

Performant silver-based biohybrids generated from orange and grapefruit wastes

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Clean technologies using the principles of *green chemistry* have attracted the attention of scientists in the last decade. Our work was focused on building advanced silver-based materials using an eco-friendly and convenient method. Two types of silver nanoparticles were synthesized using aqueous extracts of orange (*Citrus sinensis*) and grapefruit (*Citrus paradisi*) peels. Hybrid entities consisting of these bionanosilver particles and biomimetic membranes were “green” prepared and studied by spectral methods (UV-Vis absorption and emission, and FTIR-ATR spectroscopy). The morphology of silver-containing materials was analyzed by AFM images and their physical stability was checked by zeta potential measurements. Also, their antioxidant and antimicrobial properties were evaluated.

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1. Introduction

Citrus plants belonging to the *Rutaceae* family are marketed under many varieties of fruits, such as oranges, grapefruits, mandarins, lemons or limes. Medical, cosmetic or pharmaceutical applications of *Citrus* (such as grapefruit and orange) peels is steadily increasing [1]. These fruit residues (discarded as waste in the environment) can act as potential dietary supplements/functional food resources [2]. *Citrus* peels contain large amounts of compounds with many biological activities (antioxidant, antibacterial, anticancer), such as polyphenols, pectin, vitamins, carotenoids [3, 4]. In the same time, it has been discovered that phenolics have antioxidant effect (including free radical scavenging and metal chelation [1, 5, 6]), anti-carcinogenic, anti-inflammatory activity, and an important role in the defence against microorganisms. In the food sector, they are responsible for pigmentation and food organoleptic properties [7]. It is necessary to increase the interest for natural antioxidants obtained from vegetables and fruits to use them in medicine, cosmetic or pharmaceutical applications, due to their properties of free radicals'

scavenging and slowdown the progress of aging, cancer, diabetes or degenerative diseases (Alzheimer's disease, Parkinson's disease, etc.). Several studies [8-10] have found that orange and grapefruit peels are important natural sources of antimicrobial and antioxidant components due to their major content of carotenoids (β -carotene, α -carotene, lycopene, lutein, and zeaxanthin), vitamin C, essential oils, and flavonoids (naringin, hesperidin, hesperetin, narirutin, eriocitrin, quercetin, etc.) - phenolic compounds with many biological activities like antioxidant, antibacterial, antimicrobial, and bioprotection, just to name a few. *Citrus* pectin [11] exerts physiological effects on the gastrointestinal tract [12], enhancing hypocholesterolemia effect [13], and delaying gastric emptying [14]. In addition, the oligosaccharides obtained from *Citrus* pectin [15] were found to offer protection against pathogenic *Escherichia coli* [16].

Clean technologies using the principles of *green chemistry* have attracted the attention of scientists in the last decade [17, 18]. Plant-mediated synthesis of nanoparticles (silver, gold, etc.) is a very promising area because the plant itself acts as both reducing and capping agent [19]. The use of plants in the production of metallic

nanoparticles (NPs) is an eco-friendly, non-pathogenic, economical and rapid protocol [19-22].

The noble metal nanoparticles (AgNPs, AuNPs, PdNPs or PtNPs) are very attractive in various applications such as solar cells [23], sensors [24, 25] or other optoelectronics' devices [26], due to their optical features.

This paper aimed to design *Citrus*-based architectures for bio-applications. Two kinds of silver nanoparticles were *green* generated from aqueous extracts of orange (*Citrus sinensis*) and grapefruit (*Citrus paradisi*) peels. These metallic nanostructures were used to design two hybrid systems based on biomimetic membranes labelled with chlorophyll *a* (Chl*a*). This photopigment was used by many researchers for sensitization of nanoparticle surface utilized in fabrication of optical devices [27].

In our previous studies [28-32], Chl*a* was used as an antioxidant & antimicrobial agent, and also as a spectral marker in order to monitor the biohybrids' formation. The obtained silver-based biohybrids were studied by spectral methods (UV-Vis absorption & emission, and FTIR-ATR spectroscopy) and their topography, by AFM analysis. The physical stability of the samples was checked by performing zeta potential measurements. Also, the antioxidant and antimicrobial properties of these materials were evaluated.

