-NPs in the same dilution condition demonstrate that the most intense absorption and PL bands locate at 242 and 355 nm, respectively. These results are in good agreement with the literature [36–39].

Concerning the antibacterial properties of CuO-NP against *S. aureus*, the dose–response curve shows a clear reduction in the bacteria's percentage viability (V) as a function of the increase in the agent concentration (0.4– 848.0 µg/ml; see Figure 3A). The curve fitting was performed employing two approaches. The first is based on the single Hill equation (Equation 1), in which a monophasic regime is assumed, with V being characterized by the associated IC_{50}, the Hill coefficient (h), and the overall amplitude (A). As demonstrated in Figure 3A, although the single Hill approach can fit the dose–response curve, yielding $r^2 = 0.9595$ and IC_{50}

of 158.2 µg/ml, several experimental points cannot be fitted properly, thus casting doubts on the fitting accuracy.

$$V = \frac{A}{1 + \left(\frac{IC_{50}}{C}\right)^{h}} \quad \text{(Single Hill)} \qquad \text{(Equation 1)}$$

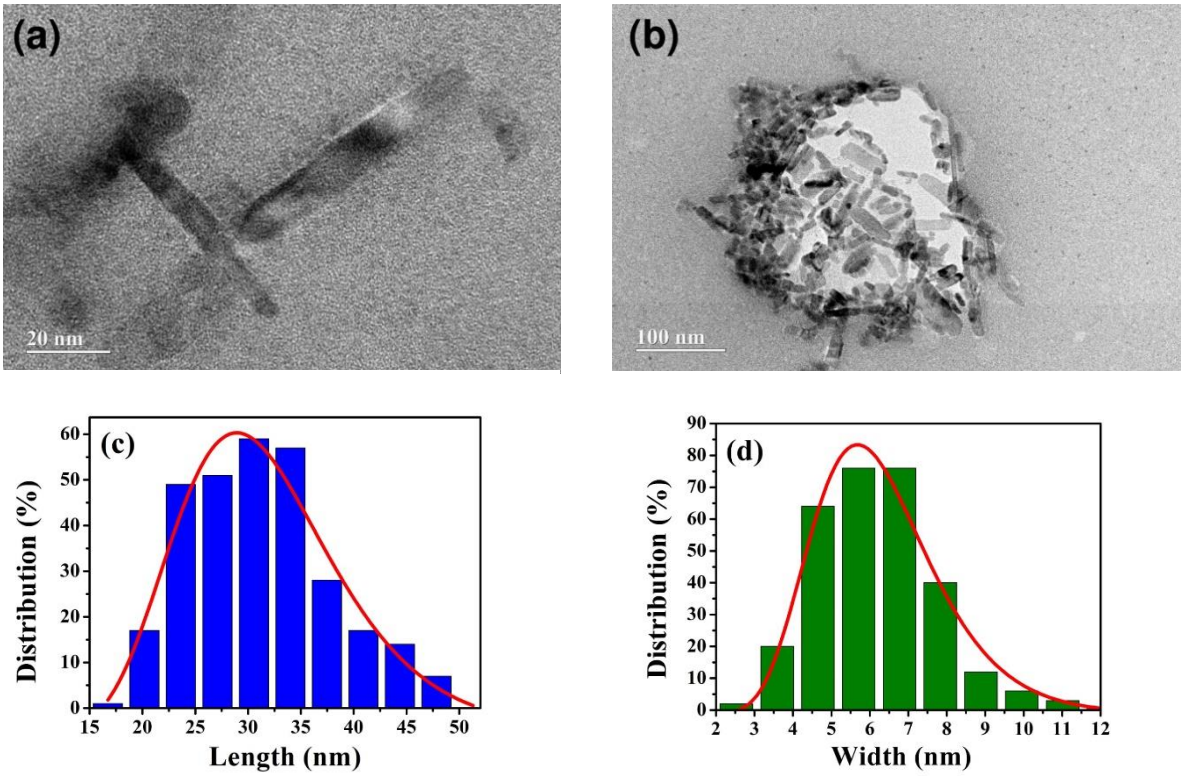


Figure 1. Microscopic characterization of CuO-NP. (A & B) Transmission electronic microscopy micrographs of the rod-shaped copper oxide nanoparticles (CuO-NP) in different scales. **(C)** Length and **(D)** width distribution of CuO-NPs measured with transmission electronic microscopy.

