SILESIAN UNIVERSITY OF TECHNOLOGY PUBLISHING HOUSE

SCIENTIFIC PAPERS OF SILESIAN UNIVERSITY OF TECHNOLOGY 2023
ORGANIZATION AND MANAGEMENT SERIES NO. 188

A NEW METHOD OF CORONAVIRUS VIRION DETECTION – INNOVATIVE METHOD OF IDENTIFICATION TRANSFORMING

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Purpose: the article presents the result of an attempt to assess the possibility of using scanning electron microscopy and energy dispersive spectroscopy (SEM/EDS) to identify silica nanoparticles (SiO_2), which, due to the size of individual particles (200 nm), can be used as coronavirus markers in simulation tests.
Methodology: SEM/EDS evaluation was performed using three different media types; namely membrane filters, sponge filters and graphite discs.
Result: SEM/EDS studies, consisting in determining the morphology and grain size of SiO_2 markers and X-ray microanalysis of their elemental composition, proved that this technique can be successfully used to identify markers.
Originality: due to their particle size, ease of handling with various types of surfaces, and biological and physicochemical neutrality, silica markers can act as coronavirus substitutes in experimental studies.
Keywords: SEM-EDS, silica nanoparticles, microbiological markers, virus marker, virus surrogate, Sars-CoV-2.

1. Introduction

COVID-19 pandemic, which originated in December, 2019 in the Hubei Province, China, afflicted over 298 million of people all over the world and seriously affected the socio-political situation of more than 213 countries (Bhardwaj et al., 2021; Lin et al., 2021). SARS-CoV-2 (*Severe Acute Respiratory Syndrome Coronavirus 2*) belongs to the betacoronaviridae genera which includes *inter alia* the highly pathogenic in human viruses such as SARS-CoV (*Severe Acute respiratory Syndrome Coronavirus*) or MERS-CoV (*Middle-East Respiratory Syndrome Coronavirus*) (Kwak et al., 2021). Epidemiological data proves the SARS-CoV-2 virus is much more contagious than SARS-CoV or MERS-CoV responsible for the epidemics in 2003 (SARs-CoV-1) and 2012 (MERS-CoV), respectively. According to WHO and CDC US, virus SARS-CoV-2 is transmitted predominantly by airborne droplets or as a result of the contact with the respiratory secretion of a COVID-19 infected people; however, the most recent evidence shows that contaminated air may constitute yet another mechanism of the pathogen transmission (Rieseberg et al., 2001; Zamora, Aguilar, 2018; King et al., 2020). The emergence of new airborne transmitted diseases (e.g. COVOD-19) is the rationale for conducting research studies to better understand the scale of the phenomenon f the airborne transmission of the pathogens. The measurement of airborne transmitted pathogenic viruses constitutes an interdisciplinary research area which necessitates the understanding of both the aerosol techniques and microbiological issues (Śliwińska, 2002). The majority of field literature concerning the detection of pathogens in bio-aerosols focuses on the presence of bacteria, fungi or allergens, while the reviews of virus sensors concentrate on the detection of the viruses in water solutions rather than in the air. What complicates the experimental research in this area is the fact that the SARS-CoV-2 virus, due to its rapid spread among the population and the high virulence, was considered as a particularly harmful biological agent (category III). This fact practically precludes using the virus in studies under *in-situ* conditions. The application of physico-chemical markers which may be used for marking of the pathogens (fluorescent markers) or which, due to their corresponding size, may constitute pathogen surrogates in laboratory research (the so-called markers) constitutes an alternative solution in airborne transmission studies. In the case where fluorescent markers are used in microbiological research, it is possible to apply small organic particles ($\leq$ 20 atoms), small particles of sizes in the range of 100-100 000 atoms (2÷10 nm) – the so called quantum dots and fluorescent proteins (e.g. green fluorescent protein, GFP 26kDa, 238 aa). The fluorescent markers are applied in order to mark selected pathogen structures or to build complexes with other proteins, like in the case of GFP. In virological research, fluorescent markers are employed to create virus vectors to enable the observation of the created structures using such techniques as fluorescent microscopy of flow cytometry (Brickey, Zydneya, Gomezab, 2021). Apart from the fluorescent ones, the silica markers (SiO_2) may be also applied. The silica markers constitute nontoxic

particles which are safe for the research staff and the environment. Their sizes are in the range of 40-400 nm; homogenous solutions with silica markers may be sprayed in the form of aerosols in a dedicated space or on a research object in real conditions and in a non-isolated environment. Unlike viruses whose identification in laboratory scale is performed by means of ELISA tests, titration tests, *Western Blot* analysis or RT-qPCR method, the silica markers may be identified on the given carrier using both the qualitative and quantitative methods. It is worth mentioning that more and more often within the course of microbiological and virological studies, microscopic techniques are used because they facilitate detailed morphological characteristics of the pathogen. The Transmission Electron Microscopy (TEM), Epifluorescence Microscopy (EFM) and electron microscopy are examples of the techniques which have found application in research studies on virus particles. When a physico-chemical marker (silica marker) is used in the investigations, there is a possibility to apply Scanning Electron Microscopy (SEM) with an Energy Dispersive Spectroscopy system (EDS) for the purpose of the identification tests. This technique is mostly used in materials science in order to draw up the characteristics of surfaces or surface layers of the examined objects and materials, in particular the morphology and elemental composition. To date, the SEM technique has been widely applied in material engineering and metallurgy (Li, Liu, Du, Li, 2020; Bernardi et al., 2018). What is important, especially in terms of the phenomena discussed in this paper, literature sources confirm the possibility of silica nanoparticle imaging by means of the SEM and TEM techniques. For instance, Huseynov (Huseynov et al., 2011) used the SEM technique for the imaging of the adhesion phenomenon utilizing neutron irradiated nanoparticles of silica. Additionally, other author also carried out an analysis of the nanomaterial structure based on the techniques such as the Transmission Electron Microscopy (TEM) and Selected Area Electron Diffraction (SAED) (Kling et al., 2008; Schamm et al., 2008). In numerous publications, the authors emphasize the possibility of applying the TEM technique as a tool to observe and quantitatively identify nanomaterials (≤100 nm). However, it is worth mentioning that the authors point out certain limitations in applying the TEM technique to nanoparticle imaging which result from the problem of observing the distant background of samples caused by the high energy electrons of the TEM devices. In such cases, it is recommended that SEM systems should be used which operates with the stream of electrons of maximum energy in the range of 30-50 keV (Matuszewski, Sintorn, 2021). Rades demonstrated that techniques such as SEM (Scanning Electron Microscopy), T-SEM (Transmission Scanning Electron Microscopy, EDX (Energy Dispersive X-ray Spectroscopy) and SAM (Scanning Auger Microscopy) constitute effective and relatively inexpensive tools which enable to carry out a comprehensive morphological and chemical characteristics of a single nanoparticle of silica and titanium, representative materials recommended by OECD Guidelines because they are frequently applied as commercial nanomaterials (Rades et al., 2014). The experimental approach using Scanning Electron Microscopy, Transmission Electron Microscopy and FIB/SEM tomography was also applied to studies on the "evolution" of Mg-silicate particles during the process of producing optical