2. Experimental part

2.1. Materials

Silver nitrate (AgNO₃), monopotassium dihydrogen phosphate (KH₂PO₄), and disodium hydrogen phosphate (Na₂HPO₄), were purchased from Merck (Germany). The 2,2-diphenyl-1-picryl-hydrazyl-hydrate stable free radical (DPPH) and L- α -Phosphatidylcholine (1,2-Diacyl-sn-glycero-3-phosphocholine, PC) from dried egg yolk were supplied by Sigma-Aldrich (Germany). Chl*a* was in-house extracted from fresh spinach leaves as previously described [33, 34].

The antibacterial activity was tested against *Escherichia coli* ATCC 8738 bacteria which were grown in Luria Bertani Agar, LBA, plates at 37°C with the medium composition containing: peptone, 10 g/L; yeast extract 5 g/L, NaCl 5 g/L and agar 20 g/L. The stock culture was then maintained at 4°C.

2.2. Preparation of the samples

Preparation of biomimetic membranes. The biomimetic lipid bilayers were obtained by hydration of a thin film of egg yolk phosphatidylcholine (prepared using a BIOBLOCK SCIENTIFIC – Heildolph 94200 rotary evaporator, 90 rpm) with a phosphate buffer solution (KH₂PO₄–Na₂HPO₄ pH 7.4) as previously described [28, 30, 33, 34]. The biomimetic membranes were labelled with Chl*a*, in a molar ratio Chl*a* /Lipid of 1%.

Preparation of *Citrus paradisi* and *Citrus sinensis* extracts. Oranges and grapefruits were washed in distilled water and dried at 50°C. An amount of 30 g of peels of

these fruits were weighed and transferred into a 500 mL Erlenmeyer flask containing 300 mL of distilled water and boiled for 5 minutes in order to release the intracellular material into solution. The aqueous *Citrus* peels' extracts thus obtained were then cooled and filtered through a filter paper to obtain clear extracts.

“Green” synthesis of silver nanoparticles. The bioreduction of silver ions was visually observed, first time, by changing the colour (from pale yellow to brown) of *Citrus* extracts after the addition of 1 mM AgNO₃ solution, in a volume ratio of 1:1. Then, the formation of AgNPs was further confirmed by spectral studies (UV-Vis absorption and FTIR-ATR spectroscopy) and AFM images.

Preparation of silver-based biocomposites. Two types of biohybrids were obtained by ultrasound irradiation (Hielser Ti probe sonicator, UP 100 H) of a mixture containing *Citrus paradisi* and, respectively, *Citrus sinensis* – generated silver nanoparticles and liposomal suspension in a ratio of 1:30 (v/v).

The experiments were carried out in dark to avoid the samples' photo-deterioration.

All the samples are summarized in the Table 1.

Table 1. The abbreviations of the samples prepared

Sample	Abbreviation
Orange extract	OE
Orange-AgNPs	ON
Orange-AgNPs/liposome hybrids	OL
Grapefruit extract	GE
Grapefruit-AgNPs	GN
Grapefruit-AgNPs/liposome hybrids	GL
Chl <i>a</i> -Liposomes	L

2.3. Characterization methods

The absorption spectra of the extracts and of the silver-based samples were recorded using an M 400 Carl Zeiss Jena UV-Vis spectrophotometer, in the wavelength range of 230-430 nm.

Fourier transformed IR (FTIR) spectra were collected using a Perkin Elmer Spectrum GX instrument with Attenuated Total Reflectance (ATR) diamond crystal in the wavelength range of 400–4000 cm⁻¹.

The values of electrokinetic potential were used to assess the physical stability of the prepared hybrid systems. Zeta potential (ZP) measurements were performed in triplicate, by applying an electric field across the analyzed aqueous dispersions in an appropriate dispositive of Zetasizer Nano ZS (Malvern Instruments Ltd., UK).

Atomic Force Microscopy (AFM) images of silver-based samples were obtained on an Integrated Platform SPM-NTegra model Prima in tapping mode using a NSG01 cantilever with a typical curvature radius of 10 nm.

The antioxidant activity of the samples was spectrally assessed by using the DPPH radical scavenging assay described in scientific literature [35, 36] with minor changes. A volume of 0.5 mL of each *Citrus*-based

specimen was mixed with 1 mL of freshly prepared 0.02 mg/mL DPPH solution. The samples were shaken vigorously for 30 minutes and preserved in the dark for other 30 minutes, at room temperature. After that, the absorbance of the residual DPPH solution in each sample was measured at 517 nm on the M 400 Carl Zeiss Jena UV-Vis spectrophotometer. A solution prepared by mixing 0.5 mL of distilled water with 1 mL of DPPH solution (0.02 mg/mL), was used as a blank. The antioxidant activity (AA%) was calculated using the formula:

$$AA\% = [(A_{Control} - A_{Sample})/A_{Control}] \cdot 100 \quad (1)$$

where: $A_{Control}$ is the absorbance of the DPPH solution without sample, A_{Sample} is the absorbance of the sample with DPPH.

