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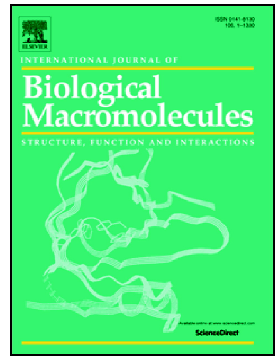

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Zein-based nanostructured coatings: A Green Approach to Enhance Virucidal Efficacy of Protective Face Masks

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Abstract

Face masks represent a valuable tool to prevent the spreading of airborne viruses; however, they show poor comfort and scarce antiviral efficacy. Zein-based coatings are herein exploited to enhance antiviral performance. Zein functionalization is done through acidifying agents (lactic acid, LA). Coatings are characterized in terms of morphological, mechanical, breathability, and cytotoxicity analyses. The antiviral efficacy is tested in vitro against four viruses (Human Coronavirus OC43, Herpes Simplex Virus type 1, Human Adenovirus type 5, and MPox Virus) according to a biological assay on cell cultures. Zein/Zein LA antiviral activity seems to be linked to its positive surface charge that enables to form electrostatic interactions with negatively charged-viruses, resulting in the highest activity (reduction > 2 Log) on Human Coronavirus OC43 and Herpes Simplex Virus type 1, with efficacy comparable or higher than that of copper/copper oxide-based- coatings. No significant activity is observed against Human Adenovirus type 5 and MPox Virus, due to their high resistance to inactivating treatments. Zein-based systems are not cytotoxic and their water vapor permeability is reduced of 26 % compared to those of not-coated systems. These promising results offer interesting insights into design of antiviral and sustainable coatings for personal protective equipment.

Keywords: Face masks, antiviral, nanostructured coatings, dip-coating, zein-based coatings, sustainable materials.

1. Introduction

Airborne viruses can be easily transmitted through micro-droplets (size <5 μm), aerosols, or dust particles [1,2], during exhalation, inhalation, coughing, speaking, or sneezing [3]. Protective face masks represent a wide class of Personal Protective Equipment (PPE) enabling to shield from the spread of droplets carrying airborne viruses and microorganisms [4–7] and thus reducing the risk of transmission of infectious diseases [8,9]. Indeed, the use of face masks was found extremely important in the framework of the SARS-CoV-2 pandemic outbreak, along with other preventive actions such as social distancing or lockdown [9].

Protective face masks are generally divided into two main categories: filtrating masks and surgical masks [10-14]. Filter-Facing Respiratory (FFR) masks such as N95, FFP1, FFP2, FFP3, and KN95 belong to the first category. They exhibit different efficiency filtrating either air particles (known as Particulate Filtration Efficiency, PFE) or bio-aerosols (whereas Bacterial Filtration Efficiency, BFE, can be also estimated). Usually, this class of PPE achieves PFE (i.e., ≥95 %) for particles/microorganisms having size <0.3 μm) [6, 10]; some drawbacks can be highlighted such as poor comfort and short effective time (max 8 h), temperature increase or sweating as well as long-term effects such as low blood oxygen levels or acute respiratory distress [9, 10].

Surgical masks have been designed as PPE in medical settings to protect the wearer from infected droplets (filtration efficiency equal to 95 %). However, they show low external protection, consequently, limiting only the spreading of larger droplets (≥5 μm) [11] or splashing and sprays during medical routes. In contrast, they are not well effective on small droplets.

Face mask consists of three layers: an external hydrophobic layer made of non-woven fabrics (conventionally made of thermoplastics polymers such as polypropylene, polystyrene, or high-density polyethylene HDPE or low-density polyethylene, LDE), obtained through spun-bond technology [12]; an intermediate layer acting as an electrostatic filter (obtained *via* melt brown process) of particles having size less than 10 μm [13, 14], and an inner layer, in contact with face, providing shape and comfort. However, there are several issues with the use of face masks that can be pointed out, including poor protection from external contaminants and viruses, poor comfort (inadequate fit and seal), and reduced sustainability (leading to environmental concerns such as waste management or dispersion of micro-plastics) [15-17]. For this reason, sustainable polymers, mainly biopolymers, have also been suggested as candidates for manufacturing face mask coatings due to their ability to capture viruses and microorganisms electrostatically, as well as for sustainability, high availability, biodegradability, non-toxicity, and biocompatibility [18]. As a matter of fact, polysaccharides such as various cellulose-based materials (bacterial nanocellulose [19], wipes [20], cationized cellulose nanofibers (kat-CNF)[21-23]) lignocellulosic systems [24-26] or chitosan [22, 27], gluten [13], starch [28] and polyphenols [29], have been widely employed in the recent literature. Natural proteins such as those from silk and soy [30], or zein (a maize-derived protein) possess functional ionizable groups, able to capture air pollutants and biological hazards [31]. Among them, zein has been extensively used in biomedical and drug delivery applications [32-34]. This protein is a combination of polypeptides α-, β-, γ-, and δ-zeins, consisting of either non-polar amino acids (such as alanine, leucine, and prolamine) or polar amino acids i.e., cysteine, conferring amphipathic behavior [35–38]. Besides, it shows a high number of cationic charges both on its main (ammonium II and III) and lateral backbones (I) [36], which are capable of electrostatically interacting with negative-charged viruses [39]. Moreover, zein is poorly soluble in water-based solvents, resulting in enhanced barrier properties to water vapor and solutes [40].

Recently, zein has been employed in air filtration applications together with Ag NPs (silver nanoparticles) and polycaprolactone in the fabrication of nanocomposites through electrospinning [41] or combined with hard carbon aerogels [42]. The resulting materials displayed remarkable antibacterial and antiviral characteristics as well as excellent air filtration capabilities. Moreover, it was found that the functionalization of textiles through zein nanoparticles enhanced the antimicrobial features of the examined systems towards both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacterial strains [43]. Nevertheless, to the best of the authors' knowledge, it has never been directly utilized for the realization of protective equipment coatings.

To date, the most widely used coatings have been those containing metal-based antiviral solutions. However, despite their proven effectiveness in combating airborne viruses and pathogens, there is great concern about the release of metal ions or reactive oxygen species (ROS) into the environment during washing, as well as potential exposure through skin contact, inhalation, or ingestion, which could lead to toxicity and the development of microbial resistance [44].

In this context, the objective of this study is to develop sustainable antiviral coatings utilizing zein i.e., a corn-based protein. Indeed, this approach enables to avert the above-mentioned issues since its antiviral mechanism is based on electrostatic interactions between protein-positive charges and negatively charged viral systems; moreover, the biodegradability and sustainability of such materials overcome both environmental and health concerns.

These innovative zein-based coatings are designed for application on protective devices, such as disposable face masks intended for single use, to enhance their antiviral efficacy. Newly coated-face masks were prepared through a simple dip-coating procedure of the developed zein-based nanostructured formulation. To improve the uniformity of the resulting coatings, functionalization was carried out using an acidifying agent (lactic acid, LA), allowing to reduce pH solution and acting as a plasticizer for zein. The virucidal activity of the resulting nanostructured coatings against Human Coronavirus OC43 (HCoV-OC43, surrogate of SARS-CoV-2), Herpes Simplex Virus type 1 (HSV-1), Human Adenovirus type 5 (AdV-5), and MPox Virus (MPoxV) was evaluated. Conventional chemicals based on active compounds such as $CuCl_2$, CuO, and ZnO-based formulations were also prepared and investigated for comparison.