fibers. In these studies, the research objective was to characterize quantitatively the composition, size and shape distribution of Mg-silicate particles within the initial silica-based preform and the final product – the fiber (Cabiéa, Neisius, Blanc, 2021). The application of SEM technique in studies where silica nanoparticles were used became the subject of works conducted by Reghioua (Reghioua et al., 2019) and Cairns (Cairns, 2020). However, it must be stated that in none of the abovementioned works silica nanoparticles were used as specific surrogates of the harmful microbiological agents (microbiological markers), which is particularly important while taking into consideration the different methodology of preparing and spraying the samples, the concentrations of the nanomaterials and different types of surfaces on which the marker was being identified. Within the framework of this study, an attempt was made to assess the possibility of applying Scanning Electron Microscopy (SEM/EDS) to identify a silica marker of a size corresponding to the sizes of single coronavirus virions (200 nm) sedimented on different types of carriers. The research will enable to identify silica markers of the coronavirus virion size on sponge filters, membrane filters and graphite discs.

2. Methods and materials

The research process was designed in such a way which would facilitate to assess the possibility of applying the SEM/EDS technique to qualitatively identify the silica marker as well as to assess the applicability of this method in the case of different types of abiotic surfaces of various structure, porosity and shape. The possibility of applying the silica marker as a potential marker of virus pathogens, along with the option of its further identification on different types of carriers, constitutes a significant issue concerning simulation tests under laboratory and real conditions in terms of the pathogen spread as well as its presence on different types of surfaces. Considering the above, the idea of this research study was based on the assumption that the examined solutions would be sprayed by means of an atomizer on different types of carriers in a manner simulating the direct transmission route of the coronavirus (i.e. cough and sneezing) and next the carriers with the sedimented material would be subject to microscopic analyses using the SEM/EDS technique.

Two types of solutions were used in this study as research material; namely, the control solution (physiological saline solution constituting the equivalent of human saliva) and the silica marker solution (constituting the surrogate of the SARS-CoV-2 virus). The following three types of abiotic carriers were employed in the research: a membrane filter SKC, a sponge filter CIP and a graphite disc. The solutions were sprayed onto the carriers under laboratory conditions in the form of aerosols produced by means of a Turn'n'Spray atomizer (Bürkle, GmbH) (https://www.burkle-inc.com…, 2022). The experiment was conducted within the

framework of two research cycles for each type of the carrier. Each cycle consisted of three series during which the two solutions were applied on each of the carriers; the control solution (cycle 1) and the silica marker (cycle 2). In the next step, the carriers were subject to the SEM/EDS microscopic analysis.

2.1. The examined solutions

2.1.1. Control solution

The 0.9% solution of sodium chloride commonly referred to as a physiological saline solution (NaCl, molecular weight 58.44) was used as the control solution. The physiological saline solution was selected for the purpose of the research due to the fact that its chemical composition approximates the chemical composition of the secretion generated in the mouth during talking or sneezing. The solution is available on the market in the form of a chemical reagent (CHEMPUR, WE: 231-598-3; CAS: 7647-14-5). It is an odorless and colorless liquid with a relative density of 1.01 g/cm^3 (20°C). According to the Regulation (EC) no 1272/2008 of the European Parliament and of the Council of 16 December 2008 on the classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006, the solution used in the research is not classified as a dangerous substance.

2.1.2. The solution of silica marker

The silica marker solution used in the study was a mixture of distilled water and nanoparticles of silica (SiO_2) which performed the role of coronavirus surrogates. The silica marker has the form of solid spherical silica nanoparticles of 200 nm diameter (General Engineering & Research, USA) suspended in water (basic SiO_2 solution). According to the product characteristic, the silica nanoparticles were synthesized using the Stöber method (www.geandr.com, 2022) which enables to obtain the final product of a very high purity (+99.999%) with a narrow size distribution. The characteristic of the nanoparticles used in the tests was drawn up on the basis of the manufacturer data and presented in Table 1. In the course of the research, the working solution was prepared by means of transferring 4 ml of the basic silica marker solution into a 100 ml volumetric flask and filling it up with distilled water to reach the total volume of 100 ml. In the initial phase of the research works, an attempt was made to prepare the silica solution based on physiological saline solution. However, due to the process of agglomeration of the NaCl particles and silica nanoparticles (SiO_2) which impedes the identification of the material sedimented on the carriers, it was decided that the marker solution would be prepared using distilled water. The adopted approach allowed for the imaging of the nanoparticles sedimented on the carriers.

Table 1.
Characteristic of the silica (SiO_2) marker solution used in the study

Composition/ formula		**SiO_2 suspended in H_2O**	
Components of the basic solution (SiO_2 suspended in H_2O)		Water	> 80.0%
		Colloidal silica	< 15.0%
The aspect and the form of the basic solution	Form	Liquid	
	Colour	Transparent to white	
pH [-]		7.0-8.0	
Relative density of the solution [g/cm^3]		1.00-1.20	
Density of SiO_2 [g/cm^3]		2.65	

2.2. The carriers of the examined solutions

Three types of abiotic carriers were employed in the research, i.e. membrane filters (cellulose), sponge filters and graphite discs. The picture below (Fig. 1) illustrates the carriers used in the study while the comprehensive characteristics are presented in Tables 2 and 3.