Antibacterial assay was performed by the agar disc diffusion method [37, 38]. Using a sterile Durham tube 6 mm diameter, the wells were made according to the number of samples. The wells were inoculated with 50 μ L of sample. All the test plates were incubated at 37°C, for 24 h to allow microbial growth. The antimicrobial activity was evaluated by measuring the zone of inhibition (ZOI) against test organism. Experiments were performed in triplicate.

3. Results and discussion

3.1. Spectral characterization of the samples

The phyto-reduction of Ag^+ to Ag^0 was firstly demonstrated by UV-Vis absorption spectroscopy (Fig. 1), which revealed the appearance of a single Surface Plasmon Resonance (SPR) band at 455 nm for GN, and at 440 nm for ON, characteristic for the synthesis of spherical silver nanoparticles [39].

The UV-Vis absorption spectra (Fig. 2) revealed the chlorophyll fingerprint at 671 nm, in liposomes and in biohybrids, and also the incorporation of biosilver in hybrid systems. The light scattering is more pronounced in the case of GL, as compared to OL, indicating that the size of OL are less than GL, a fact confirmed further by AFM analysis.

The biohybrids' synthesis was monitored also by fluorescence spectroscopy (Fig. 3). The emission peak of Chla is situated at 679.5 nm in liposomes and in orange-based biohybrids and at 679 nm in grapefruit-based biohybrids. A fluorescence quenching process was observed, due to an energy-transfer process when the fluorophore (Chla) is directly bound to the surface of AgNPs [40] indicating that a structural reorganization of the biomimetic membranes occurred in the presence of biogenic nanosilver.

The FTIR-ATR spectra of the samples (Fig. 4) revealed strong broad IR bands at 3391 cm^{-1} in orange (OE) and at 3388 cm^{-1} in grapefruit (GE) extracts, attributed to the stretching vibration of O-H groups of polysaccharides and polyphenolic compounds [41]. After reduction of silver ions, these bands are shifted to 3417 cm^{-1} in orange-based AgNPs (ON) and to 3423 cm^{-1} in grapefruit-based AgNPs (GN), demonstrating the

involvement of polyphenols and polysaccharides in Ag^+ bio-reduction.

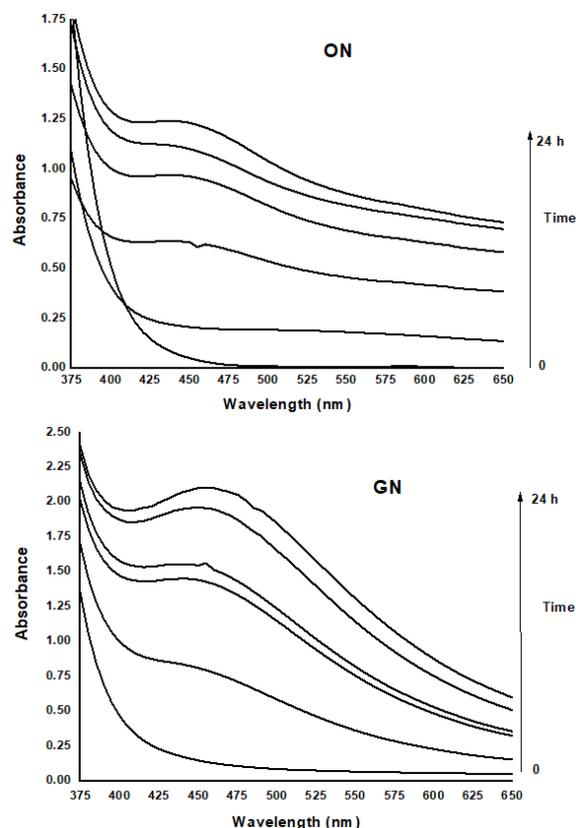


Fig. 1. The bioreduction of Ag^+ to Ag^0 by using peels' extracts of oranges (ON) and grapefruits (GN)