2. Experimental section

2.1. Materials

Corn-based zein powder (CAS 9010-66-6, 25 000 Da) was purchased from Sigma Aldrich, Italy. Lactic acid (85 % w/w solution, Sigma Aldrich, Milan, Italy) was used as an acidifying agent for zein solutions. Copper (II) chloride-di-hydrate ($CuCl_2$ X $2H_2O$) and Zinc Nitrate hexa-hydrate ($Zn(NO_3)_2$ X 6 H_2O) were purchased from Sigma Aldrich and Titol Chimica, respectively, and used as references and starting chemicals for oxide-based antiviral solutions. Ethanol (98 wt.% purity, Carlo Erba, Milan, Italy) was selected as a solvent for zein-based systems. NaOH, in the form of pellet, was purchased from Sigma Aldrich. Streptomycin 100 µg/mL, penicillin 100U/mL (used as antibiotic solutions), and L-glutamine were supplied by Sigma Aldrich along with fetal bovine serum (FBS), non-essential amino acid (NEAA), L-glutamine and Alamar blue test. Dulbecco's Modified Eagle's Medium (DMEM) was supplied by Thermo Fisher Scientific Inc. (Waltham, MT, USA).

2.2. Cell lines and viral strains

L929 cells were chosen for the cytotoxicity assay and Vero cell line for the viral tests employing HSV-1, AdV-5, and MPoxV. Conversely, HCoV-OC43 was cultivated on human diploid fibroblasts.

The selected HSV-1, AdV-5, and MPoxV strains were clinical isolates, identified by monoclonal antibodies and laboratory-adapted through serial passages (>50) on Vero cells. HCoV-OC43 strain was purchased from the American Type Culture Collection, ATCC (VRI-558).

2.3. Preparation of the antiviral solutions

Three antiviral solutions based on *active chemicals* $CuCl_2$, $Cu(OH)_2$, and $Zn(OH)_2$ and two based on *sustainable Zein solutions* were prepared.

Antiviral solutions (named as AS) based on *active chemicals* were prepared by alternatively dissolving 25g of $CuCl_2x2H_2O$ and 25g of $Zn(NO_3)_2x6H_2O$ in 1L of bi-distilled water, to obtain Cu-based solution (namely $CuCl_2$-AS) and Zn-based solution (namely $Zn(NO_3)_2$-AS), respectively.

Furthermore, NaOH 1M in bi-distilled water was used to obtain metal hydroxide solutions (namely $Cu(OH)_2$-AS and $Zn(OH)_2$-AS respectively), according to the following reactions:

$$\mathbf{CuCl_2}x2H_2O + 2NaOH \rightarrow Cu(OH)_2 + 2NaCl + 2H_2O \quad (1)$$

$$\mathbf{Zn(NO_3)_2}x6H_2O + 2NaOH \rightarrow Zn(OH)_2 + 2NaNO_3 + 6H_2O \quad (2)$$

Two antiviral solutions based on the above-mentioned *sustainable Zein solutions* were prepared by dissolving 25 g and 150 g of zein powder in 1L of 98 % wt. ethanol at 80°C for 3h according to the procedure described in [45] to obtain a Zein solution equal to 25g/L (namely Z-AS25) and 150g/L (namely Z-AS150) respectively. Subsequently, to these antiviral solutions, 0.05g and 0.3g of lactic acid solution (85 % wt./wt., LA) were added to reduce pH (the final solutions were named as Z-AS25-L and Z-AS150-L) [46-49]. Zein-based ethanol solutions were prepared as reference solutions at the above-reported concentrations.

2.4. Preparation of the nanostructured coatings

Commercial PP-based-non-woven fabric coupons (synthesized according to Italian standard UNI EN 14638-2019, BFE≥95 %, differential pressure<40 Pa/cm^2, microbial disifection≤30 colonies forming units (cfu)/g, whose Fourier Infra-Red, FT-IR spectrum is shown in Figure S1, Supplementary materials), cut to a predefined size (2.5 cm x 2.5 cm), were coated using a dip coating technique at standard room temperature, relative humidity, and pressure conditions (**Figure 1**).

Non-woven fabrics (namely NWF) were preventively purified in ethanol and immersed in each antiviral solution. These include both *active chemicals* (the coated masks were named as NWF-$CuCl_2$ and NWF-ZnN) and *sustainable Zein solutions (*masks were coded NWF-Z-25, NWF-Z-150, NWF-Z-25-L and NWF-Z-150-L). NWF-$CuCl_2$ and NWF-ZnN were further soaked in NaOH solution to obtain metal hydroxide coatings. The coated masks were coded as NWF-$Cu(OH)_2$ and NWF-$Zn(OH)_2$, respectively.

Finally, NFW samples (NWF-$CuCl_2$, NWF-Z-25, NWF-Z-150, NWF-Z-25-L, and NWF-Z-150-L) were dried in air at standard room temperature, relative humidity, and pressure conditions. Conversely, NWF-$Cu(OH)_2$ and NWF-$Zn(OH)_2$ were dried at 60°C for 24h to allow the formation of the nanometric metal oxide coatings on the mask surface according to the following reactions:

$$2Cu(OH)_2 \rightarrow 2CuO + 2H_2O\ (\uparrow) \qquad (3)$$

$$2Zn(OH)_2 \rightarrow 2ZnO + 2H_2O\ (\uparrow) \qquad (4)$$

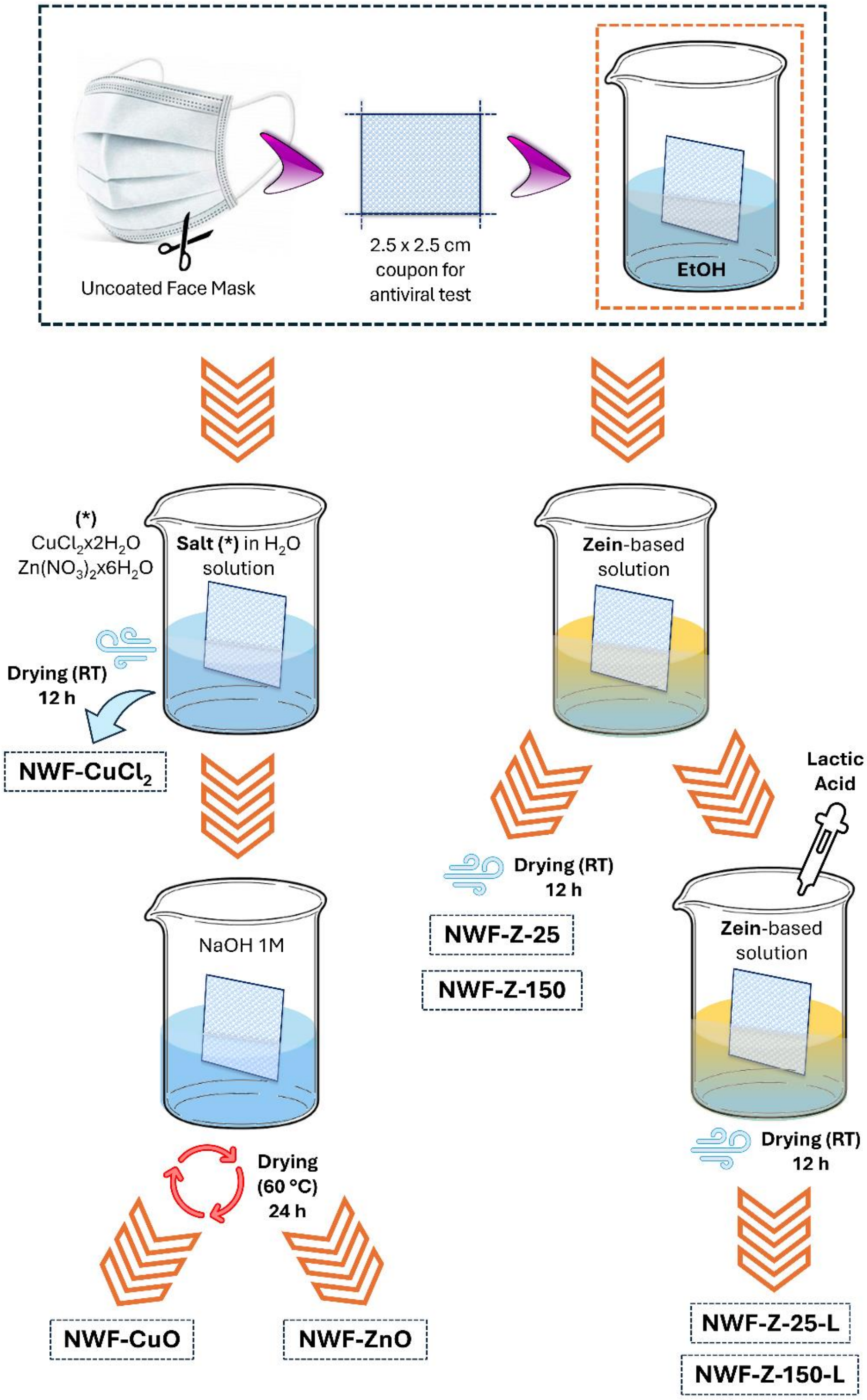

Figure 1. Schematization of the dip-coating procedure used to fabricate the examined non-woven fabric coatings. Left) Preparation of NWF-$CuCl_2$, NWF-CuO and NWF-ZnO. Right) Preparation of NWF-Z-25, NWF-Z-150, NWF-Z-25-L and NWF-Z-150-L.

The resulting coated masks were coded NWF-CuO and NWF-ZnO. It should be noted that NWF-Z-AS150 was not analyzed due to high brittleness caused by the high amount of zein; conversely, the mask named as NWF-Zn-AC was not synthesized because $Zn(NO_3)_2$ was meant to be only as a medium throughout ZnO-based coatings were obtained.

2.5. Characterization of the antiviral solutions

It is well established that common viruses have negatively charged surfaces when placed in solutions with pH values typical of the human body [50]. Consequently, they can readily interact with positively charged systems like lipids or electrodes. To verify the average external charge of the particles (in terms of ζ-potential) dispersed in the examined solutions, and to evaluate their potential application as virus gatherers, Laser Doppler Microelectrophoresis (LDME) analysis was performed through a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, Malvern, UK). Measurements were performed in triplicate at 25°C. Samples were diluted 100-fold before being measured.

2.6. Coatings characterization

The morphological characteristics of the coatings were examined through Scanning Electron Microscopy (SEM, FEI ESEM Quanta 200, the Netherlands), operating at 30 kV under different magnifications. Fabrics were opportunely coated with Au/Pt sputter coater (SC500, emScope-now Quorum Technologies Ltd) before imaging. Face mask-coupon mass was gravimetrically measured before and after coating formation, through an analytical balance (Pioneer OHAUS, Germany) having 6-decimal accuracy and estimated as the mass of deposited antiviral solution per unit of surface (mg/cm^2). Data are expressed as average and standard deviations of three independent measurements.

Tensile tests were carried out according to ASTM D 1708-02 standard and using a universal testing machine (model CMT4304 from Shenzhen SANS Testing Machine Co. China), equipped with 5 kN load cell. Specimens in the shape of stripes (100 mm x 10 mm, with thickness ranging between 0.14 and 0.22 mm) were cut from face masks, then tested as-cut for uncoated samples, while, for those coated with each of the selected solutions, they were first soaked and subsequently left for casting. Tests were performed imposing a velocity of 20 mm/min and at 25 ± 2 °C and RH= 50 ± 5%. The maximum tensile strength (σ_{max}, MPa, i.e., stress at the break) and the elongation at break (ε_{break}, mm/mm) were evaluated from stress-strain curves [36]. The elastic modulus (E, MPa) was estimated as the slope of the linear region of the stress-strain curve. For each sample, results were expressed as average and standard deviation of five independent measurements.

Oxygen permeability was determined using the coulometric sensor technology using an OxTran 2/22 (Mocon, Germany). Oxygen permeation tests were performed by setting the relative humidity at the downstream and upstream sides of the film to 50%. Each test was carried out in duplicate.

Water vapor permeability (WVP, g/m·s·Pa) was determined using the infrared sensor technology using a Permatran W3/34 (Mocon, Germany). Samples with a surface area of 5 cm^2 were tested at 25 °C. Permeation tests were performed by setting the relative humidity at the downstream and upstream sides of the film to 0% and 50%, respectively. For both tests, a mask sample (A=5 cm^2) consisting of all three layers was used: the tested multilayer material is composed by the outer layers, made of conventional non-woven fabrics, and by the intermediate layer, made of the developed coated non-woven fabric. A conventional (not modified) mask was used for comparison purposes.

2.7. Cytotoxicity assay

The coated fabrics were thoroughly washed with cell culture medium before the cytotoxicity test. L929 cells were grown in a 75 cm^2 flask in DMEM supplemented with 10% FBS, 1% NEAA, antibiotic solution (streptomycin 100 µg/mL and penicillin 100U/mL), and 2 mM L-glutamine. Cells were maintained in culture at 37°C, 5% CO_2, and 95% humidity to achieve a confluent layer. Before the experiments, all samples were sterilized using UV light for 30 min for each side. Cytotoxicity was assessed by indirect test (ISO 10993-5) using 0.1g/mL as the extraction ratio (mass/volume) (ISO 10993-12 for membranes, textiles [51]) and with elution and exposure time of 24 hours, respectively. After 24 hours of elution, 200 µL of the conditioned medium was pipetted onto a 96-well plate seeded with L929 at 80% confluence and cultured for an additional 24 hours. The cell viability was assessed using the Alamar blue test. This latter is a rapid, sensitive, and inexpensive test for measuring cell proliferation and cytotoxicity [52] in a wide range of human and animal cell lines [53]. It is based on the mitochondrial respiratory chain in live cells, which converts oxidized non-fluorescent blue resazurin into a red fluorescent dye (resorufin). Resorufin synthesis is directly proportional to the number of living cells [54].