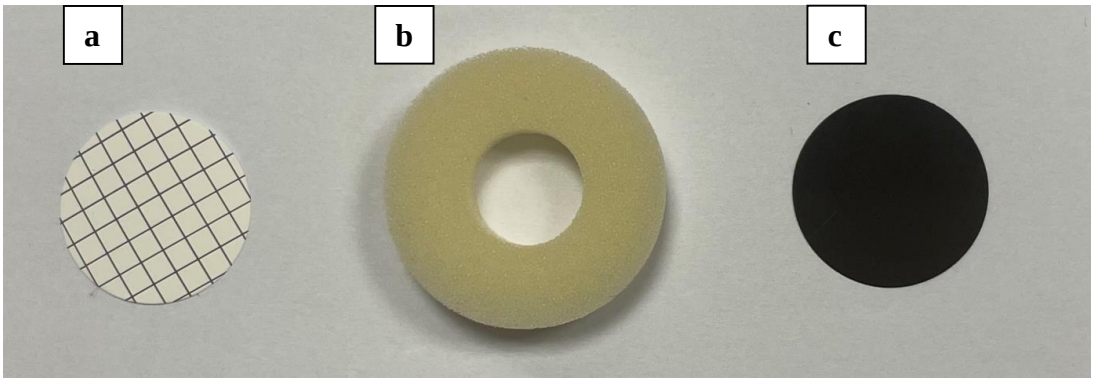

Figure 1. The carriers of the SARS-CoV-2: (a) membrane SKC; (b) sponge filter CIP; (c) graphite disc.

2.2.1. Membrane filter SKC

The first carrier used in this research was a cellulose nitrate membrane filter (SKC) with a 25 mm diameter, black grids and a 0.8μm pore size produced by Sartorius Stedim Biotech GmbH, Göttingen, Germany. The characteristic of the cellulose nitrate filter based on the manufacturer specification is presented below in Table 2. The membrane filters used for the purpose of this research are commonly applied as pads for vacuum filtration, or as filtration membranes for the assessment of water, sewage and air quality. The filters used in this study were exposed to the aerosols using clean tweezers taken from an airtight box.

Table 2.
Technical data – cellulose nitrate membrane filters

Material	**Cellulose nitrate**
Sterilization	Gamma irradiation or ethylene oxide
Pore size	0.8 μm
Thickness	Approx. 6 mil (150 μm) ± 10 μm
Size	25 mm
BSA protein bond	Approx. 160 μg/cm^2
Extractable substances	< 4%
Maximum operating temperaturę	356°F (180°C)
Color	White
Sealing compatibility	Ultrasonic, heat, radio frequency and insert moulding

2.2.2. Sponge filter CIP

The second carrier was a sponge filter CIP developed on the basis of polyurethane foam (TCR TECORA, France). This kind of material finds application as absorbent filters, *inter alia* in devices measuring air dustiness (e.g. dust meter CIP 10). Such devices are typically equipped with a small size rotating cup containing sponge filters (absorbent material) for the air sampling.

The measurement of air dustiness by means of this device may be performed on the basis of the so-called dosimetric method or the stationary method (Courbon, Wrobel, Fabriest, 1988). The device facilitates the sampling of a broad aerosol spectrum starting from the respirable, through the thoracic to the inhalable fractions by means of the gravimetric method and a dedicated head (selector). Widely used to determine the exposure to particles that are health risk, the gravimetric method of air sampling with a dedicated selector finds application in mining, wood and textile industries (Gero, Tomb, 1988). In standard laboratory research, this material ensures very effective and thorough filtration of biological water contaminants. Sponge filters successfully separate solids from liquids and gases stopping them mechanically and operating like a sieve. Thanks to their spongy structure (based on open and closed tubules/ pores constituting 97% of the volume of the material), the filters are most effective for capturing contaminants and impurities present in water solutions. Sponge filters are characterized by high moisture and thermal resistance, recovery capabilities, high flow of air and/or liquid, high elasticity as well as tensile strength. The technical parameters of the filter used in this study are compiled below in Table 3 (Raimbault et al., 2021).

Table 3.
Technical data – Polyurethane foam sponge filters CIP

Material	Polyurethane foam PU
Density	0.029 [g/cm^3] (according to PN-EN-ISO 845)
Tensile strength	5-60 [kPA] (according to PN-EN-ISO 1798)
Elongation at break	Min. 50 [%] (according to PN-EN-ISO 1798)
Air flow	Very high
Thermal resistance	245 [°C]
Color	Nude
External diameter	36 [mm]
Internal diameter	15 [mm]
Thickness	10 [mm]
Weight of the rotating element with the filter	Approx. 3 [g]
Weight of the filter	Approx. 0.3 [g]
Sponge filter grade (number of pores per centimeter)	25

2.2.3. Graphite disc

Micro to Nano high purity vitreous carbon discs (planchets) of 25 mm diameter produced by Agar (Fig. 1c) were used in the research (www.microtonano.com/Vitreous..., 2022). The double sided adhesive discs are dedicated to examining samples by means of Scanning Electron Microscopy (SEM). Used in electron microscopy to mount the samples on the SEM stub, graphite discs are characterized by mechanical and chemical stability and durability under different operating conditions. Their use facilitates the observations of objects in high vacuum conditions as well as at various temperatures. However, their most important feature is the

electrical conductivity as the carbon content ensures removing the electric charges from the sample. Therefore, the effect of electron accumulation on the surface of the sample placed in vacuum is nullified. In addition, the specimens subject to SEM observation should be accordingly prepared. Namely, they should be of the smallest possible volume in addition to being durable, resistant and eclectically conductive. The biological specimens require fixation, dehydration and drying prior to being mounted on the stub. In the case of non-conductive specimens, they are coated with a thin layer of a conductive material (e.g. gold, platinum or carbon) by means of a vacuum sprayer.

2.3. Spraying of the solutions

The application of the solutions onto the three types of the carriers was performed under laboratory conditions. The examined solutions were applied in the form of aerosols generated by means of a Turn'n'Spray atomizer (Bürkle, GmbH). The device consists of a multiple use 250 ml bottle and a spraying nozzle with a 0.6 mm (0.02 in.) diameter. The amount of the liquid sprayed per stroke (measurement series) was 1.2 ml ± 0.1 ml (0.04 oz. ± 0.003). Within the course of the experiment, the nozzle generating the aerosol stream was vertically directed downwards, at a distance of 30cm from the carriers on which the drop-lets of the examined solutions were collected (Lee et al., 2010).