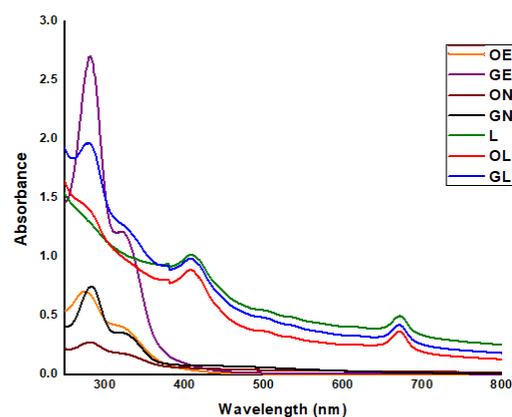


Fig. 2. The comparative UV-Vis absorption spectra of the samples

The bands at 2934 cm^{-1} and 2933 cm^{-1} belonging to the FTIR-ATR spectra of orange and grapefruit extracts, respectively, are characteristic for C-H asymmetric stretching alkyl vibrations of saturated C (sp^3) [42]. This band is shifted to 2933 cm^{-1} in ON spectrum, and to 2931 cm^{-1} in OL (in this case, the band strong weakened). For grapefruit-based samples, after addition of silver ions to GE, this band was weakened and shifted to 2940 cm^{-1} in

the case of GN, and to 2924 cm^{-1} in the spectrum of GL, respectively. In addition, a new band at 2896 cm^{-1} attributed to the C–H symmetrical stretch vibration of alkyl chains [42], appeared in the case of GN. In the spectrum of GL biohybrids, this band is shifted to 2851 cm^{-1} .

The weak band at 1739 cm^{-1} present in GE spectrum, attributed to amide I (arising from stretch vibration of carbonyl C=O of proteins) [43], is shifted to 1735 cm^{-1} in silver nanoparticles GN, and strong weakened and shifted to 1740 cm^{-1} in the case of GL biohybrid.

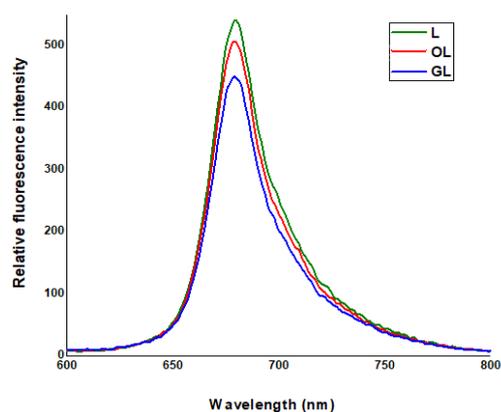


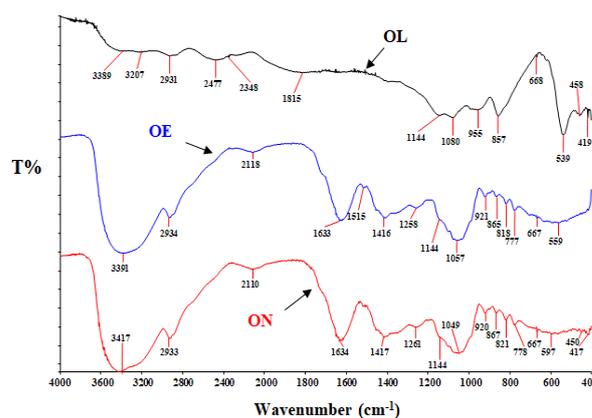
Fig. 3. Fluorescence emission spectra of Chla in mimetic biomembranes (L) and in orange- (OL) and grapefruit-based (GL) biohybrids (the excitation wavelength was 430 nm)

FTIR-ATR sharp weak bands located at 1633 cm^{-1} for orange extract (OE) and at 1630 cm^{-1} for grapefruit extract (GE) are assigned to amide I (arising due to carbonyl stretch in proteins), stretching vibration of phenolic aromatic rings [44], and to stretching bands of COO⁻ of pectin [45, 46].

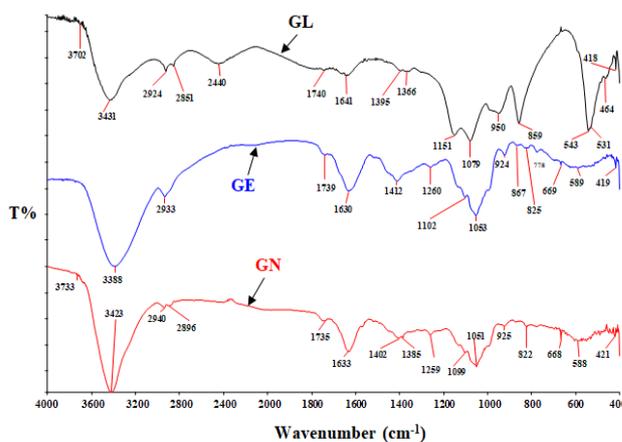
The band at 1416 cm^{-1} occurred in orange extract IR spectrum, arising from O–H in plane deformation due to the aromatic ring stretching vibration of phenolic compounds [47], is shifted to 1417 cm^{-1} in orange-silver (ON) and strong weakened in OL hybrid.

