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Article

Hydrophilic silver nanoparticles loaded into niosomes: physical-chemical characterization in view of biological applications

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Abstract: Silver nanoparticles (AgNPs) are widely used as antibacterial agent and anticancer drug. However, in many cases the low AgNPs stability limits their massive production and broad applications. The use of niosomes as carrier, to protect and envelop AgNPs, can solve these problems. In this study, AgNPs were functionalized with sodium 3-mercapto-1-propanesulfonate (3MPS) to induce high hydrophilic behavior, improving

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their loading in in,Tween 20 and Span 20 niosomes. ~~niosomes. AgNPs were loaded in two different niosomes, Tween 20 and Span 20, and investigated.~~ Their entrapment efficiency was evaluated by UV analyses and is around 1-4%. Dimensions were investigated by means of DLS ($<2R_H> = 140 \pm 4$ nm and $<2R_H> = 251 \pm 1$ nm respectively for NioTw20+AgNPs and NioSp20+AgNPs) and ~~the nanosize~~were ~~was confirmed~~compared with those by AFM and SAXS analyses. Moreover, stability was assessed in water up to 90 days, and both in Bovine Serum (BS) and Human Serum (HS) up to 8 hours. In order to characterize the local structure of niosomes, SAXS measurements have been performed on Tween20 and Span20 empty niosomes and loaded with AgNPs. The release profiles of hydrophilic probe calcein and lipophilic probe Nile Red were performed in ~~H~~HEPES~~EPES~~ and in human serum. All these features contribute to deeply ~~define~~ characterize the two systems, NioTw20+AgNPs and NioSp20+AgNPs, suitable and promising in the field of biological applications.

Keywords: niosomes; silver nanoparticles; liposomes; plasmonic materials; drug delivery.

1. Introduction

It is well known that the body barrier to external pathogenic attacks is represented by the skin, that prevents microbial invasion [1], so every damage or wound can provide an environment for microbial growth, leading to infection and prolonged wound healing [2,3]. The efficacy of antibiotics is superior to that of other drugs, and antibiotics are thus widely used [4,5]. However, the antibiotic resistance is currently a health emergency so drug-resistant bacterial infections are becoming more common with a consequent increase in public spending [5]. Therefore, new materials for the treatment of wound infections are needed [6,7].

Small sizes, nanoparticles have the advantage to be characterized by a larger contact surface with bacteria, able to enhance their penetration and bactericidal effects [Porcaro, F.; Carlini, L.; Ugolini, A.; Visaggio, D.; Visca, P.; Fratoddi, I.; Venditti, I.; Meneghini, C.; Simonelli, L.; Marini, C. Synthesis and structural characterization of silver nanoparticles stabilized with 3-mercapto-1-propansulfonate and 1-thioglucose mixed thiols for antibacterial applications. Materials 2016, 9, 1028.].~~[8].~~ A good candidate is represented by silver nanoparticles (AgNPs) that show antibacterial properties and reduce inflammatory response [Li, Y. et al Inflammatory responses to micro/nano-structured titanium surfaces with silver nanoparticles: In vitro; Journal of Materials Chemistry B Volume 7, Issue 22, 2019, Pages 3546-3559], with no drug resistance in gram-negative bacteria [Sanyasi S et al. Polysaccharide-capped silver Nanoparticles inhibit biofilm formation and eliminate multi-drug-resistant bacteria by disrupting bacterial cytoskeleton with reduced cytotoxicity towards mammalian cells ; Scientific Reports Volume 6, 29 April 2016, 24929].

AgNPs are one of the most used metals in nanotechnology, due to their amazing chemical physical features. They have a striking anti-microbial activity. In fact, AgNPs are known to cause oxidative lesion that destroys the bacterial cell wall, due to Ag^+ ions release, facilitating the entry into the cell and the disruption of crucial metabolic processes and halts cell proliferation.

Therefore, AgNPs are widely used in several medical devices, such as in coating of bone prosthesis and chirurgical stents, in orthodontic materials, in bandages [8-11].