2.8 Antiviral assay

Cell lines were cultured at 37°C in 5% CO_2 atmosphere in DMEM containing 10% (growth medium) or 5% (maintenance medium) FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM). Cell lines were maintained by bi-weekly passages in fresh medium. Viral suspensions, used as *inocula*, were obtained from centrifuged lysates of virus-infected cell cultures. Virus batches were titrated on cell cultures, aliquoted, and stored at -80ºC.

Face mask coupons, previously sterilized under UV light for 15’/side [55] were soaked in a tube containing 2 mL of viral inoculum and incubated for 2 minutes at room temperature. Mask fragments were then removed, and the residual infectious viral load of the supernatants was quantified by end-point titration on cell cultures. Briefly, from each sample, 10-fold dilutions were prepared and seeded onto 24 h growth cells in a 96-well culture plate. After 3 days, the viral titer of each sample was estimated as Tissue Culture Infectious Dose 50 i.e., the concentration required to infect 50 % of cell culture (TCID50/mL), considering the last dilution still showing the typical virus cytopathic effects (CPE) under an inverted optical microscope [55]. In each experiment, masks coated with only the solvent (Ethanol) used to suspend the nanomaterials were used as controls. All mask samples were tested at least 3 times in duplicate with each virus.

2.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism®, version 5.00 (GraphPad Software, La Jolla California USA, www.graphpad). A *p*-value < 0.05 was considered statistically significant. Student's t-test, one-way analysis of variance (ANOVA) with the Bonferroni and Kruskal-Wallis tests were applied when appropriate.

3. Results and discussion

3.1. Characterization of antiviral solutions

As anticipated in paragraph 2.5, LDME analyses revealed positive zeta potential (ζ-potential) values for all the examined solutions, making possible the interaction with viruses (thus to be considered as antiviral), which are typically negatively charged [39] (**Table 1**).

Table 1. ζ-potential of antiviral solutions expressed as a mean value of three independent measurements ± Standard Deviation (SD).

Legend: copper chloride-antiviral solution, $CuCl_2$-AS, copper hydroxide antiviral solution, $Cu(OH)_2$-AS, zinc hydroxide antiviral solution, $Cu(OH)_2$-AS, zein 150 g/L antiviral solution- Z-AS150, zein 25 g/L antiviral solution- Z-AS25, zein/LA 150 g/L antiviral solution, Z-AS150-L, zein/LA 25 g/L antiviral solution, Z-AS25-L.

Entry	**LDME** **ζ-potential (mV)** Mean ± SD
$CuCl_2$-AS	27.0 ± 2.8
$Cu(OH)_2$-AS	6.0 ± 1.7
$Zn(OH)_2$-AS	6.1 ± 2.2
Z-AS150	9.3 ± 0.7
Z-AS25	8.4 ± 0.3
Z-AS150-L	13.9 ± 0.5
Z-AS25-L	12.7 ± 1.5

In the case of Cu-based active chemical solutions, higher values were observed for $CuCl_2$-AS compared to the corresponding metal hydroxide. This implied that a more positive charge was exposed, which could be more effective for antiviral purposes. The resulting colloids were also more stable than their hydroxide counterparts, as the ζ-potential values were between 20 and 30 mV [56]. Moreover, $CuCl_2$-AS is based on the ionic bond, which is more labile than $Cu(OH)_2$-AS, whereas the chemical interactions are covalent-based. In this regard, a higher positive charge was distributed along the particle surface.

For the zein solutions, it should be noted that the ζ-potential increased when lactic acid was added to the solution. Once again, the result indicates an increase in the exposed positive charge and thus enhanced electrostatic activities with viruses and microorganisms compared to the bare zein-based solutions. Indeed, the physical/chemical characteristics of zein and the resulting films are strongly affected by the solvent chemical nature i.e. ethanol/ethanol-water solutions, and by the presence of acidifying agents, resulting in different solubility and thus in pH and ζ-potential [56]. It is also important to argue that, within the explored conditions, no important effect of zein concentration on ζ-potential was observed.

3.2. Coatings characterization

Morphological analysis was carried out to highlight the impregnation of the antiviral solutions into non-woven fabrics (**Figure 2**). As insets, cross-section images at higher resolution are displayed.
NWF presented tubular like-fibers of average length of 600 μm and diameter of 20 μm. The selected nanostructured coatings did not alter the morphology of non-woven fabrics upon dip coating; however, differences in terms of deposition of the employed antiviral components could be noted. CuO particles present a leaf-like structure with dimensions less than 1 μm and appear as aggregates completely covering the individual fibers, this result is in agreement with previous works [14, 57, 58], where CuO-based coatings for textiles were obtained through sonochemical routes. ZnO particles exhibited a rod-like structure with a length of 2.9 µm and a width of 1.2 µm, approximately [57]. Similar to CuO structures, it was found to be completely deposited on the individual non-woven fabrics, by increasing the roughness of the systems, [14] with a higher tendency to agglomeration, as demonstrated in previous works [58, 59]. Conversely, the deposition of $CuCl_2$ led to homogenous and smooth coatings; also in this case, the morphological features of non-woven fabrics were not altered by the deposition of the active compounds. More specifically, $CuCl_2$ presented a lamellar-like structure with a size lower than 1 micron, inducing surface roughness in the nanometric scale and thus resulting applicable as a powerful antiviral agent (as confirmed by antiviral tests) [14]. On the other hand, the tested zein-based coatings were homogenous, whereas its particles assumed a ribbon-like structure [60]. This is in agreement with the work of Torres-Gigner *et al.,* where zein nanofibers at different concentrations (from 18 wt. % to 25 wt. %) were synthesized through electrospinning routes [61].
The effect of the acidifying agent on the morphology of zein-coated NWF can be pointed out. In particular, the addition of a low amount of LA induced an important variation of the morphology from ribbon-like to globular-like shape, with an average size < 1 µm.