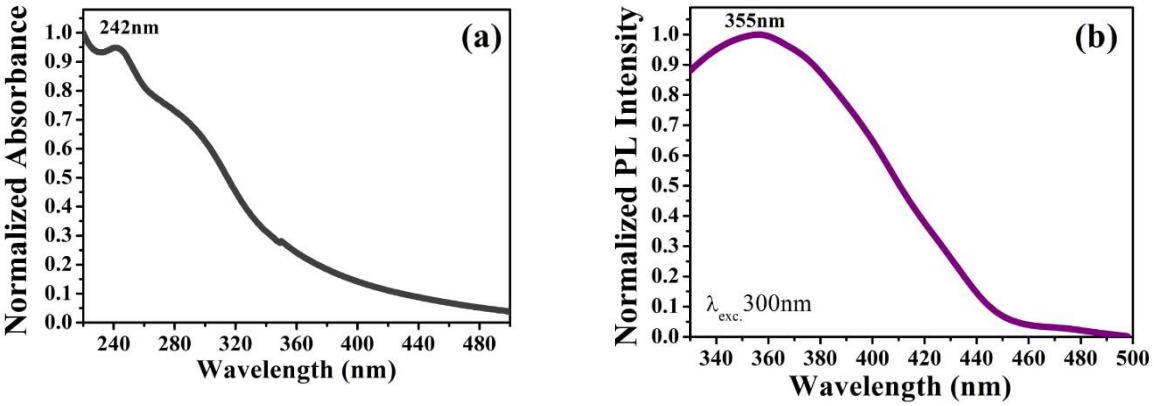


Figure 2. Spectroscopic characterization of CuO-NP. (A) UV-Vis absorption spectrum of copper oxide nanoparticles (continuous dark gray line) with maxima centered at ~242 nm and **(B)** steady-state photoluminescence spectrum of the sample (continuous purple line) with maxima centered at ~355 nm for excitation wavelength set to 300 nm. The sample is dissolved in ultra-pure water.

To test the monophasic character of the obtained Hill coefficient, a complementary equation describing the bacteria's percentage inhibition (I) is written as follows: $I = (IC_{50}/C)^{h}$ /

$1+(IC_{50}/C)^h$, with the relation I = 1 − V being valid [23,27]. The logarithm of the ratio between viability (V) and inhibition (I) leads to theHill plot [23,27], written in Equation 2, which implies that log(V/I) varies linearly as a function of $\log(C/IC_{50})$, with h behaving as an angular coefficient. In this case, the employed IC_{50} parameter is the same as obtained from the single Hill fitting.

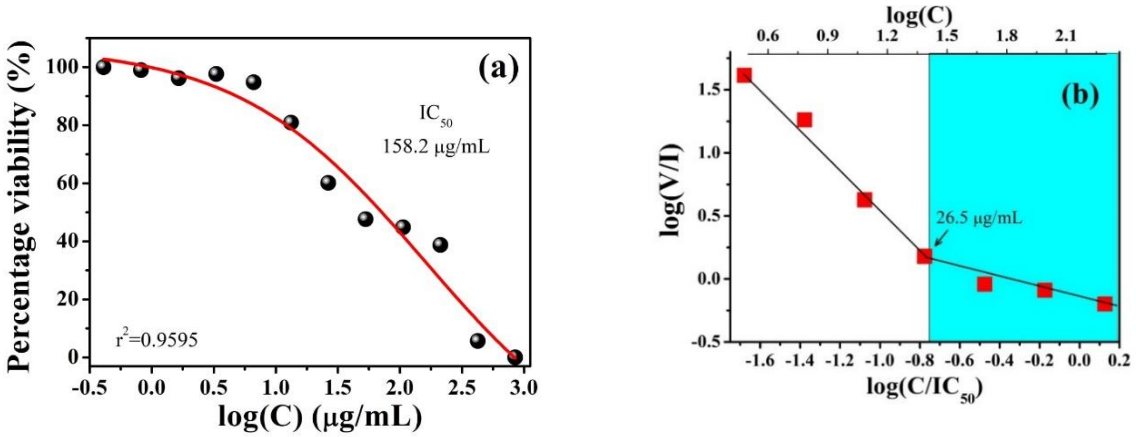


Figure 3. Dose–response curve of *Staphylococcus aureus* treated with copper oxide nanoparticles. (A) The curve is fitted with a single Hill equation, with the black spheres representing the experimental points and the continuous red line accounting for the theoretical curve used in the fitting. **(B)** Hill plot for the same conditions demonstrates the evolution of the Hill coefficient in two distinct phases.

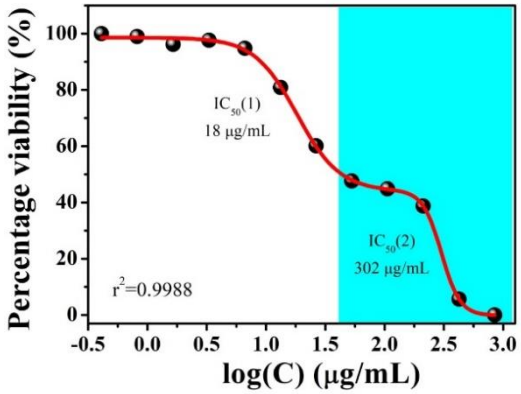


Figure 4. Dose–response curve of *Staphylococcus aureus* treated with copper oxide nanoparticles fitted with the double Hill equation. The black spheres represent the experimental points, whereas the continuous red line accounts for the theoretical curve used in the fitting.

$$\log\left(\frac{V}{I}\right) = h \log\left(\frac{C}{IC_{50}}\right) \qquad \text{(Equation 2)}$$

Applying Equation 2 to the experimental data, we observe that the plot develops into two distinct phases, each comprising a proper linear Hill coefficient (Figure 3B). The crossing

between phases occurs when CuO-NP concentration reaches 26.5 µg/ml. This behavior reveals the incapacity of the single Hill equation to describe our dose–response curve properly.