~~Moreover, recently~~ In the last decade, the research field of AgNPs has moved to the possibility of their use as anticancer drug, due to their inherent cytotoxic effect on cancer cells [12].

However, instability of silver nanoparticles limits their industrial application in several cases and most of the methods to prepare Ag-NPs, causing environmental pollution and low production efficiency. To overcome this problem, silver nanoparticles are usually loaded onto carriers like [13].

Among various antibacterial agents, silver ions and nanosilver (i.e., Ag nanoparticles (NPs)) show impressive antimicrobial activity against a broad spectrum of bacteria by destroying infectious microorganisms, and have an attractive feature of relatively low tendency to cause such bacterial resistance against antibiotics [2,7]. The bactericidal effect of Ag NPs is mainly attributed to their small nanoscale sizes and high surface-to-volume ratios, which allow them to relatively quickly release Ag ions and interact with microbial membranes. Recently, hybrids/composites with Ag NPs dispersed on carriers or supports have attracted much attention due to their enhanced antibacterial activity compared with sole Ag NPs [2]. Furthermore, it is of very significance to seek for the optimal choices of carriers to combine with AgNPs in order to construct ideal antibacterial agents. Various AgNPs-based nanocomposites with different structures and morphologies have been developed up to now, such as amorphous silica matrix dispersed with AgNPs [Das.A. et al.; Confinement induced formation of silver nanoparticles in self-assembled micro-granules Colloids and Surfaces A: Physicochemical and Engineering Aspects Volume 577, 20 September 2019, Pages 185-193], AgNPs core@silica shell [Huang, J. et al. Unveiling the growth mechanism of SiO 2 /Ag hybrid nanospheres and using for Surface Enhanced Raman Scattering detection; Applied Surface Science Volume 463, 1 January 2019, Pages 115-120], ~~silica core@silver shell [17e20],~~ mesoporous silicas loaded with Ag NPs [Mofakham, E.B. et al.; Progesterone Release from PDMS-Modified Silica Xerogels Containing Ag Nanoparticles Silicon Volume 11, Issue 2, 15 April 2019, Pages 703-711;

Arun Kumar, K.V.et al; Surface plasmon response of silver nanoparticles doped silica synthesised via sol-gel route; Applied Surface Science Volume 472, 1 April 2019, Pages 40-45], hollow mesoporous silica spheres with Ag NPs in cavity [Lin L. et al; Preparation and antibacterial activities of hollow silica-Ag spheres; Colloids and Surfaces B: Biointerfaces Volume 101, 1 January 2013, Pages 97-100

Nishanthi, S.T. et al; Nanostructured silver decorated hollow silica and their application in the treatment of microbial contaminated water at room temperature; New Journal of Chemistry Volume 43, Issue 23, 2019, Pages 8993-9001], fibers coated with Ag NPs [Bonan R.F. et al; In vitro antimicrobial and anticancer properties of TiO2 blow-spun nanofibers containing silver nanoparticles;Materials Science and Engineering C Volume 104, November 2019, Article number 109876

Gao A. et al; Efficient antimicrobial silk composites using synergistic effects of violacein and silver nanoparticles; Materials Science and Engineering C Volume 103, October 2019, Article number 109821],etc.

Although extensive efforts have been devoted to fabricating a lot of these AgNPs-based nanocomposites involving different carriers' structures, there are still few systematic investigations on the effects of structures on antibacterial performance [14].

The use of drug delivery systems (DDS) has been proposed to overcome important issues in active pharmaceutical molecules release, such as unfavorable pharmacokinetics and biodistribution with consequent decrease of side effects. Nanocarriers represent an innovative approach to overcome these issues [I. Venditti; Engineered gold-based nanomaterials: morphologies and functionalities in biomedical applications. A mini review; Bioengineering 2019, 6(2), 53; doi.org/10.3390/bioengineering6020053;]. Moreover, the niosomes systems allow to modulate the silver concentration loading in a range of interest for the biological applications (0,3-5,0 µg/mL) and the modulation of release [16].