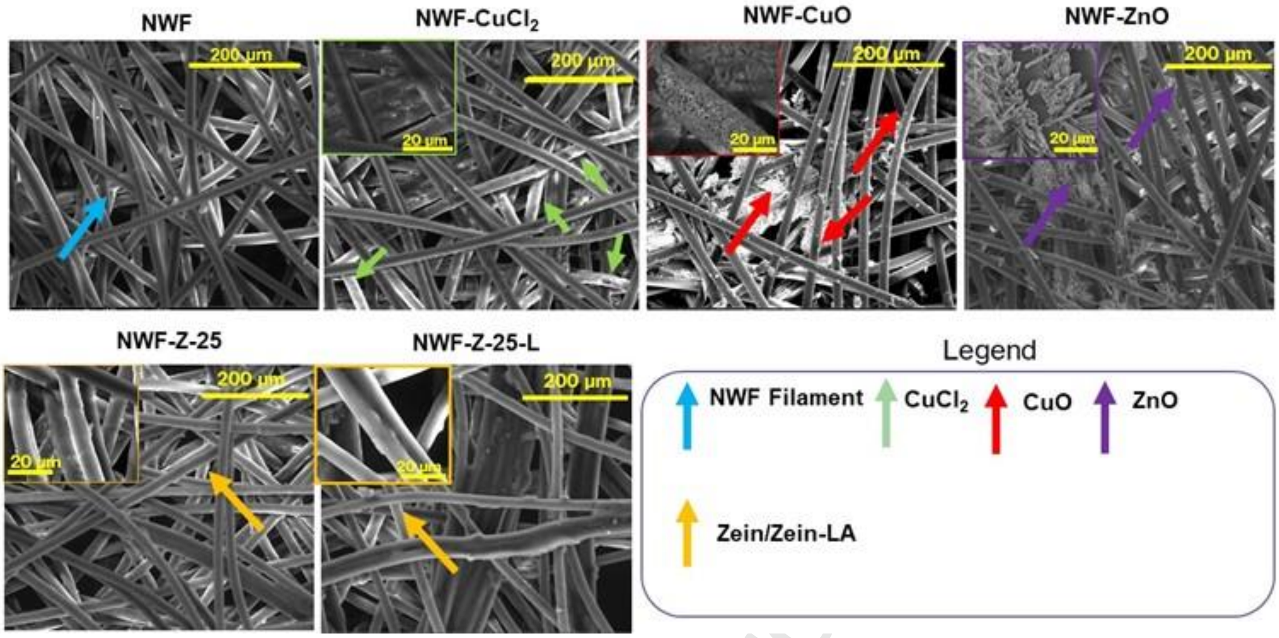

Figure 2. SEM images of the examined nanostructured coatings. Scale bar=200 µm. Magnification:500X. Acceleration voltage=20 kV. Insets: higher-magnification images meant to analyze the deposition of the active compounds onto the non-woven fabrics (scale bar=20 µm, magnification=5000X).

As expected, the examined nanostructured coatings exhibited different amounts of antiviral solutions per unit of fabric surface **(Table 2**). As a reference, the mass per unit of surface of net NWF is shown. All the investigated coated NWF possessed more than 40 % more coating mass than the control system. On the other hand, the highest mass deposition per unit of surface was found for the case of NWF-Z-150-L, being 3 times more than the mass per unit of surface of NWF.

Table 2. Results of mass deposited per unit of mask surface upon dip coating. Data are shown as average and Standard Deviation (SD) of six independent measurements.

Entry	Mass deposited per unit of face mask surface (mg/cm^2)
NWF	5.3 ± 0.3
NWF-$CuCl_2$	8.5 ± 0.9
NWF-CuO	8.8 ± 1.3
NWF-ZnO	8.8 ± 0.9
NWF-Z-25	7.7 ± 0.6
NWF-Z-25-L	9.2 ± 0.1
NWF-Z-150-L	14.4 ± 0.7

Mechanical tensile tests were performed on both coated and uncoated NWF samples. As can be seen (**Table 3**), in all cases the coating was applied, the maximum strength (σ_{max}) increased, as well as the elastic modulus (E) while the deformation at break (evaluated in correspondence of the maximum strength, $\boldsymbol{\varepsilon_{break}}$) decreased.

For the copper and zinc-based coatings, used as references for the viral tests, σ_{Max} had a slight increase with respect to the uncoated NWF. A similar trend was noticed for the case of elastic modulus, whereas the maximum increase compared to the uncoated material was attained for the case of CuO (38 %, approximately). Such results suggest that metal-based coatings slightly enhance strength and stiffness, but reduce the ductility of the material; indeed, the usage of oxides was crucial to provide additional strength and rigidity. The following results are in agreement with some relevant works focusing on the use of CuO nanofibers/nanoparticles for electrospun polyacrylonitrile (PAN)/Copper oxide (CuO)-based nanocomposites for respiratory masks [62].

Table 3. Tensile properties of the selected coated-face masks.

Entry	E (MPa)	σ_{max} (MPa)	ε_{break} (at break) (mm/mm)
NWF	32.3 ± 5.5	7.0 ± 0.2	0.70 ± 0.10
NWF-$CuCl_2$	42.4 ± 5.6	7.3 ± 0.1	0.43 ± 0.04
NWF-CuO	51.9 ± 7.3	8.8 ± 0.5	0.42 ± 0.02
NWF-ZnO	51.2± 5.3	8.0 ± 0.2	0.40± 0.03
NWF-Z-25	86.1 ± 11.7	11.4 ± 0.8	0.43 ± 0.05
NWF-Z-150	194.9 ± 7.1	18.9 ± 3.5	0.38 ± 0.03
NWF-Z-25-L	43.4 ± 4.6	7.01 ± 0.40	0.61 ± 0.07
NWF-Z-150-L	155.5 ± 12.0	15.1 ± 2.0	0.52 ± 0.02

Moreover, the pure-zein-based samples (NWF-Z-25 and NWF-Z-150) exhibited an increase in maximum strength and elastic modulus (Table 3), and a reduction in deformation evaluated at maximum strength, meaning that a substantial increase in strength and stiffness occurred, making the material less flexible but more resistant to deformation. In particular, where the treatment with 25 g/L solution has almost doubled the (11.4±0.8 MPa) and almost tripled *E* value (86.1±11.7 MPa), with respect to the pristine one, the coating obtained with the 150 g/L solution gave an even higher σ_{max} value (18.9±3.5 MPa) and *E* six times higher (194.9±7.1 MPa) than the uncoated NWF. In both cases, though, the elongation was reduced. These outcomes indicate that the presence of zein provides the best reinforcement, producing a material that is both strong and rigid, though slightly less ductile than the uncoated sample. However, the mechanical values changed when the further modification of both zein solutions utilizing lactic acid was operated. For the case of NWF-Z-25-L, the mechanical properties partly recovered their original value, getting more similar to those of the pristine sample, suggesting that the LA modification sacrifices stiffness and strength (σ_{max} equal to 7.0±0.4 MPa and E equal to 43.42±4.6 MPa), but improves deformability (0.61 mm/mm). The same trend was also observed when concentrated zein solution was used (NWF-Z-150-L), with reduced elastic modulus and maximum strength, and increased deformation. Considering the final use of such materials, these outcomes might result in better comfort for the user, combined with a more effective antiviral activity (see section 3.4).