2.4. SEM/EDS analysis

Scanning Electron Microscopy (SEM) with the system of Energy Dispersive Spectroscopy (EDS) constitutes a technique applied in material science research whose aim is to draw up a characteristic of the surface or the surface layer of the examined objects and materials. This technique enables to recognize the morphology of the examined structures including the elemental composition. In the course of the analysis, a given surface area is subject to a high energy focused beam of electrons. In the first stage of the investigation, the beam of electrons hits the surface layer of the material and induces signals coming from the examined layer. As a consequence of the reactions taking place, the induced and analyzed signal of the secondary electrons (SE) enables the imaging of the examined surface. The analysis of the X-ray radiation carried out during the measurement makes it possible to determine the elemental composition of the layer of the examined material (object). Thereby, the SEM technique facilitates the analysis of the sample surface, the assessment of the morphology as well as the shape of the biological material microstructures in huge magnification. The basic requirement concerning the samples subject to the SEM analysis is the ability to conduct electricity; therefore, in the case of organic samples, whose ability to conduct electricity is very low, special preparation of the samples is required. In addition, highly hydrated biological objects (e.g. microbiological specimen, plant or animal tissue) due to the possible vapor emission during the analyses require the conditions of controlled vacuum (from 6 to 650 Pa). In the SEM/EDS technique, the electron beam is generated from a tungsten

filament cathode. The controlled electron beam is focused on the sample surface by means of condenser lenses. The magnification may be altered within the range of X 5 to X 300 000, which makes it possible to observe the surface in the scale of macro and micro areas. The achievable resolution depends on the following factors: the type of the sample, the accelerating voltage of the electron beam, the current of the electron beam, the working distance between the sample and the objective lens, the adjustment and the correction of astigmatism. The microscope has a very good resolution with the possible voltage of 3-30 kV. The achievable resolution of the imaging equals 4nm at the acceleration voltage of 30kV. Thanks to the large sample chamber, there is a possibility to analyze samples of relatively big sizes at different distances and different tilts.

2.4.1. The scope of the microscopic analysis

The study involved the following two research cycles: (i) a qualitative identification of salt crystals (NaCl) sedimented on various carriers after spraying the control solution (cycle I), and (ii) a qualitative identification of silica markers sedimented on the carriers after spraying the silica marker solution (cycle II). The investigation was conducted based on a SEM analysis supported with an X-ray EDS microanalysis. The SEM/EDS analysis was performed using a variable vacuum SEM SU3500 Hitachi electron microscope (Hitachi High-Tech Corporation, Japan) which was coordinated with an EDS UltraDry (Thermo Scientific) X-ray spectrometer with energy dispersion. The microscope is equipped with two detectors, which allows conducting the observations of the sample surfaces in two operating modes; namely, SE (secondary electrons) image recording and BSE (backscattered electrons) image recording.

Dedicated software enables to perform the measurements of the observed objects directly on the screen of the microscope and to digitally record the photographic documentation. The study of the salt crystals and the silica markers involved determining the morphology, the grain size and the elemental composition on the basis of the observations of the grain surfaces and the X-ray microanalysis. The parameters of the X-ray microanalysis were as follows: (i) acceleration voltage – 15 keV, (ii) working distance (WD) – 10 mm, (iii) pressure – 30 Pa, and (iv) vacuum – variable. In each of the research cycles, within the par-ticular measurement series for each specimen, a sequence of microanalyses were per-formed. The scope of the microanalyses involved several dozens of chemical composition measurements of the characteristic particles in order to determine the dominant chemical forms of the occurrence of particular elements.

3. Results

3.1. The SEM/EDS technique – the imaging of physiological saline solution

Fig. 2 presents the morphology of the physiological saline solution on the examined carriers (a membrane filter SKC, a sponge filter CIP, a graphite disc). The tests concerning the presence of the physiological saline on all the types of the carriers using Scanning Electron Microscopy (SEM) demonstrated regular, polyhedral shapes occurring individually or in the form of agglomerated particles with the size range of 50-500 μm. The shape of the particles was determined using the descriptive method. The chemical composition in the micro-areas of the examined samples of the physiological saline showed that sodium chloride (NaCl) is the dominant component (Fig. 3).

The NaCl crystals (monocrystals) identified on all the carriers used in the research are characterized by a regular crystalline structure. Exemplary crystallized forms of NaCl occur on all of the applied carriers (Fig. 2a, 2b, 2c). A detailed analysis of microscope data showed that the grains of the sodium chloride (NaCl) sedimented on the carriers reach the size of a few to several dozen micrometers. The spectrum of the chemical composition in the micro-area confirms the presence of NaCl in the sample of the physiological saline (Fig. 3).

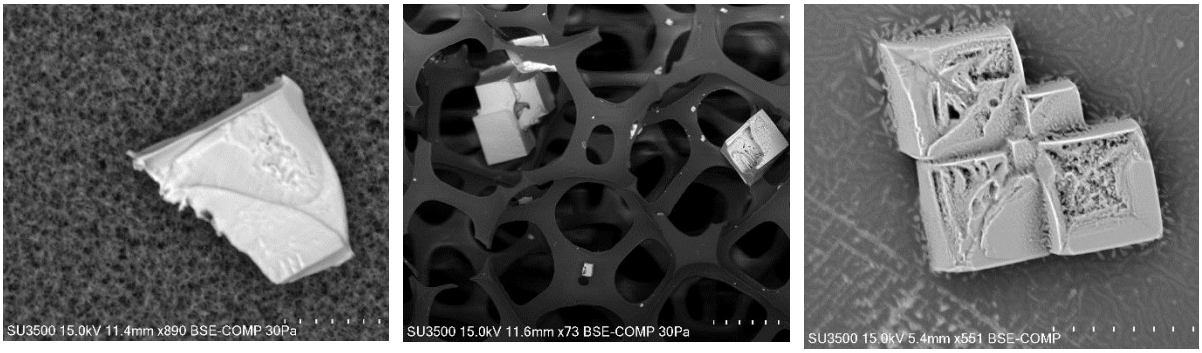

Figure 2. Illustrative SEM images of physiological saline grains on the carriers: (a) membrane filter SKC; (b) sponge filter CIP; (c) graphite disc.