The second employed approach is based on the use of the double Hill equation [32], defined in Equation 3. In this case, the dose–response curve is fitted assuming a percentage distribution between two correlated phases, each characterized by their own IC_{50} (from now, referred as $IC_{50}(1)$ and $IC_{50}(2)$, Hill coefficients (h1 and h2) and relative amplitudes (A1 and A2) (given in percentages). In Figure 4, it can be observed that the double Hill equation considerably improves the quality of the fitting as endorsed by the increase in the r^2 parameter, which reaches 0.9988.

$$V = \frac{A_1}{1 + \left(\frac{IC_{50}(1)}{C}\right)^{h_1}} + \frac{A_2}{1 + \left(\frac{IC_{50}(2)}{C}\right)^{h_2}} \quad \text{(Double Hill)} \qquad \text{(Equation 3)}$$

The obtained parameters derived from the biphasic dose–response can be grouped into two well-defined concentration ranges: phase I (low concentration) and phase II (high concentration). Notably, the crossing between both phases occurs again at 26.5 µg/ml, which is

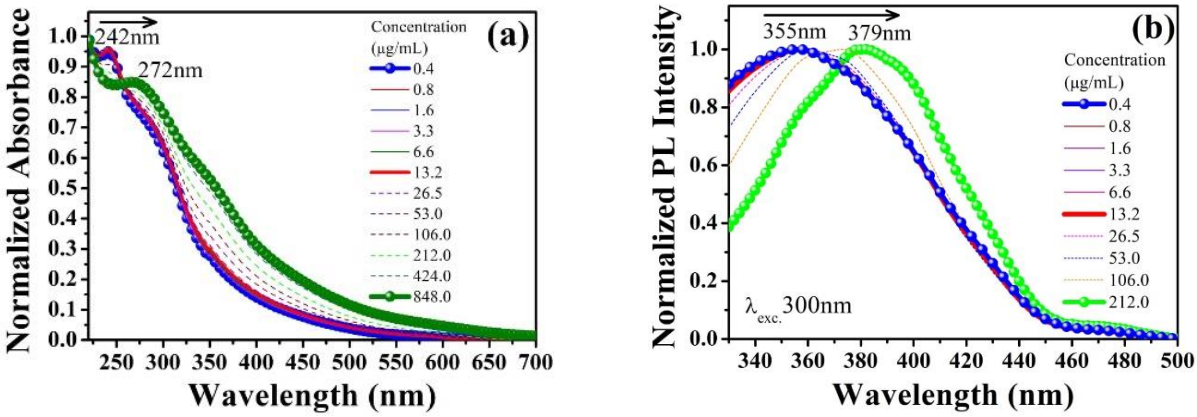


Figure 5. Concentration-triggered modification of CuO-NP colloidal system. Evolution of the normalized **(A)** UV-Vis absorption and **(B)** steady-state photoluminescence spectrum of CuO-NP. The sample is dissolved in ultrapure water.

in total agreement with the double-phased Hill plot previously depicted in Figure 3B.

Phase I comprises 57% of the total curve's amplitude, with a corresponding Hill coefficient of h_1 = -2.39 and calculated IC_{50} (1) of 18 µg/ml. phase II, in contrast, comprises 43% of the total amplitude, displaying a Hill coefficient of h_2 = -5.56 and IC_{50} (2) of 302 µg/ml. The fact that these phases have very different Hill coefficients suggests the occurrence of a concentration-triggered modification in *S. aureus* sensitivity. This assumption is also verified by taking the ratio between the Hill coefficients ($\frac{h_2}{h}$) = 2.32, which leads to the conclusion that

at higher concentrations (phase II), *S. aureus* sensitivity to dose increases, becoming twice as higher as at low concentrations (phase I).

Discussion

The double Hill equation has already been used to model target effects in the antiproliferative activity of dasatinib and similar molecules of interest in cancer pharmacology [32,40,41]. In these cases, the agent is assumed stable under the tested concentrations [32,40,41]. Although a similar interpretation could help to assign the biphasic dose–response observed herein, with the obtained phases reflecting the on-target and off-target interactions between CuO-NPs and *S. aureus*, the instability of the colloidal system to thermodynamic and physicochemical features (including concentration) [18–22,42] is found to play a predominant role in our results.

By preparing a CuO-NP sample with an initial concentration set to 824.0 μg⁄ml and submitting the sample to the dilution steps used in the dose–response curve, we were able to track possible correlations between the biphasic dose– response and a concentration-triggered modification in the agent structure using optical spectroscopy techniques. UV-Vis absorption and steady-state PL spectroscopies showed that the CuO-NP spectra remain unaltered when the sample concentration locates at phase I (0.4–13.2 μg⁄ml), that is, the system is stable in this regime. However, at concentrations greater than 26.5 μg⁄ml (phase II), the former most intense absorption (PL) bands originally located at 242 nm (355 nm) progressively undergo a redshift (Figure 5A & B), evolving to 272 nm (379 nm) at the highest concentration (848.0 μg⁄ml). This trend confirms that concentration causes modifications in the colloidal system. According to the literature, the occurrence of concentration-dependent redshifts in the absorption and PL spectra of MONPs can be assigned to the emergence of an aggregation process [43–45]. Because the crossover between phases and the aggregation thresholds are equivalent, we believe that the aggregation of CuO-NP is the most probable cause behind the biphasic dose response.

In addition to size modifications [43–45], the biological activity of MONPs can also be affected by aggregation [46]. Considering that larger (smaller) CuO-NP sizes are associated with an improvement (reduction) in cytotoxicity [46], the observation of CuO-NP aggregates endorses the hypothesis that the obtained Hill coefficients are related to specific interactions of *S. aureus* with the dispersed CuO-NP in phase I and the aggregated CuO-NP in phase II. This interpretation enables an understanding of the threshold-guided sensitivity of *S. aureus* to CuO-NP doses.