Oxygen permeability measurements showed that for all the investigated samples the oxygen transmission rate (OTR) values were higher than the evaluation limit of the instrument (>2000 mL/m^2 day). This feature was maintained also for all the investigated coated face mask samples, thus showing that the breathability of the face mask tissue was not affected by the presence of the coatings. On the other hand, differences in water vapor permeability were noted. It was found that the examined coated-face masks exhibited differences in water vapor permeability compared to the uncoated non-woven fabrics. More specifically, NWF-Z-150-L exhibited the lowest permeability value, being 26 % lower than that of unloaded material (passing from $1.2{\cdot}\pm0.2{\cdot}10^{-12}$ g/m s·Pa to $8.9{\cdot}\pm0.9 \cdot 10^{-13}$ g/m s Pa). This result supports the idea that the examined face masks are more effective in preventing the risk of spreading of small droplets from the inner to the outer layers of the face mask [63], i.e. during breathing, speaking, or coughing, representing a valuable solution from antimicrobial/antiviral point of view. Copper-based coatings (NWF_CuO) presented comparable water vapour permeability compared to the unloaded non-woven fabrics ($9.5{\cdot}\pm0.6{\cdot}10^{-13}$ g/m s·Pa). Conversely, higher permeability values were attained for the case of NWF_$CuCl_2$ ($1.5{\cdot}\pm0.5{\cdot}10^{-12}$ g/m s·Pa) It has been demonstrated by the work of Benjan et al., [64], where the use of copper oxide nanoparticles provided enhanced water permeability to the biobased nanocomposite fibers compared to the reference materials.

3.3 Cytotoxicity assay

Cell viability of the selected systems was estimated as a percentage of the reference cell line (**Figure 3**). L929 fibroblasts in contact with NWF-Z-25-L, NWF-Z-150-L, NWF_$CuCl_2$ and NWF_CuO exhibited higher or comparable viability in comparison with the controls (L929 and NWF). Conversely, NWF-Z-25 and NWF-ZnO led to a cell viability reduction of 50% and 60% respectively (p<0.01).

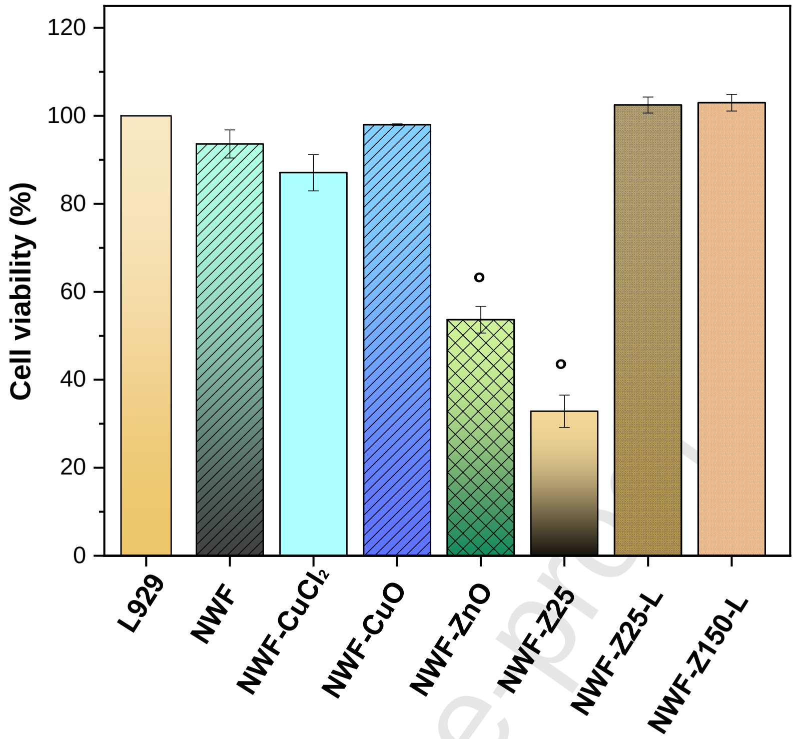

Figure 3. Cell viability of the selected nanostructured coatings expressed as a percentage of cell viability of the reference cell line (L929), °*p*<0.001 vs L929 plate control.

3.4 Antiviral assay

The virucidal activity of nanostructured coatings was assayed against HSV-1, AdV-5, CoV-OC43, and MPoxV and compared with those of conventional metal-based ones. The selection of such viral strains was driven by different reasons and characteristics: i) transmission through respiratory droplets, ii) distinct morphology i.e. naked vs enveloped virus with consequent different resistance towards the environment, and the inactivating treatments (low for HSV-1, intermediate for HCoV-OC43, extremely high for the two non-enveloped viruses, AdV-5 and MPoxV) and iii) ease of cultivation [55]. HCov-OC43 presents an extremely high homology of structure with CoV-SARS-2, from both a phylogenetic and a molecular point of view. They both belong to the β-Coronavirus group, in an extremely close position of the phylogenetic tree. Since germicidal treatments act by non-specific mechanisms, viruses with a high morphological similarity likewise respond to inactivation [65]. Therefore, HCoV-OC43 has been used in several studies focusing on viral persistence/inactivation as a substitute for the highly pathogenic Coronaviruses SARS-1, SARS-2, and Middle-East Respiratory Syndrome Coronavirus (MERS) [65-67]. MPoxV causes monkeypox, a disease with symptoms similar to smallpox, although less severe. While smallpox was eradicated in 1980, monkeypox has kept occurring in countries of central and West Africa. Since May 2022, an outbreak has involved many countries outside Africa, without previously documented MPoxV transmission, for a total of 95,226 cases and 185 deaths [68]. Furthermore, in autumn 2023, a new variant of MPoxV (clade Ib) was discovered in the Democratic Republic of Congo: this strain displays a higher transmissibility in comparison with the strain responsible for the 2022 epidemic and is associated with a more severe disease. Due to the rapid diffusion in central Africa, also in countries with no previous cases of MPoxV, on Aug 14 th 2024, the World Health Organization (WHO) declared MPoxV Public Health Emergency of International Concern (PHEIC)[69].

The viral load present on the mask was quantified through biological assay rather than molecular approaches. As a matter of fact, molecular methods cannot discriminate whether the genetic material possibly detected derives from infectious or inactivated particles. Conversely, the biological method herein proposed, though less sensitive than the molecular one, provides essential information about the infectivity of the examined viruses. Both NWF-$CuCl_2$ and NWF-Z-150-L provided a remarkably significant viral load reduction (2.4 Log, **Figure 4a**), while for NWF-CuO the reduction was 1.2 Log. In the case of HCoV-OC43, NWF-$CuCl_2$ and NWF-Z-150-L gave the best reductions i.e., 1.8 Log and 2.1 Log respectively ($p<0.01$, **Figure4b**), while no statistically significant activity against AdV-5 and MPoxV was demonstrated for any of the treatments, the best performance being a 0.7 Log reduction by NWF-$CuCl_2$ against both of them (**Figure 4c** and **d**).