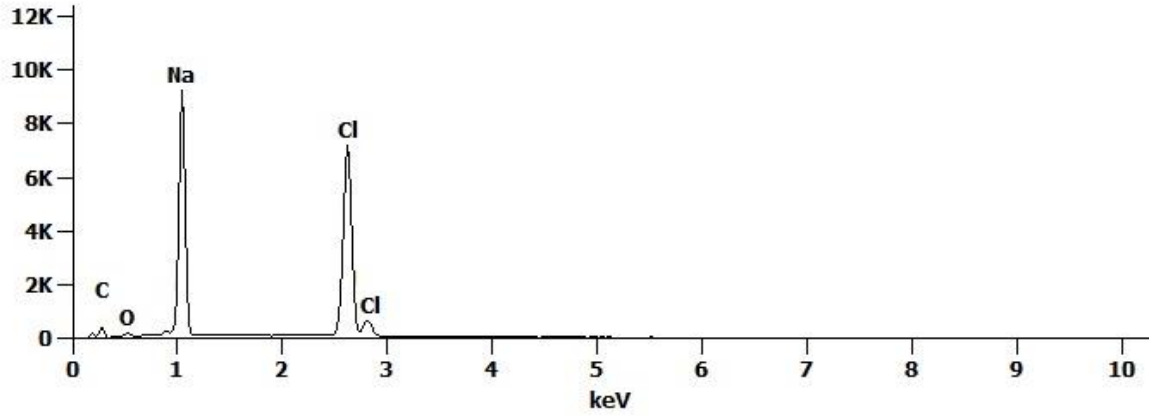

Figure 3. Illustrative chemical composition spectrum in the micro-area of physiological saline sample.

3.2. The SEM/EDS technique – the imaging of the silica solution

The morphology of the silica marker grains sedimented on all of the carriers used in this study is presented in Fig. 4, Fig. 5 and Fig. 6). As discussed in a previous section (2.1.2 Silica Marker), the grains of the marker are characterized by a spherical shape with the size of approximately 200 nm, which corresponds with the sizes of single SARS-CoV-2 virions. As can be seen in Fig. 4, Fig. 5 and Fig. 6, regardless of the carrier type, the grains of the marker occur in agglomerated forms, whereas the shape of the examined particles is decidedly rounded. The shape of the particles was determined by means of the descriptive method. Based on the performed analysis of the chemical composition spectrum, it was found that in the micro-areas of the marker samples, Si (silica) constitutes the dominant element (Fig. 7), which confirms the presence of the sprayed silica marker on all of the carriers used in the study.

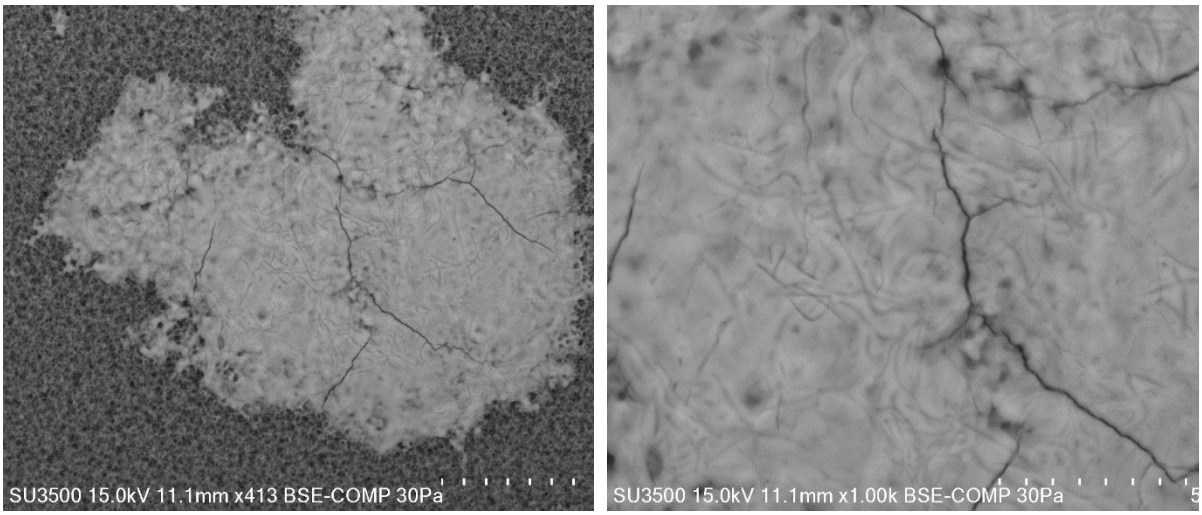

Figure 4. Scanning Electron Microscopy (SEM) imaging: (a) and (b) silica marker with the particle size of 200 nm sprayed on the membrane filter SKC.

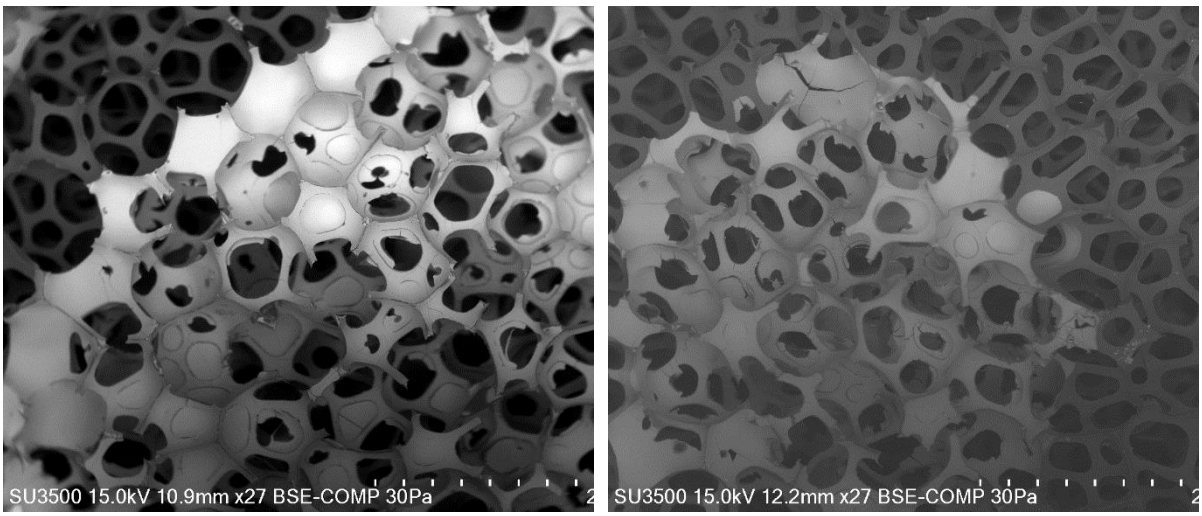

Figure 5. Scanning Electron Microscopy (SEM) imaging: (a) and (b) silica marker with the particle size of 200 nm sprayed on the sponge filter.