In this research study AgNPs were synthetized using 3-mercapto-1-sodium propanesulfonate (3MPS) to induce hydrophilic behavior improving niosomal entrapment efficiency and reducing bilayer destabilization. AgNPs were loadea in two different niosomes, Tween 20 and Span 20 loaded with AgNPs, producing two different systems, namely NioTw20+AgNPs and NioSp20+AgNPs. A deep physical chemical characterization was carried out to obtain information on hydrophilic AgNPs and their influence on preparation and characterization of Nio+AgNPs. Moreover, stability

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Un dubbio… la nostra conc. È 0,5 mg/mL poi messa il 1 L, giusto? Ne intrappola il 4%...Quindi siamo nel range 1-5 µg/mL ?!

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studies were performed in water, bovine serum and human serum to assess their use in biological compartment. Hydrophilic and lipophilic probe ~~The~~ release profiles ~~of hydrophilic probe calcein~~ were ~~performed~~obtained~~.~~ in ~~HEPES~~HEPES and in human serum. ~~manca una frase conclusiva tipo:~~ Both systems ~~loaded with AgNPs~~ proved to be able to entrap AgNPs, are stable and maintain the ability to entrap also hydrophilic or lipophilic model molecules so are promising systems for biotechnological applications.

2. Experimental

2.1 Materials and Methods

Silver nitrate ($AgNO_3$, 99.5%, Sigma-Aldrich, St. Louis, MO, USA) and sodium borohydride (NaBH4, 98%, Sigma-Aldrich, St. Louis, MO, USA), were used for the synthesis of the nanoparticles. 3-mercapto-1propanesulfonic acid sodium salt ($C_3H_7S_2O_3Na$, 3MPS, Sigma Aldrich, 98%) was used as a capping agent. For all of the solutions, we used deionized water (electrical conductivity less than 1 μΩ/cm at room temperature) obtained from a Millipore Milli-Q water purification system. ~~HEPES~~HEPES salt (Sodium 2-(4-(2-hydroxyethyl) piperazin-1-yl) ethanesulfonate), Cholesterol, Sephadex G75, Pyrene, DPH (1,6-diphenyl-1,3,5-exatriene), Span 20 (sorbitan monolaurate), Tween 20 (polyoxyethylene sorbitan monolaurate), Human/Bovine serum, Calcein and Nile Red were purchased from Sigma-Aldrich (Milan, Italy). All other products and reagents were of analytical grade. All of the reagents were purchased from Sigma Aldrich and were used without further purification.

2.2 Preparation of AgNPs loaded niosomes

The AgNPs-3MPS synthesis consisted in a wet reduction of silver nitrate to metallic silver by means of sodium borohydride in presence of 3MPS [Prosposito, P.; Mochi, F.; Ciotta, E.; Casalboni, M.; De Matteis, F.; Venditti, I.; Fontana, L.; Testa, G.; Fratoddi, I. Hydrophilic silver nanoparticles with tunable optical properties: Application for the detection of heavy metals in water. Beilstein journal of nanotechnology 2016, 7, 1654-1661.

Venditti, I.; Testa, G.; Sciubba, F.; Carlini, L.; Porcaro, F.; Meneghini, C.; Mobilio, S.; Battocchio, C.; Fratoddi, I. Hydrophilic metal nanoparticles functionalized by 2-Diethylaminoethanethiol: a close look at the metal–ligand interaction and interface chemical structure. The Journal of Physical Chemistry C 2017, 121, 8002-8013.

Fratoddi, I.; Benassi, L.; Botti, E.; Vaschieri, C.; Venditti, I.; Bessar, H.; Samir, M.A.; Azzoni, P.; Magnoni, C.; Costanzo, A. Effects of topical methotrexate loaded gold nanoparticle in cutaneous inflammatory mouse model. Nanomedicine: Nanotechnology, Biology and Medicine 2019.].