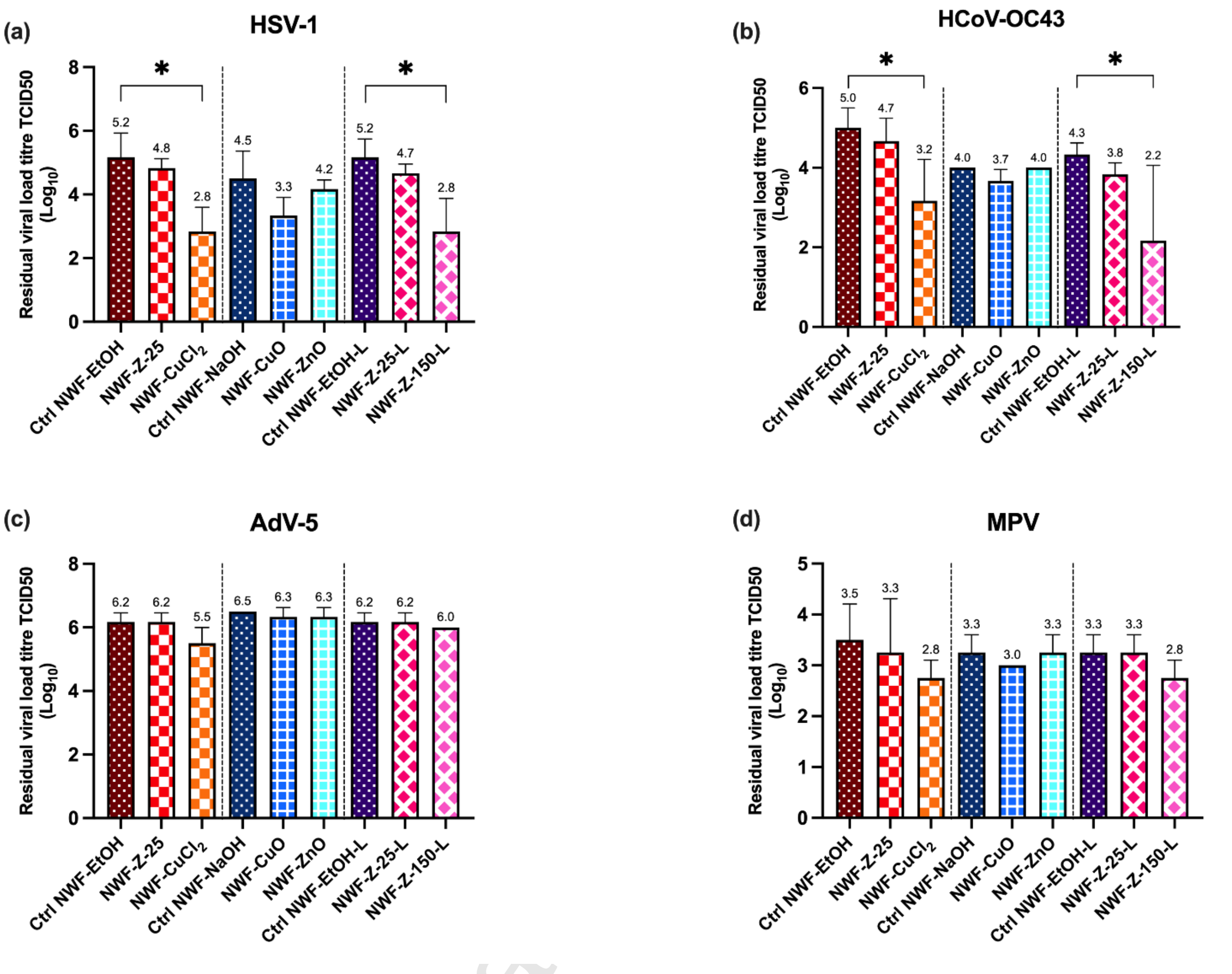

Figure 4. Evaluation of the antiviral efficacy of the coated face masks on the selected viral strains: a) HSV-1, b) HCoV-OC43, c) ADV-5, and d) MPoxV. The results are expressed as the Log_{10} of the residual viral titer after 2 min exposure. At least three experiments, each in duplicate, were carried out for each virus. *$p< 0.01$.

Not surprisingly, the best results were obtained with HSV-1 and HCoV-OC43 which are enveloped viruses with limited resistance outside the host, whereas AdV-5 and MPoxV are extremely stable retaining their infectivity up to several months in the environment and are also resistant to many disinfectants, including ethanol.

The possible mechanisms of this antiviral activity can be associated with the different chemical nature of the antiviral solutions. Copper/zinc can be easily oxidized in aqueous solutions, becoming oxides with subsequent release as ions: therefore, copper in the form of salts ($CuCl_2$) seemed to improve its antiviral activity owing to the larger ion availability compared to the oxidized form. Different antimicrobial action mechanisms are proposed for metal particles, mainly attributed to their oxidative behavior [70]. The most probable one involves membrane depolarization corresponding to surface interaction among positive-charged metals, and virus negative-charged surface molecules, leading to the formation of ROS with envelope oxidation as well as viral nucleic acid degradation [71, 72]. Indeed, the considerable virucidal activity against the two enveloped viruses (HSV-1 and HCoV-OC43) supports such a hypothesis.

For zein-based systems, it can be assumed that electrostatic interactions represent the principal mechanism of action [73]. It might be possible that the positive charge of zein can bind with surface proteins of viruses, interfering with the virus-host cell interaction to block fusion and entry, and/or virus budding from membranes of the host cell [71] (**Figure 5**). This antiviral activity seems more appealing than that of $CuCl_2$-based coatings because it overcomes some issues of conventional metal-based coatings such as leaching in the environment, possible antiviral resistance as well as poor sustainability [74].

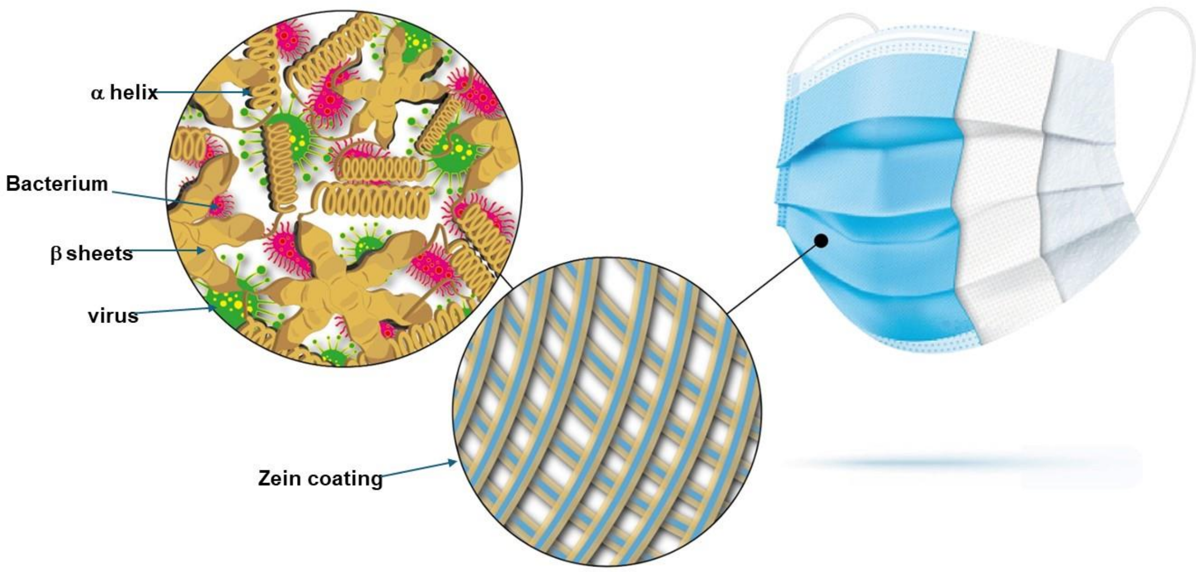

Figure 5 Schematic representation of the antiviral mechanism of zein-based coating on non-woven fabrics.