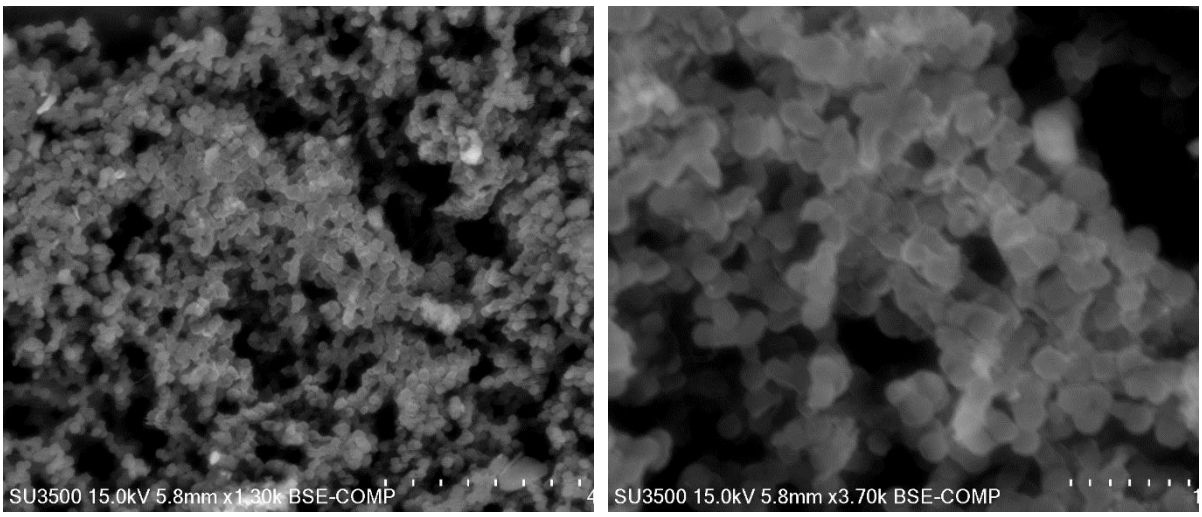

Figure 6. Scanning Electron Microscopy (SEM) imaging: (a) and (b) silica marker with the particle size of 200 nm sprayed on the graphite disc.

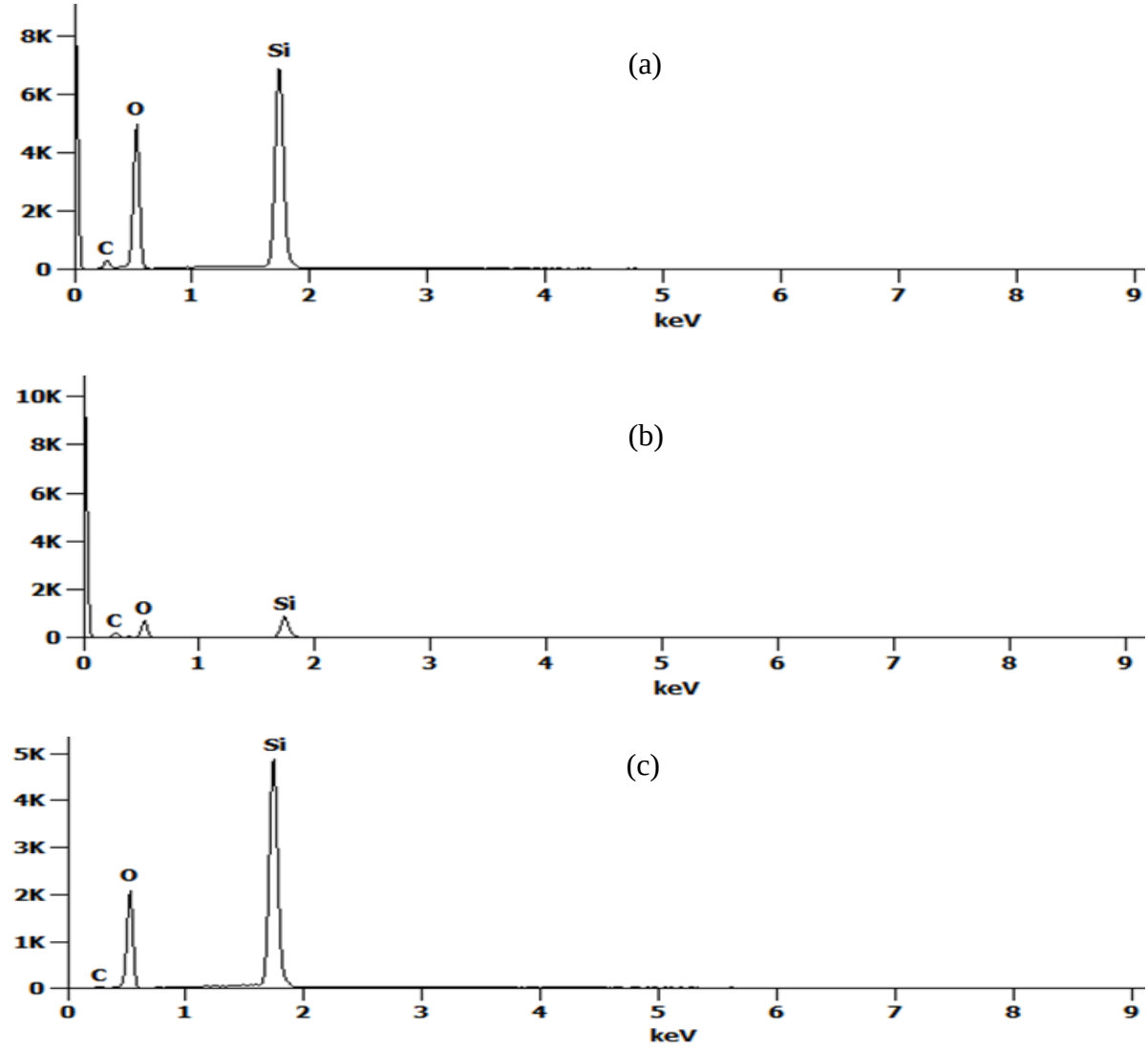

Figure 7. Illustrative chemical composition spectrum in the micro-area of the silica marker sprayed on: (a) membrane filter SKC, (b) sponge filter, (c) graphite disc.