Conclusion

We have demonstrated that the dose–response profile of CuO-NPs against *S. aureus* cannot be satisfactorily under- stood by employing a single Hill fitting. Our results bring novel insights into the use of multiphasic mathematical interpretation of *in vitro* assays. The combination of this interpretation with optical spectroscopy techniques was helpful to assign the concentration-triggered aggregation of CuO-NPs as the most probable cause behind the biphasic dose–response pattern. The findings also suggest that modifications in colloidal-derived antibacterial agents such as CuO-NPs can impose dose-related changes in *S. aureus* sensitivity. This result allows choosing an ideal concentration range to maximize the antibacterial action of CuO-NPs and offers great opportunities for future evaluation of other MONPs as optimized antibacterial agents. Finally, to the best of our knowledge, this is the first discussion on the biphasic dose–response interpretation of the antibacterial activity of MONPs.

Summary points

- Colloidal copper oxide nanoparticles (CuO-NPs) were synthesized and characterized.
- A biphasic dose–response is observed when CuO-NPs are used as an *in vitro* antibacterial agent against *Staphylococcus aureus*.
- Single Hill equation's incapacity to adequately describe the dose–response curve is discussed.
- The double Hill equation enables the quantitative investigation of two distinct concentration-dependent phases.
- *S. aureus* sensitivity to dose is greater at higher CuO-NP concentrations.
- Concentration-dependent evolution of CuO-NP optical spectroscopy signals is assigned to the formation of nanoparticle aggregates.
- The concentration threshold required for aggregation matches the second phase's emergence.
- *S. aureus* interaction with the nonaggregated (phase I) and the aggregated (phase II) CuO-NPs addresses the biphasic dose response.

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Bioanalytical Method Validation for Copper Quantification in Skin Samples by Atomic Absorption Spectroscopy

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Abstract

Recent discoveries about the therapeutic potential of copper as a potent antimicrobial stimulate the development of new pharmaceutical forms. Therefore, the copper quantification is relevant due to its potential as an antiseptic additive in future formulations. The detection of this analyte is commonly performed by spectroscopic methods, so the objective of this work was to validate a simple bioanalytical method capable of quantifying copper in skin matrices aiming at the development of topical antiseptic formulations. For this, the skin was digested with a mixture of acids in different proportions of H_2O_2, HNO_3, H_2SO_4 and HCl. The method was validated in Flame Atomic Absorption Spectrometry (FAAS) and the detection was at 324.8 nm. The bioanalytical method was selective, linear (0.9931) and tested for robustness, precision and stable assays for reading samples after 8 days, with recovery rates greater than 88% ± 3,292. Finally, the method was applied on a porcine total skin permeation, which showed retention due to stable skin. The validated method is therefore suitable for recovering and quantifying copper, favoring the development of new topical antiseptic formulations intended for these administration sites.

Keywords: Bioanalytical method; Copper oxide nanoparticles; FAAS; skin permeation

1. Introduction

Detecting of metal ions is important in several areas of study, as they are essential in several biochemical processes and their lack can cause relevant diseases, so it is important to control food and environmental analyzes (DUGANDŽIĆ et al., 2019). In the human body, copper actively participates in the wound healing process and helps in the formation of the skin, which makes it an essential trace element involved in several human physiological and metabolic processes (DUGANDŽIĆ et al. 2019; BORKOW et al., 2009). In this scenario, metal oxide nanoparticles derived from copper have also proved to be effective auxiliary alternatives in the wound healing process due to their antimicrobial activity (SÁNCHEZ-LÓPEZ et al., 2020).

The popular use of this metal dates back thousands of years, and even today patients with arthritis use copper jewelry to relieve inflammation and musculoskeletal disorders (RAMTEKE et al., 2020). However, studies that prove the real effect of these copper jewelry on these diseases or a placebo effect are still lacking (HOSTÝNEK et al., 2006). On the other hand, the main skin reactions to metals, such as nickel (LÍDEN; CARTER, 2001) and copper (KARLBERG et al., 1983), have already been reported.

The result of contact with coins and other articles of daily use indicates the beginning of the penetration process in the short term for skin reactions. Although, it is believed that one of the critical factors to determine whether a metal will penetrate the subcutaneous tissue thru the epidermis and dermis is related to the formation of soluble compounds and, only then, to observe whether there is a local or systemic effect (HOSTÝNEK et al., 2006).

The use of nanomaterials is common in consumer products, such as cosmetic and dermatological preparations. However, they present a specific environmental risk and must be carefully controlled and evaluated (COHEN et al., 2013). It is relevant to know the toxicology of copper oxide nanoparticles (CuO-NP), since studies reveal that the release of these copper ions in the growth medium was significant (MIDANDER et al., 2009). CuO-NP is capable of releasing ions inside cells after penetration, accumulating in organelles such as lysosomes, where they must dissolve quickly due to the acidic pH (STUDER et al., 2010) and can still accumulate in the body, causing long-term toxicity (ERMINI & VOLIANI, 2021; ZHOU et al., 2018).