Briefly, a solution of $AgNO_3$ in deionized water (0.200 g in 10 mL) was added in a flask with 3MPS water solution (0.830 g in 10 mL) and the mixture was maintained under stirring in argon atmosphere at room temperature for 10 min. Then $NaBH_4$ water solution (0.220 g in 10 mL) was finally added dropwise, under vigorous stirring. The reaction mixture was allowed to react for 2 h. The obtained black product was centrifuged with deionized water tree times (20 min, 5000 rpm) and then characterized.

Several niosomal formulations by Tween 20 or Span 20 were prepared using AgNPs-3 MPS at different concentrations. Only results obtained by selected samples in terms of size, ζ-potential, entrapment efficiency and stability features were reported and discussed.

AgNPs-3 MPS were loaded into Span 20 and Tween 20 niosomes, through a protocol already used to internalize chemicals [17]. Niosomes were prepared using thin film hydration method [18]. Span 20 or Tween 20 (15 mM) and cholesterol (15 mM) were dissolved in organic solvent mixture (chloroform/methanol 3:1 v/v). The solvent was evaporated using rotary evaporator (VV2000, Heidolph, Schwabach, Germany) to form a thin "film". The film was hydrated using 5 mL of AgNPs solution, vortexed and sonicated at 60 °C and 18%/16% amplitude for 5 min using ultrasonic microprobe (Vibra-Cell VCX-400, Sonics & Materials, Newtown, CT, USA). The unilamellar

vesicular suspension was purified by gel filtration chromatography using Sephadex G75 (glass column of 50 × 1.2 cm) with ~~HEPES~~HEPES buffer as the eluent. The purified vesicles were filtrated, by using cellulose filters in order to obtain desired dimensions

2.3 Characterizations

The AgNPs water solution has been characterized by means of UV-VIS spectra of water suspensions were collected using a Perkin-Elmer Lambda 19 spectrophotometer. The dynamic light scattering (DLS) measurements on the AgNPs colloidal suspensions (0.200 mg/mL) at T = 25.0 ± 0.2°C were performed by the Malvern Zetasizer Nano ZS90 instrument (Malvern,UK), as reported in previous studies [17]. The ζ -potential was calculated from the measured electrophoretic mobility by means of the Smolukovsky equation [19]. Uv-vis: λ_{max} (nm) 415; DLS: $<2R_H>$ 5 ± 2 nm; ζ-potential: -35 ± 2 mV.

Mean size, size distribution, and ζ-potential of empty and AgNPs-loaded niosomes were characterized by using DLS. UV-Visible spectroscopy was employed to evaluate the amount of AgNPs entrapped in niosomal formulations.

Bilayer characterization has been carried out on empty Span 20 or Tween20 Niosomes and on AgNPs loaded ones, employing DPH and pyrene (lipophilic probes) that provided different bilayer information (fluidity, microviscosity, and polarity)

Span 20/Tween 20 (15 mM), cholesterol (15 mM), and DPH solution (2×10^{-4} M) were codissolved in chloroform/methanol, which was removed using rotatory evaporator (VV2000, Heidolph, Schwabach, Germany), hydrated with ~~HEPES~~HEPES buffer or AgNPs solution (0,5 mg/ml), with the same preparation methods above mentioned. Cellulose filter of 450 nm cut off was used to purify the DPH-niosomal formulations. Fluorescent measurements were performed (λ = 350–425 nm) using luminescence spectrometer (LS5013, PerkinElmer) in order to obtain Florescence Anisotropy. The florescence anisotropy (r) was determined by using Equation (1) [20-22].

$$A = \frac{I_{vv} - I_{vh} \; x \; G}{I_{vv} + 2I_{vh} \; x \; G} \qquad (1)$$

where I_{VV}, I_{VH}, I_{HV} and I_{HH} are fluorescent intensities, and subscript V (vertical) and H (horizontal) represent the orientation of polarized light. G factor is ratio of sensitivity of detection system for vertically and horizontally polarized light.