4. Conclusions

The SARS-CoV-2 pandemic dramatically posed the urgent need for efficient, low-cost, and environmentally friendly strategies to restrain the spread of infectious agents, especially those air-borne-transmitted. On this account, surgical face masks are widely used not only in medical settings but also in the social community as a preventive tool. They represent a valid PPE that contributes significantly to the containment of infectious diseases; however, they display some important limitations, mainly negligible germicide/virucide activity as well as poor sustainability (leading to remarkable waste management issues). Therefore, this work aimed at setting up a proof of concept based on nanostructured coatings for disposable face masks with enhanced virucidal activity by selecting for the first time a sustainable antiviral agent based on zein, a biopolymer derived from corn. This approach provides several key advantages, including biocompatibility, non-toxicity, sustainability, and biodegradability, offering valuable solutions to the environmental and health challenges linked to face mask usage.

Coatings were obtained through an easy route based on dip coating and subsequently characterized from chemical/physical and morphological points of view. This procedure allowed to obtain uniform coatings with both low and high concentrations of active compounds. A comparison with metal-based coatings i.e., copper, copper oxide, and zinc oxide, was done. In this latter, an easy route was employed to prepare metal oxide coatings: i) oxido-reduction reactions were employed to attain metal hydroxides; ii) mild temperature conditions (i.e., 60°C for 24 h) were used to remove water and thus to get metal oxides. Moreover, for the case of zein-based coatings (obtained at room temperature conditions), lactic acid was used as an acidifying agent in the zein/ethanol solutions, allowing to suitably functionalize the resulting deposited materials, overcoming the high brittleness of zein-based coatings.

The following conclusions can be drawn from this study:

- ζ-potential revealed that all the antiviral solutions were exposed to positive external charge, enabling to electrostatically interact with negative-charge viruses.
- Functionalization of zein solutions through lactic acid seemed to positively impact on mechanical and functional characteristics of the resulting coatings.
    - Zein-based coatings displayed homogenous structures, whereas metal-based ones exhibited an aggregate-like morphology, with improved tensile properties compared with the uncoated NWF achieving a value of elastic modulus six times more than that of uncoated fabrics.
    - The use of lactic acid allowed to partially recover the original mechanical characteristics of the uncoated masks, although with improved deformability.
    - The breathability, measured through oxygen and water permeability, of the resulting coatings was comparable or slightly lower with that of uncoated face mask, preventing the risk of biological contamination *via* the spreading of small droplets through the face masks.
- The cytotoxicity analysis revealed that zein-lactic-based coatings along with Cu-based coatings were not cytotoxic at the investigated conditions.

- The highest antiviral activity of zein-lactic-based coatings was attained (2.1 Log and 2.4 Log) on HCoV-OC43 (the SARS-CoV-2 surrogate) and HSV-1, respectively, whereas no significant effect was observed against MPoxV and AdV-5.

Overall, the examined zein-based coatings represent an effective tool to improve the antimicrobial protection provided by surgical masks. Compared to conventional metal-based coatings, the antiviral action of zein is mainly based on the electrostatic interactions between positive charge-zein and negative charge viruses, whereas, in this latter case, more complex phenomena are concerned (i.e. formation of ROS), leading to some relevant leaching effects and possible resistance to treatments.

4.1 Future Perspective

As a future perspective of this work, investigations of the antimicrobial features (on bacterial strains relevant in healthcare applications) of the selected nanostructured materials along with filtrating capabilities will be accurately examined. Additionally, the antiviral properties of combined zein with other antiviral agents can be examined along with the assessment of its durability, hypothesizing possible scenarios such as aging owing to saliva or sweat as well as folding or washing cycles. Moreover, a dedicated study about molecular dynamic (MD) simulations will be required to gain deeper insights into the antiviral mechanism(s) of zein. In particular, it will clarify how the supramolecular structure of the zein protein might interact with the viral envelope or proteins.

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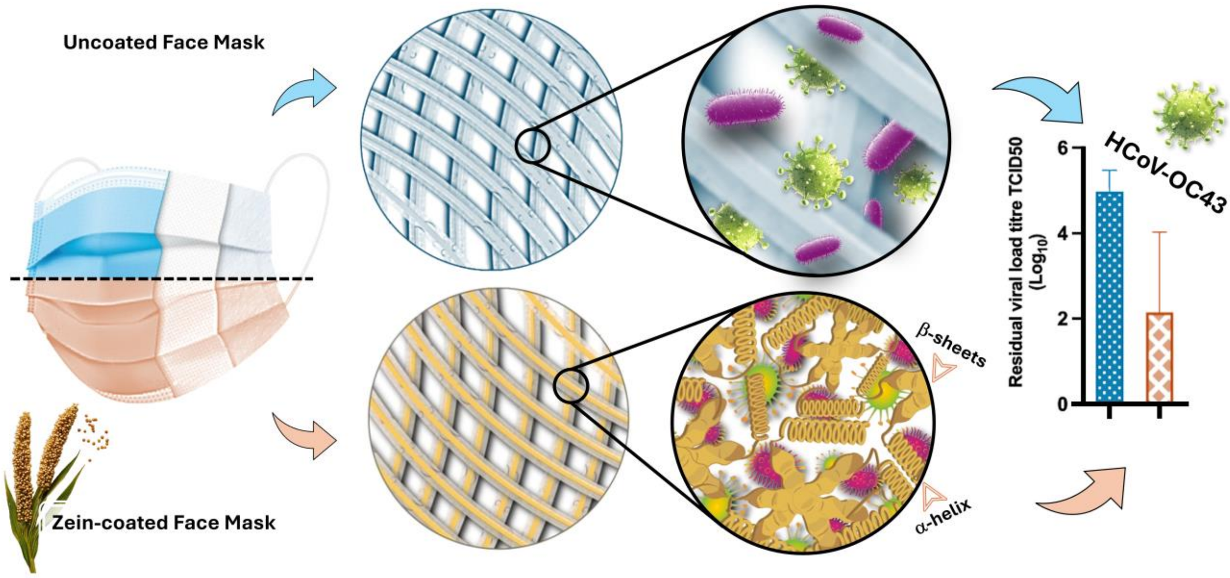
Declaration of interests

XThe authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Graphical Abstract

Highlights

- Innovative and sustainable zein-based coatings for face masks were developed
- The antiviral efficacy was studied on 4 respiratory viral strains.
- Zein provides higher or comparable antiviral activity compared to active compounds.
- Zein antiviral properties are linked to electrostatic interactions with viruses.
- The selected zein coatings are not cytotoxic.