Therefore, it is necessary to control the concentration of metal ions in environmental analysis, consumer products such as food, and diagnostics (DUGANDŽIĆ et al., 2019). For this, the development of analytical methods using atomic absorption spectroscopy (AAS),

inductively coupled plasma-spectrometry (ICP-MS), as well as voltammetry methods, which exhibit good detection limits and reliable working range, is necessary (DUGANDŽIĆ et al. 2019; LIU et al. 2008). In addition, through spectroscopy techniques such as ICP-MS, it is possible to analyze hair and nail samples, which makes them non-invasive techniques, where only a small sample size is required for analysis and allows easy long-term storage (GOULLÉ et al., 2009).

This study aimed to develop and validate a bioanalytical method of efficient and simple atomic absorption spectrometry capable of quantifying copper in skin matrices to support the development of new topical formulations for antiseptic formulations to reduce microbial contamination.

2. Materials and Methods

2.1. Materials:

Nitric acid, hydrochloric acid, and hydrogen peroxide were obtained from Dinâmica (São Paulo, Brazil) and sulfuric acid was purchased from Vetec (Rio de Janeiro, Brazil). All experiments were carried out with ultrapure water (Milli-Q®, Millipore, lllkirch-Graffenstaden, France). The copper (II) chloride dihydrate ($CuCl_2.2H_2O$) (≥ 99.0%) and copper standard for ICP 1 g/L were obtained from Sigma-Aldrich (Steinheim, Germany) and trisodium citrate solution was purchased from Merck KGaA (Darmstadt, Germany). Flame Atomic Absorption Spectrometry (FAAS) (Varian AA 240FS - Fast Sequential) was used to analyze the samples. Via Carnes (Formosa, Brazil) provided the skin of porcine ears used in the studies of validation.

2.2. Synthesis of CuO-NPs

The CuO-NPs were synthesized as described by (FERREIRA et al., 2023), briefly: 75.0 ml of aqueous $CuCl_2.{}_2H_2O$ solution (0.02 mol/l) prepared with ultrapure water was heated under magnetic stirring until boiling. Then, 0.4 g (0.01 mol) of NaOH was added to the former solution, which was kept under heating and magnetic stirring for an additional 15 min. The resulting black precipitate of CuO-NPs was isolated, washed with ultrapure water and centrifuged three times. The CuO-NPs were suspended in 50.0 ml trisodium citrate solution at 1% (w/v) and sonicated for 10 min at 125 W in pulsed mode (1 s). Afterward, the sample was purified by dialysis for 48 h using cellulose regenerated bags (molecular weight cutoff = 12,000 g⁄mol).

2.3. Sample Preparation - Skin digestion

Porcine skin (2cm^2 and ~0.8 g) was cut into small pieces and submitted to the digestion process. To adjust the actual amounts of acids, some tests were performed with different proportions. Initially, potassium permanganate was replaced as an oxidizing agent, then

hydrogen peroxide was added, which together with sulfuric acid formed the piranha etch (piranha solution), considering that the objective was to digest the skin. Subsequently, the amount of nitric acid was increased proportionally to the weight of the skin, and, finally, more hydrochloric acid was added to speed up the process. Finally, the defined proportions of the acids were by adding 1.5 mL of hydrogen peroxide and 4.5 mL of sulfuric acid for 10 minutes under magnetic stirring, generating a strongly exothermic reaction. Then, 2.0 mL of nitric acid was added and, after 15 minutes, 10.0 mL of hydrochloric acid was added. The sample was heated at 80º C for 1.5 hours or until the reaction ended. After the skin digestion process, the sample solutions were transferred to a 25.0 mL volumetric flask and completed with MilliQ water. After the first dilution, 5.0 mL was transferred to a 10.0 mL volumetric flask and completed with acid nitric 2.0%. The final solution was filtered with PTFE 0,45 µm hydrophobic filter and analyzed. The skin digestion method was adapted from the organic materials digestion method of INORGANIC VENTURES, 1996.

2.4. Method validation

The analytical method was validated based on The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Bioanalytical Method Validation and Study Sample Analysis (M10) Guideline (ICH, 2022).

2.4.1. Linearity

The linear ratio from 1.50 to 9.00 µg/mL of copper was performed in triplicate at six different concentrations (1.50, 3.00, 4.50, 6.00, 7.48 and 9.00 µg/ mL) prepared in nitric acid 2%. Data were analyzed considering the analysis of correlation coefficient (R), homoscedasticity, normality by Shapiro Wilk test, and Grubbs test for outlier detection using GraphPad Prism® Software, version 9.5.1 (San Diego, CA, USA). The significance level was set at $p < 0.05$ and was used to reject the null hypothesis.

2.4.2. Limits of detection (LOD) and quantification (LOQ)

The minimal theoretical concentration of copper to be detected (LOD) and quantified (LOQ) was calculated based on the standard deviation (σ) and slope (S) of the calibration curves according to Eqs. (1) and (2).

$$LOD = 3{,}3\ x\ \frac{\sigma}{S} \quad (1)$$

$$LOQ = 10\ x\ \frac{\sigma}{S} \quad (2)$$

2.4.3. Selectivity

The ability of the method to differentiate and measure the analyte in the presence of the porcine skin was evaluated using six blank samples (prepared as described in item 2.2) and the copper standard at 1.5 µg/mL, the lowest concentration of the calibration curve.