Pyrene loaded niosomes were prepared by adding the probe (4 mM) to niosomes (Nio) components in order to obtain empty and Nio-AgNPs following the same preparation method as above. Pyrene is a florescence probe, whose monomer exhibited a spectrum characterized with five emission peaks (from I1 to I5) and excimer has only one peak (IE). The monomer and the excimer have different fluorescence signals, and the ratio between the several fluorescence intensities is directly related to the probe distribution in the bilayer. In particular, the ratio I1/I3, corresponding to the first and third vibration bands in the pyrene spectrum, is related to the polarity of the probe environment. Pyrene can form intramolecular excimer based on the viscosity of the probe microenvironment [23], and it is estimated with the ratio IE/I3, where IE is the excimer intensity. The fluorescence signals emitted by pyrene loaded niosome suspension was scanned (λ = 350–550 nm) using luminescence spectrometer (LS5013, PerkinElmer) and intensities of excimer florescence (IE), first (I1), and third (I3) peak were recorded [24].

Atomic force microscopy (AFM) study of the morphology of the niosomes has been performed using a standard AFM setup (Dimension Icon, Bruker Inc.) equipped with Si cantilevers suitable for tapping mode imaging. Samples have been prepared by depositing a droplet of solution on a clean monocrystalline Si surface and waiting until partial dehydration. AFM imaging has been performed in air and at room conditions.

SAXS experiments were performed at ID02 high-brilliance beamline at the ESRF (Grenoble, France). The measured SAXS profiles report the scattered radiation intensity as a function of the momentum transfer, q = (4π/λ)sin(θ/2), where θ is the scattering angle and λ the x-ray wavelength (0.1 nm).

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Analysis was carried out to obtain information on dimension, homogeneity, shape of the particles in solution. The form factors of niosomes have been reconstructed as uni-lamellar or multi-lamellar closed bilayers. Details are reported in the Supporting information.

Biological studies were also carried out in the presence of Bovine or Human serum to evaluate the in vitro stability. Mixtures of empty and AgNPs loaded Nio and 45% of Bovine or Human serum, were prepared and incubated at 37 °C. Analyses were performed at different time intervals (0, 0.5, 1, 2, and 3 h) by DLS (to evaluate variations of particle size and ζ-potential [25].

Stability studies up to 90 days at different temperatures, room temperature (RT) and 4°C, were carried out by DLS, to asses dimension and ζ-potential of empty niosomes and Nio -AgNPs [25].

Release studies were carried out following the release calcein from empty niosomes and Nio--AgNPs using Fluorimetric apparatus.

The hydrophilic probe (calcein 10^{-2}M) was added to formulation during film hydration and the excess of calcein was purified with the same technique above mentioned [25].

In vitro release experiments were performed at 37°C, defined volume of vesicle dispersions was included in dialysis sacs (cut-off 8.000) with a fixed diffusing area (5.5 cm^2) adding to niosomal formulation 45% of human serum or ~~Hepes~~HEPES (in order to maintain the same probe concentration). The probe concentration was detected in the outer solution at fixed time intervals (0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h) by means of the Fluorometric apparatus taking into account the dilution factor.

Results and discussion

AgNPs were functionalized by 3MPS to induce high hydrophilicity and to control the shape and dimension in the range of 2-5 nm, as already report [F. Mochi, L. Burratti, I. Fratoddi, I. Venditti, C. Battocchio, L. Carlini, G. Iucci, M. Casalboni, F. De Matteis, S. Casciardi, S. Nappini, I. Pis, P. Prosposito; Interaction of colloidal silver nanoparticles with Co2+ and Ni2+ in water for sensing application; Nanomaterials 8 (2018) 488; doi:10.3390/nano8070488www

Corsi, P.; Venditti, I.; Battocchio, C.; Meneghini, C.; Bruni, F.; Prosposito, P.; Mochi, F.; Capone, B. Designing an Optimal Ion Adsorber at the Nanoscale: The Unusual Nucleation of AgNP/Co2+–Ni2+ Binary Mixtures. The Journal of Physical Chemistry C 2019, 123, 3855-3860]. In fact, the hydrophilicity is a crucial feature to obtain the insertion in the aqueous core of the niosomes. Even the small dimensions are a key parameter to ensure the inclusion and above all the stability of the final hybrid system, as also reported in the literature [Yusuf 2017]. The AgNPs were loaded into niosomes ~~by adding nanoparticles during the typical preparation protocol of niosomes~~, as schematized in Figure 1.