The IR bands at 1057 cm^{-1} in OE, and at 1102 cm^{-1} & 1053 cm^{-1} in GE, assigned to C–N stretching vibrations of aliphatic amines and to C–O stretching vibrations of alcohols/phenols [43] which are originated from different phytoingredients present in the peels' extracts (polyphenols, polysaccharides and proteins), were shifted to 1049 cm^{-1} in ON & to 1080 cm^{-1} in OL, to 1099 & 1051 cm^{-1} , in GN, and to 1079 cm^{-1} in GL hybrid system, respectively.

After incorporation of silver nanoparticles ON and GN in liposomes, new sharp bands appeared: at 857 & 539 cm^{-1} , and at 859 , 543 & 531 cm^{-1} in the fingerprint region of FTIR-ATR spectra of OL and GL, respectively, highlighting a structural reorganization of the artificial lipid membranes in the presence of bionanosilver. These observations are in agreement with fluorescence emission and absorption spectroscopy data.



(a)



(b)

Fig. 4. The FTIR-ATR spectra of samples derived from orange (a) and grapefruit (b) extract

3.2. Morphological characterization and physical stability evaluation of silver-based samples

AFM pictures (Fig. 5) revealed the nanometric dimensions of the samples with the size values increasing in the order: ON < GN < OL < GL. These images confirmed the synthesis of nanosilver particles. AgNPs derived from orange (ON) are well dispersed as compared to those derived from grapefruits (GN).

The sizes of orange-based hybrids (OL) were less than grapefruit-based hybrids (GL), as predicted by the absorption spectra (Fig. 2). The sample OL was more dispersed than GL.

The physical stability of the biohybrids was rapidly estimated in terms of zeta potential, ZP (Fig. 6). The orange-AgNPs/liposome biohybrids (OL) exhibited the highest stability (ZP = -30.2 mV), due to the strong interparticle repulsive forces, while the silver nanoparticles ON showed moderate stability (ZP = -18.3 mV). Grapefruit-derived biohybrids (GL) were quite stable (ZP = -29.8 mV), as compared to GN (ZP = -17.7 mV).

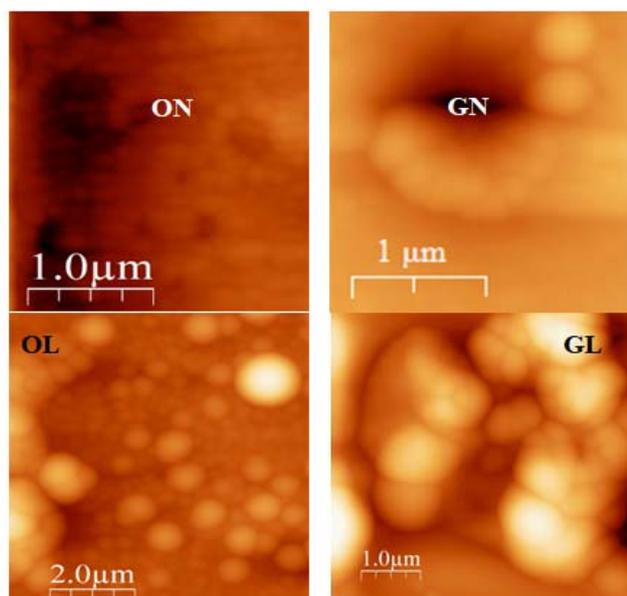


Fig. 5. AFM images of Citrus-based samples

The topographic images together with ZP values provide an insight about the synthesis mechanism of the biohybrids: the metallic nanoparticles are encapsulated in a biological matrix, which stops the growing process and reduces and stabilizes the metallic nanoparticles.