As regards the membrane carrier, the grains of the silica marker occur in the form of solid agglomerates (Fig. 4a, 4b). In the case of the sponge filter, the silica markers cling to the polyurethane foam wall forming shell agglomerations, and thus reflecting the surface of the filter which is characterized by a larger specific surface area in comparison to the other two carriers (Fig. 5a, 5b). Concerning the graphite disc, the silica marker is present in the form of visible silica agglomerate grains of a spherical structure (Fig. 6a, 6b). In the chemical composition spectrum induced in the micro-area using the EDS technique, the maxima of silica and oxygen which differ in terms of intensity are distinctly visible and confirm the presence of the silica markers (Fig. 7). The induced EDS signal of the elemental composition analyzed in the micro-area coming from the silica marker applied to the membrane filter (Fig. 7a) as well as on the graphite disc (Fig. 7c) is stronger than the induced signal of the elemental composition obtained from the silica marker sedimented on the sponge filter (Fig. 7b). The bigger strength of the EDS elemental signal coming from the silica marker is most probably connected with the bigger accumulation of the silica marker sprayed on the membrane filter in the form of solid structures and the agglomerates of the silica marker on the graphic disc. The lower intensity of the EDS elemental signal coming from the spherical shell structures clinging to the sponge filter is most likely associated with the smaller amount and the smaller thickness of the silica marker on the sponge filter.

4. Discussion

The objective of this research study was to assess the possibility of using Scanning Electron Microscopy (SEM/EDS) to identify the silica marker (silica nanoparticles of SiO2) which may be applied as a potential surrogate of the pathogens in laboratory and field simulation tests, especially in the case of SARS-CoV-2. The selection of the marker used in this research study was based on two key features of the silica particles; namely, their neutral character in terms of the microbiological and physico-chemical properties (the silica particles are neutral both for the environment and the research staff performing the experiment) and the fact that a single silica nanoparticle ($\leq$ 200 nm) corresponds to the size of a single coronavirus virion. The concurrence regarding the size of silica particles (SiO_2) and the size of the pathogen (SARS-CoV-2 virion) were the foundation to assume that after spraying, the silica nanoparticles will behave in a similar manner to the coronavirus particles present in the body fluids (consisting of 99% of water) which are exhaled by COVID-19 infected people while coughing or sneezing. According to literature data, viruses of the particle size in the range of 25-400 nm, including SARS-CoV-2 (40-200 nm), may exist in the air as single virions or in the forms of agglomerates due to the process of aerosolisation. Clinical tests proved that the size distributions of virus aerosol particles generated in the breathing and coughing of people with

upper respiratory tract infections demonstrated big similarity and range from several dozen nanometers to several dozen micrometers with a simultaneous domination of the particle size < 5μm (Lee et al., 2011; Verpaele, Jouret, 2013). The hazards resulting from the aerosol transmission of pathogens are confirmed in research on the spread of the seasonal flu virus conducted with the application of a slot sampler with condensation or the quantitative measurements of virus concentrations by means of RT-qPCR. The research demonstrated that the particles of the size ≤ 5 μm contain 8.8 times more virion copies than the ones of the size > 5 μm (Milton et al., 2013). Among the participants of the study who had respiratory symptoms (coughing and sneezing), the flu virus RNA of particle size ranges 0.65-4.7 μm and > 4.7 μm was detected in 80% of children and 58% of adults (Issa et al., 2023). In addition, the measurements of viruses responsible for respiratory tract infections performed at hospital wards showed that a significant number of infection particles had the aerodynamic diameter Ø ≤ 4.8 μm (Milton, 2013; Fennelly, 2020). In their research studies, Gralton (Gralton et al., 2011; Duan et al., 2021; Fennelly et al., 2020) point out that with the increase in the number and size of the generated aerosol particles, it is possible that accumulated structures (agglomerates) are created in which the number of the microorganisms increases due to the effect of accumulation. The authors also emphasize that the soluble components present in the bio-aerosol may provide specific protection against the surrounding environment, thus maintaining the contagiousness of the pathogen. The effects of nano-silica accumulation indicating the phenomenon of the agglomeration of the nanoparticles present in the bio-aerosol were also observed during the conducted experiment. The markers applied on the surfaces of the carriers form agglomerates, accumulated structures, in a similar way as the viruses present in the aerosol in the air. The occurrence of the agglomerates of the silica marker sprayed by means of an atomizer, was observed on the carriers subject to microscope analysis; see the results of the SEM imaging presented in (Fig. 5) and (Fig. 6). The photographs show agglomerations clinging to the structure of the CIP sponge filter (Fig. 5) as well as accumulated structures/particle clouds sedimented on the graphite disc (Fig. 6). Despite numerous attempts, it was impossible to image single particles of the SiO_2 marker on the selected carriers because during the falling of the sprayed solution mist, the particles clustered and formed agglomerates. Nevertheless, the chemical composition analysis confirms the presence of silica in the analyzed micro-area indicating the presence of the marker on each of the three carrier types. Therefore, the obtained results prove that even if it is impossible or difficult to identify single nano-silica particles based on the results of the imaging, the presence of the particles may be confirmed by means of the elemental composition analysis using induced X-ray radiation. The results of the physico-chemical analysis based on the examination of the spectra chemical composition (Fig. 7) demonstrate that such analyses bring positive outcomes and enable to identify the marker on each type of the surfaces used in this research. On the basis of the SEM observations, it was found that the quantitative SEM/EDS analysis of single silica marker grains on the cellulose and sponge carriers is hampered or impossible. These limitations are caused by the

occurrence of the silica nanoparticles in the form of solid agglomerations on the membrane carrier and in the form of shell agglomerations on the sponge filter. During the analysis of the results of the imaging (photographs), it was impossible to identify single grains of the silica marker. Most probably, it results from the process of agglomeration of the silica nanoparticles and the adsorption of the agglomerates on the surfaces of the filters (Fig. 4, Fig. 5). The agglomeration of the silica nanoparticles constitutes a phenomenon also observed by other researchers. For example, Rovani et al. demonstrated the agglomeration of silica nanoparticles obtained from sugar cane ash by means of the SEM and TEM techniques. The observed agglomerates of the particles were in the range of 20 to 50 nm (Rovani et al., 2018). A stronger spectrum signal of the chemical composition in the micro-area on the membrane filter (Fig. 7a) and a weaker signal of the induced X-ray radiation on the sponge filter, resulting from the smaller amount of the silica marker clinging to the walls of the filter (Fig. 7b), constitute the consequence of the silica marker agglomerations. On the other hand, the silica marker grains sedimented on the graphite carrier produce a stronger spectrum signal of the chemical composition in the micro-area (Fig. 7c). Due to the occurrence the of silica markers in the agglomerates sprayed on the graphite carrier, there would be a possibility of identifying the markers not only based on the qualitative but also the qualitative analysis (Fig. 6a, Fig. 6b), which would indicate better prospects for the research concerning silica markers on graphite carriers. The photographs in Fig. 4-6 demonstrate a better resolution of the SEM imaging and the possibility of counting single grains of the silica markers in the agglomeration clusters on the graphite carrier.