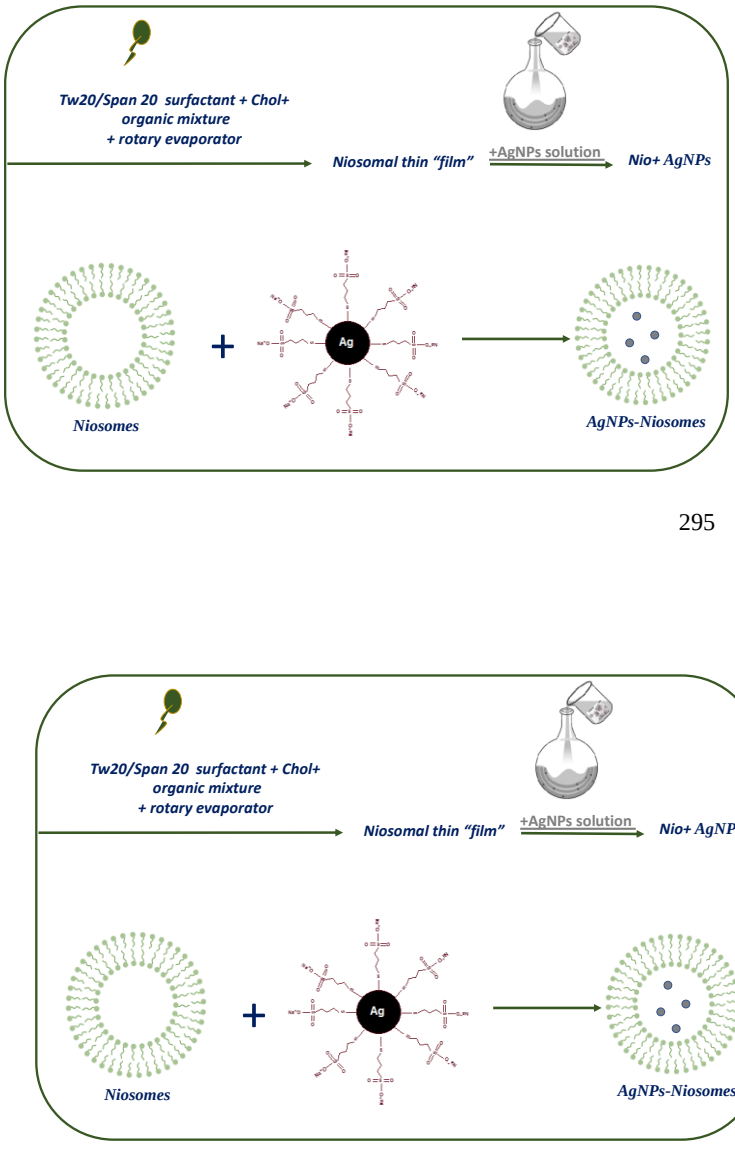

Figure 1 ~~Preparation scheme of~~ Nio -_AgNPs ~~preparation.~~

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According to characterization results obtained, the best samples selected were Tween 20/Span 20 niosomes hydrated with AgNPs at the 0,5 mg/ml concentration.

The first comparison between empty niosomes and Nio—-AgNPs was done by analyzing hydrodynamic diameter, ζ-potential and PDI (Polydispersity index) by DLS. Results are shown in table 1.

In both niosomal formulations, by Tween 20 or Span 20, all the parameters analysed by DLS are preserved after the addition of AgNPs. The empty samples show differences in dimensions, due to the different internal structures determined by the different surfactant employed for niosomal preparation. In particular Span 20 niosomes, as expected [26] are bigger than Tween 20 ones and show more negative ζ-potential.