2.4.4. Precision

The precision was evaluated by repeatability and intermediate precision at copper concentrations of 1.5, 5.0, and 9.0 µg/mL, prepared in triplicate. The intermediate precision was performed in two days on the same equipment by two different operators. The calculated Coefficient of Variation (CV%) Eq. (3) results were evaluated based on the (AOAC) limits presented in Table 2.

$$CV = \frac{SD}{average}\ x\ 100 \quad (3)$$

2.4.5. Accuracy

The accuracy of the method was performed in triplicate by the quantification of low, medium, and high concentrations (1.50, 5.00, and 9.00 µg/mL, respectively). After the skin digestion process and preparation of the samples, they were analyzed by FAAS. The recovery of the standard was determined by the ratio between the amount of copper extracted from the skin layers and the amount initially added. The calculated copper recovery (Equation (4) results were evaluated based on the (AOAC) limits presented in Table 3.

$$Recovery = concentration\frac{measured}{theoretical}\ x\ 100 \quad (4)$$

2.4.6. Analytical stability

The copper stability in the samples under storage and processing conditions was also evaluated. The standard samples with 5.00 µg/mL of copper were prepared and analyzed on day 0, stored at room temperature, and analyzed after 8 days. The calculated copper recovery was evaluated based on the accuracy limits.

2.4.7. Robustness

The robustness of the method was performed by the variation of the filter used in the filtration step of the skin digestion process. The PVDF hydrophobic 0,45 µm filter was used. The assay was performed in triplicate with 5.00 µg/mL copper concentration samples, the calculated copper recovery was evaluated based on the accuracy limits.

2.4.8. Quantification of copper in the sample

CuO-NP concentrations were measured in atomic absorption spectroscopy employing a Varian (CA, USA) AA 240FS Fast Sequential atomic absorption spectrometer flame using a flame source derived from the air/acetylene mixture. After synthesis, the colloidal sample

storage was made in the refrigerator and for discovery for the real concentration, 4.0 mL of the sample was exposed in 4.0 mL nitric acid pure and diluted in a volumetric balon of 50.0 mL for measurements. Posteriorly 1.2 mL was transferred for a volumetric balon of 25.0 mL with nitric acid 2%. Before the analysis, the calibration curve in 2% nitric acid was constructed with certified standard copper solution in a range of concentrations, from 1.5 to 9.0 ppm. (FERREIRA et al., 2023).

2.5. Skin penetration assays preparation

The copper oxide nanoparticles (CuO-NP) penetration into the porcine skin layers was determined in vitro using Franz-type vertical diffusion cells with 15.0 mL capacity of receptor compartment and 2.0 mL capacity of donor compartment. The diffusion area of the cells was 11.3 mm in diameter. A porcine skin was used as membrane into the diffusion area, separating the donor and receptor compartments of the diffusion cells. The donor compartment was filled with 0.3 mL of CuO-NP (6.09 μg/mL) and the receptor compartment was filled with 15 mL of phosphate buffer pH 7.4. The Franz-type cells were maintained at 32 ± 2 °C and under magnetic stirring for 12 hours (TOLENTINO et al., 2023 modified; ZANONI et al., 2019).

The skin was removed from the diffusion cells, placed in a vertical position, and washed with ultrapure water for 5.0 seconds to remove the sample excess. The skin was then cut into small pieces and placed in amber vials. The copper was extracted from skin layers using the optimized skin digestion procedure described in Section 2.2. All samples were analyzed for copper quantification by FAAS using the method previously described.

3. Results and discussion

3.1. Development of the skin digestion method

To develop the skin digestion method, the effect of sulfuric acid, nitric acid, hydrochloric acid and hydrogen peroxide on the digestion of skin layers was studied. We were initially based on the reference of INORGANIC VENTURES (1996), a methodology for complex matrix, which demonstrated the digestion of oils and viscous petroleum products, developed for the analysis of several metals, among them, copper. In our analyses, permanganate was replaced by hydrogen peroxide to facilitate the process, because the manganese present in this reagent would have to be removed in the other step due to its presence interfering with the analysis, moreover, after this replacement and addition of sulfuric acid to part of the skin it was almost completely digested characterized by a turbid solution. Subsequently, when adding a greater amount of nitric acid than suggested by the author (3.2 mL), it was observed that the solution became more turbid, which affects the reading by the

equipment, leading to the impression that the skin is not digested satisfactorily, and finally, when adding a larger volume of hydrochloric acid (12.0 mL) the flask got overheated, considering that this last step is already heated, it was decided to follow the suggested method. Thus, the condition of complete digestion observed was using 1.5 mL of H_2O_2, 4.5 mL of H_2SO_4, 2.0 mL of HNO_3 and 10.0 mL of HCl in contact with small pieces of swine skin. After the addition of hydrochloric acid, it was necessary to heat the solution to 80ºC to complete the reaction, and the organic matter to evaporate.

3.2. Analytical Method Validation

3.2.1. Selectivity

The copper concentration on blank skin samples, presented near-zero results, being possible to confirm that the organic material of the digested porcine skin does not interfere with the copper quantification, proving it is a selective method for copper quantification in the porcine skin. The 1.5 µg/mL Cu solutions presented an average reading of 0.1537 ± 0.001. As stated in the ICH M10 guideline (ICH), the response attributable to interfering components should not be higher than 20% of the lower analyte response, therefore, the method passes the test for selectivity and specificity. Furthermore, the wavelength of the method (324.8 nm) is specific to copper detection in these samples (PORENTO et al., 2011).

Table 1: Method selectivity of copper detection at the porcine skin layers.