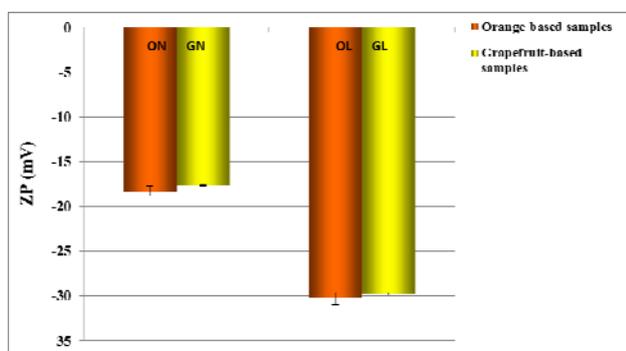


Fig. 6. Zeta potential values of silver nanoparticles and biohybrids derived from orange and grapefruit peels

3.3. The bioactivity of the orange- and grapefruit-based samples

The DPPH radical-scavenging activity analysis revealed the antioxidative characteristics of all the samples (Fig. 7). The AgNPs showed antioxidant activity values higher than *Citrus* extracts. The incorporation of *Citrus*-derived AgNPs in liposomes resulted in a high-rise of AA%. The most potent proved to be orange-based biohybrids, OL, possessing an AA% value of 77%.

The antimicrobial investigations were performed on *Escherichia coli* Gram-negative bacteria, chosen for our study because this one became more investigated, having new forms and causing various diseases [48].

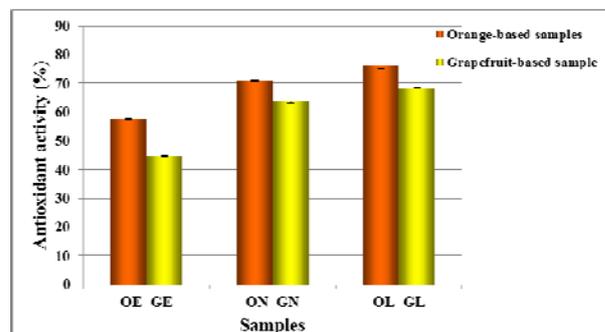


Fig. 7. The antioxidant activity values of the Citrus-based samples

The results of antibacterial susceptibility assay show promising evidence for the antibacterial effect of the *Citrus*-based samples. Non-significant differences between ZOI values of orange- and grapefruit-based samples were observed (Fig. 8).

In both cases, the addition of silver nitrate to *Citrus* extracts resulted in an increase of ZOI value of 55% for orange-biogenerated AgNPs (ON), and of 80% for grapefruit-biogenerated AgNPs (GN). Therefore, the biogenerated silver nanoparticles showed higher levels of antibacterial activity compared to those of *Citrus* peels' extracts (OE and GE).

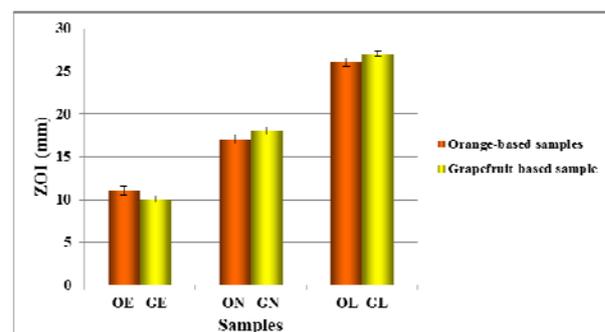


Fig. 8. Comparative presentation of antimicrobial activity of the samples against *Escherichia coli* ATCC 8738

Moreover, the lipid coating of the biohybrids OL and GL favours their attachment to bacterial cells, causing an enhancement of the biocidal effect of 53% for OL and of 50% for GL biohybrids, as compared to the silver nanoparticles alone.

4. Conclusions

This paper presented a simple, low cost and eco-friendly approach to build performant biohybrid systems based on biomimetic membranes and silver nanoparticles phytosynthesized from *Citrus paradisi* and *Citrus sinensis* peels' extracts. The artificial membranes were labelled with chlorophyll *a* in order to monitor the formation of biohybrids. This photo-active natural dye detected the changes occurred in biomimetic membranes, at molecular level.

The biosynthesis of AgNPs and of the hybrid systems was firstly monitored by UV-Vis absorption spectroscopy and then confirmed by fluorescence emission, FTIR-ATR spectroscopy and AFM analysis. Liposomes-AgNPs hybrids presented good physical stability as compared to AgNPs alone, this conclusion being highlighted by zeta potential measurements. The spectral results are well correlated with AFM images.

The obtained biohybrids presented good antioxidant properties and antimicrobial activity against *Escherichia coli* and could be used in biomedical field.

Moreover, the UV-Vis absorption spectra showed that these hybrid systems absorb the radiation from the visible range, so these materials prove to be promising candidates for optoelectronic applications.

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