Sample	Hydrodynamic diameter (nm) ± SD	ζ-potential (mV) ±SD	PDI ± SD
NioTw20	136.1 ± 2.0	-32.8 ± 0.3	0.38 ± 0.01
NioTw20+AgNPs	140.3 ± 3.9	-33.1 ± 1.4	0.40 ± 0.01
NioSp20	230.2 ± 5.9	-42.7 ± 2.3	0.35 ± 0.01
NioSp20+AgNPs	251.7 ± 6.0	-42.9 ± 1.2	0.40 ± 0.01

Table 1. Hydrodynamic diameter, ζ-potential and PDI of different niosomal formulations.

The entrapment efficiency of AgNPs in vesicular systems, was evaluated by means of the calibration curve (see SI-Fig.2) and results are reported in Table 2.

Samples ID	Entrapment efficiency (mg/mL)
NioTw20 + AgNPs	<1 %
NioSp20 + AgNPs	.4 %

Table 2. The trapping efficiency of AgNPs in niosomes

The data obtained indicate that entrapment efficiency for the two systems is not the same; better for Span 20 than Tw20 niosomes, probably related to different internal structure and or capacity (aqueous volume available to host AgNPs). Moreover, these values allow to assemble systems with a silver concentration in the range of interest for biological applications (0,3-5,0 μg/mL) as reported in literature [16]. Results obtained by bilayer characterization studies (table 3), show no variation in the evaluated bilayer properties (table 3), thus demonstrating that no interactions occur between niosomal double layer and AgNPs, that probably will be located inside aqueous compartments.

Sample	Fluidity (Anisotropy)	Microviscosity (I_E/I_3)	Polarity (I_1/I_3)
NioTw20	0.10	0.90	0.90
NioTw20+AgNPs	0.11	0.90	0.90
NioSp20	0.10	1.01	0.94
NioSp20+AgNPs	0.11	1.03	0.90

Table 3: Bilayer Characterization Results of Nio and Nio-AgNPs

Morphological studies were performed on empty niosomes and Nio–AgNPs . Representative AFM images of the samples are shown in Fig. 2. The morphological characterization indicates that niosomes have regular spherical shape. Probably due to the intrinsic limitations related to sample preparation, the size of niosomes seems highly dispersed, according to PDI values by DLS analyses, the bigger particles being probably the results of agglomeration of individual niosomes.

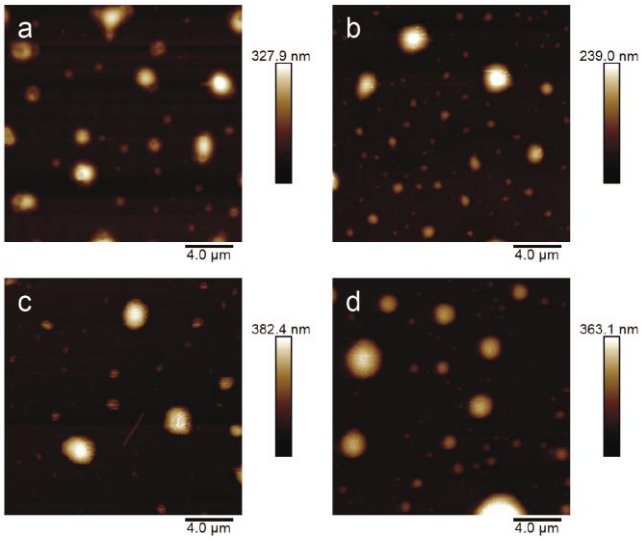

Figure 2: AFM images related to: NioTw20 (Fig 2a), NioTw20+AgNPs (Fig. 2 b), NioSp20 (Fig.2 c) and NioSp20+AgNPs (Fig. 2 d).