Sample	**Copper detection (ABS)**	**Sample**	**Copper detection (ABS)**
Skin 1	-0.007	Skin + sol. 1.5 ppm	0,1603
Skin 2	-0.004	Skin + sol. 1.5 ppm	0,1600
Skin 3	-0.009	Skin + sol. 1.5 ppm	0,1604
Skin 4	-0.004	Skin + sol. 1.5 ppm	0,1611
Skin 5	-0.004	Skin + sol. 1.5 ppm	0,1585
Skin 6	-0.004	Skin + sol. 1.5 ppm	0,1530
Average	-0.005 ± 0.002	**Average**	0.158 ± 0.003

3.2.2. Linearity

The calibration curves constructed, in triplicate, for copper quantification by FAAS exhibited linearity, as shown in Figure 1. The linear regression of absorbance versus analyte theoretical concentrations resulted in correlation coefficient values of 0.9931 and the equation:

y= 0.857 + 0.0469, this result exhibits a high correlation between variables within the established limits (ICH, 2022). Also, the method demonstrated homoscedastic behavior, residuals normality, and no outliers detected.

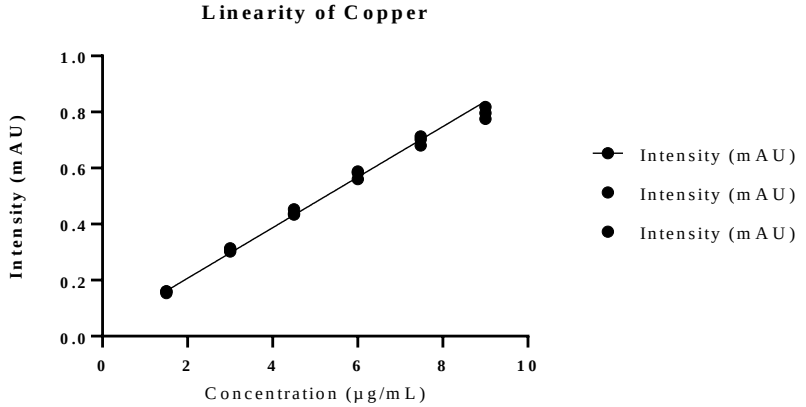


Figure 1: Analytical curve obtained from copper standard samples diluted in 2% HNO_3 at different concentrations ranging from 1.5 to 9.0 µg/mL. A calculated calibration curve equation y= 0.857 + 0.0469 with a correlation coefficient of 0.9931.

3.2.3. Limits of detection (LOD) and quantification (LOQ)

The method showed a low limit of detection (0.1709 µg/ml) and quantification (0.5178 µg/ml) for copper, demonstrating a high sensitivity. Based on that, it is possible to imply the method can determine small concentrations of copper retained in the skin layers. These values should be low enough to quantify copper in the skin after permeation assays (TOLENTINO et al., 2023). This indicates that the developed method can be used to support skin penetration studies in the development of new copper-containing dermatological products.

3.2.4. Precision

The precision of the method is used to evaluate the variability of it and intrinsic errors inherent to the process, which can be minimized, but not completely controlled, such as reagents, glassware, sensitivity of equipment and sample preparation (OLIVEIRA et al., 2020; MATOS et al., 2015).

Coefficient of variation (CV%) values for the repeatability tests were all below 2.7%, for reproducibility less than 3.8% and for the intermediate precision test (precision between days) ranged from 3.5 % to 5.7%, as showed in table 2. These results demonstrate consistency in the analytical response (TOLENTINO et al., 2023), such as this reasonable modification was not as discordant as in a bioanalytical method with the presence of a matrix effect (OLIVEIRA et al., 2020).

Table 2: Precision test results for determination of copper

	Theorical concentration (µg/mL)	Measured concentration (µg/mL)	CV%	Limits (%)[1]
Repeatability	1.500	1.353	2.7	7.3
	5.000	4.603	1.0	
	9.000	7.720	1.2	
Reproducibility	1.500	1.319	3.8	7.3
	5.000	4.214	1.8	
	9.000	6.973	3.4	
Intermediate Precision	1.500	1.335	3.5	11.5
	5.000	4.409	4.7	
	9.000	7.346	5.7	

[1]AOAC

3.2.5. Accuracy

The accuracy of the method was calculated by triplicate analyses of the samples on three different concentrations: 1.5, 5.0 e 9.0 µg/mL. These samples were submitted to the digestion process described in item 2.3. The results obtained are show at Table 3.

The average values obtained for copper recovery are superiors of 85%, 91% and 85% respectively. These values are greater than 80% recovery, which is in line with the recommended accuracy in bioanalytical methods (TOLENTINO et al., 2023; ÂNGELO et al., 2017). In agreement, the values are within the limits of 80% to 110%, which reveals adequate accuracy according to ICH, 2022.

Table 3: Accuracy test results for determination of copper

Theorical concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (%)	Limits (%)[1]
1.500	1.378	92	80-110
	1.298	87	
	1.274	85	
5.000	4.626	93	80-110
	4.637	93	
	4.549	91	
9.000	7.667	85	

	7.647	85	80-110
	7.847	87	

[1]AOAC

3.2.6. Analytical stability

After preparation (D0), the flask containing the copper solution (5,000 µg/mL) was kept at room temperature for 8 days (D8) and did not show significant losses, considering that in D0 it presented a concentration of 4.674 µg/mL and in D8 4.503 µg /mL (96.3 %). This stability ensures that samples can be prepared in one day and analyzed within 8 days, which improves the workflow, considering that permeations are usually carried out for long periods.