To date, the detection of pathogens of such fine sizes has been carried out mainly in water solutions, for example by means of flow virometry which enables to identify even single particles of the virus. According to the literature reports, the flow virometry makes it possible to detect micro-particles of the size ranging from 100 nm to 1000 nm which may constitute both the cell components (e.g. exosomes) and the unwanted intruders (viruses and bacteria) (Binder et al., 2020).

Laboratory research demonstrated that flow virometry is a far more sensitive technique of virus detection compared to ELISA tests and it is characterized by virus detectability similar to the RT- PCR tests (Hill, Pan, Williamson, Santarpia, Hill, 2013). However, the flow virometry is dedicated to liquid samples; therefore, in the case of air aerosols it may be applied only after saturating the water solutions with the air sample (Kulkarni et al., 2016). Also, there exist studies confirming the application of fluorescent markers in the research on bio-aerosols. The overwhelming majority of the cases involve works conducted in structures of small cubature or in closed containers (tubes, pipes); the aim of the research is the quantitative identification of the objects, the assessment of the particle transmission routes and the degree of their dispersion. Yet, due to the requirements concerning research with the use of fluorescent markers, they rarely find application in experimental studies conducted in real scale.

Microscope techniques constitute an alternative form of research which enables the imaging of the pathogen occurring in the analyzed samples (Yoon, Lee, Kim, Yoo, Min, Kim, 2019). Even though the microscope techniques have been used for centuries, only the advancements in electron microscopy in recent decades made virus imaging possible. Viruses with the sizes smaller than the wavelength of visible light could not be investigated until the invention of electron microscopy in the 1930s. Since that time, electron microscopy has become the method used to identify this group of pathogens on the basis of their sizes, shapes, ultrastructural features and the distribution in tissues. Electron microscopy is a powerful tool in the field of microbiology due to its resolution capability in comparison to an optical microscope. Electron microscopy significantly contributed to the development of research concerning pathogen identification (Brown et al., 2015) functions and structures; it also played the key role in rapid diagnostics of different groups of viruses in environmental samples (Van M. Hoang et al., 2016).

The results of the conducted research prove that the SEM/EDS electron microscopy enables to identify silica markers of sizes corresponding with coronavirus virions on membrane filters, sponge filters and graphite discs. Consequently, the technique constitutes a sensitive tool for identifying such tiny structures as the silica nanoparticles. Due to the simplicity of handling, the low operating costs as well as the environmental neutrality, the silica nanoparticles may perform the function of pathogen markers in dedicated experimental studies. Scanning Electron Microscopy (SEM) appears to be a reliable and potent instrument both in the diagnostics of contagious diseases and microbiological research (Golding, Lamboo, Beniac, Booth, 2016). According to (Nolte-‘t, Cremer, Gallow, 2017). Scanning Electron Microscopy (SEM) was applied in ultra-rapid microscope imaging of SARS-CoV-2. Using the high-definition scanning microscopy, the research team examined the interactions of the new coronavirus with Vero cells (Esperanto: verda reno) which represent a continuous and aneuploid cell lineage similar to fibroblasts applied in the production of vaccines against viral diseases; the lineage allows for cell passaging for extended periods of time under laboratory conditions. The authors justify the use of the SEM technique due to its capability to rapidly screen the Vero cells infected with SARS-CoV-2 and to perform a thorough ultra-structural analysis of SARS-CoV-2 in the entire infection cycle. In the course of that research study, the SEM technique allowed for the imaging of cell surface morphology and the intracellular surfaces in order to assess the degree of the virus distribution within a time period of 2-48 h which elapsed from the onset of the infection (Bonar, Tilton, 2017). Another research study with the use of Scanning Electron Microscopy was the one by Mondei; applying the SEM technique, the author demonstrated the typical shape of the crown of the new coronavirus cultivated in cell cultures (Corina et al., 1999). Scanning Electron Microscopy was also used in the research on coronavirus severe acute respiratory syndrome invading the human placenta (Chen et al., 2020; Algaet alrroba, Rekawek, Sevan, 2020; Mondeja, Valdes, 2021).

5. Conclusions

The results of the performed research works confirmed that Scanning Electron Microscopy SEM/EDS may be successfully applied as a research tool for identifying silica markers. As proven, based on the results of the SEM/EDS investigations, it is possible to qualitatively identify the silica markers on different types of carriers such as sponge filters, membrane filters or graphite discs. Therefore, it was demonstrated that the SEM/EDS technique constitutes a sensitive tool which enables the qualitative identification of structures as fine as the silica nanoparticles which thanks to their handling simplicity, low operating cost and environmental neutrality may be used as pathogen markers in dedicated experimental studies. The obtained results demonstrate that the silica marker (SiO_2) can be effectively used as a surrogate of pathogenic organisms in simulation tests. The neutral character of the marker ensuring the safety of research staff as well as the fact that the single nanoparticle of silica (Ø 200 nm) corresponds to the size of a single virion of SARS-CoV-2 (Ø 40-200 nm) grant the possibility of its application in the simulation studies of the coronavirus transmission routes conducted both under laboratory and real conditions. In addition, the study confirms the interdisciplinary character of material engineering science as a research area involving the design, production and optimization of materials for their future applications, mostly in engineering. Material engineering is based on exploring the relationships among the chemical composition, the structure and the properties of the materials as well as the parameters of the production processes. The understanding of the above issues leads to the development of new solutions as well as the refinement of the already existing ones. The investigation confirms the importance of this research area in micro-biological studies, with a special emphasis on the optimization of the materials used in personal protective equipment used during pandemics.

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