3.2.7. Robustness

The method robustness was evaluated by the congruence of the results of the chosen filter (0.45 µm hydrophobic PTFE filter) and the alternative filter (0.45 µm hydrophobic PVDF filter). The results obtained showed that even if the filter is changed, the test remains reproducible and robust, quantifying the amount of copper in the formulation, as shown in Table 4.

Table 4: Robustness test results for copper quantification using PVDF filter and PTFE filters

Filter	Theorical concentration (µg/mL)	Recovery (%)	Measured concentration ± SD (µg/mL)	CV %	Limits (%)[1]
PVDF	5.000	100	4.25	2.398	80-110
PTFE	5.000	Control	4.31	2.019	

[1]AOAC

3.2.8. Quantification of copper in the sample

After the synthesis of CuO-NP, the FAAS determined the actual concentration of the sample, revealing the concentration of 1.272 µg/mL of copper in the sample.

3.3. Skin penetration assay

The practical application of the method developed and validated in this study was performed by 12 hours *in vitro* skin permeation assay using an intact porcine skin as shown in section 2.5.1. A copper nanoparticle solution (CuO-NP) previously developed was applied on the skin and, after the 12 hours of experiment, the concentration of copper that penetrated the skin layers and the receptor medium was quantified by FAAS.

It is possible to verify the CuO-NP retention on the skin, which indicates that it is difficult to penetrate the intact skin. In addition, there is no significant copper concentration recovery at

the diffusion cells receptor medium, confirming the low CuO-NP low penetration. HOSTÝNEK et al., (2006) have demonstrated that soluble metal complexes seem to form a reservoir in the skin's outermost layers, as well as the intact epidermis acts as a good barrier against the dispersion of Cu^{2+} ions in 24 hours, avoiding the passage through the intact skin (ZANONI et al., 2019)

A critical factor in the process of metal oxidation seems to be the availability of oxygen, as this process appears to be slow under occlusion, based on the limited oxygen access. On the other hand, in the presence of sweat, oxidation leads to the formation of cupric ions, Cu^{2+} (HOSTÝNEK et al., 2006). In other words, in the absence of oxygen, this reaction is suppressed. Even with the addition of the simultaneous reduction of oxygen, which with water forms hydroxyl ions, studies showed that copper has remarkably more resistant to corrosion when compared to other materials, such as nickel (HOSTÝNEK et al., 2006; HOSTÝNEK et al., 2001).

Given all the above, it can be seen that the new method developed is validated and is selective, robust and stable, as it detects the metal without interference, quantifies it even with changes in filtration and maintains a satisfactory reading even after 8 days at rest, in addition to be validated for use in permeation tests.

4. Conclusion

Recent discoveries about the therapeutic potential of copper encourage the development of new pharmaceutical forms with this metal. Based on that, to advance in this area, it is necessary to develop an analytical method capable of accurately quantifying copper in the presence of biological matrices, such as skin layers, allowing permeation assays. This work developed and validated a robust method to recover and quantify this metal from skin layers in compatible concentrations with permeation assays. In addition, the proposed method can help design new formulations, specifically allowing *in vitro* performance assays in target tissues. Furthermore, the analytical method demonstrated selectivity with skin interferences and a high copper recovery level from skin layers. In addition, presented lower limits of detection and quantification, which would allow the performance of kinetic assays. Thinking about it, if necessary, this method can also be used as an alternative method for evaluating occupational contamination of copper in the skin.

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CONCLUSÃO GERAL

Diante do exposto, é possível confirmar a atividade antimicrobiana presente nas nanopartículas de óxido de cobre frente a *S. aureus*, uma vez que foi possível definir a concentração mínima inibitória necessária para inibir o crescimento dessa bactéria gram positiva, além de gerar discussão sobre o comportamento bidose resposta pouco debatido na literatura e novidade quanto às CuO-NP. A combinação dos testes *in vitro* com as técnicas de espectroscopia ótica foram úteis para atribuir a agregação desencadeada por concentração de CuO-NPs como a causa mais provável por trás do padrão dose-resposta bifásico.

Dessa forma, tendo em vista a aplicação tópica como uma importante via de acesso à produtos para saúde, é relevante o desenvolvimento de um método eficiente e robusto capaz de quantificar o analito para que o cobre possa atuar como aditivo antisséptico. O método revelou características desejáveis com significativos padrões de eficiência, seletividade e robustez para quantificação do analito em uma matriz biológica como a pele. Ademais, faz-se necessário mais estudos com foco na avaliação do produto, além de testes de toxicidade frente às células integrantes do meio analisado.

Por fim, nota-se que a síntese de CuO-NP é branda, tendo em vista a maior composição aquosa, além da sua produção ser simples, rápida e comprovadamente efetiva contra *S. aureus*, o que torna esse material uma alternativa promissora a ser testada contra outras espécies de bactérias.

Diante disso, para perspectivas futuras, é necessário a avalição da toxicidade do material diante de células como fibroblastos e queratinócitos, presentes na derme e epiderme, respectivamente, assim como o desenvolvimento de uma formulação contendo o material, bem como avaliar a permeação e retenção das nanopartículas na pele em diferentes condições de aplicação.