In deposited sample, large amorphous particles are visible, likely resulting from coalescence of several vesicles on the substrate surface as well as the possible partial dehydration make the vesicles lose their original spherical shape. By a visual inspection, Tween–based niosomes, i.e., NioTw20 (Fig 2a) and NioTw20+AgNPs (Fig. 2 b), are smaller than the Span-based ones, i.e., NioSp20 (Fig.2 c) and NioSp20+AgNPs (Fig. 2 d), in qualitative agreement with the measured hydrodynamic diameter reported in Table1. Conversely, the effect on the size of the presence of AgNPs cannot be appreciated in both the Tween and Span based formulation, as expected considering the relatively small variations evaluated by DLS and the dispersion of the size in AFM samples.

In order to characterize the local structure of niosomes, SAXS measurements ~~whave been~~ were performed on Tween20 and Span20 niosomes empty and loaded with AgNPs.

In Figure 3 the intensity spectra are reported for Tween20 based nanoparticles (panel A) and for Span20 based nanoparticles (panel B) in a wide q range (0.014 nm-1 ≤ q ≤ 6 nm-1-), corresponding to distances from 150 nm to the nm. Differences in the intensity profiles are clearly visible for the two systems in all regions of the spectra. Besides the feature of the form factor of the particles in solution a small diffraction peak at 1.84 nm-1, corresponding to a characteristic distance of 3.41 nm, is well known, and stems for the presence of cholesterol crystallites in surfactants/cholesterol mixtures [27].

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Crystallites could be either excluded from the bilayers, in peripheral contact, for example, or included in the bilayers, as segregated structures.

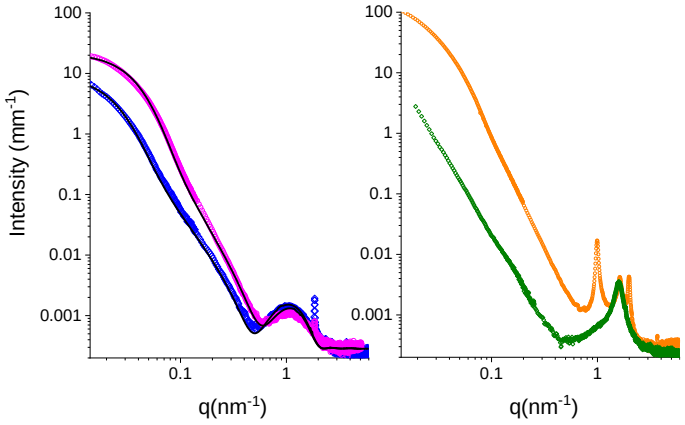

Figure 3 SAXS spectra of Tween 20 and Span 20 based niosomes. Panel A. Tween 20 based empty niosomes (blue diamonds) and ~~AgNPs~~ Nio--AgNPs (magenta dots). Fitting curves have been obtained by modelling the particle as an internal solvent core surrounded by a surfactant closed bilayer (for details see Supporting Information…….). Panel B. Span 20 based empty niosomes (green diamonds) and ~~AgNPs~~ Nio--AgNPs (orange dots).

Commentato [UdMO7]: Vi mandiamo in allegato supporting information di elena…se serve

Tween20 based particles display a niosomes shape, characterized by a local bilayer structure with thickness of about 6 nm, being the hydrophobic core of 2.5 nm and the two hydrophilic layers of 1.6 nm and of 2 nm, respectively. Moreover, the absence of peaks due to multilamellar organization, reveals that the adopted structure is unilamellar.

In the presence of AgNPs Tween 20 niosomes keep a local unilamellar structure, with unaffected structural features. On the other hand, in the low-q region of the spectrum, the scattered intensity increases by one order of magnitude. The form factor of the unilamellar closed particles can be modelled replacing the internal water with a higher electron density solvent, confirming the presence of AgNPs entrapped inside the aqueous core of the niosomes.

Span 20 based aggregates display the characteristic features of closed lamellar-type particles. Nevertheless, the local structure of Span 20 based niosomes is quite different from the Tween 20 based one. A broad intensity peak is clearly visible at q = 1.6 nm-1, together with a very broad left shoulder. The position of the peak corresponds to a characteristic distance d = 3.9 nm, compatible with twice the length of a Span 20 (sorbitan monolaurate) molecule. Results indicate that Span 20 niosomes are multilamellar closed particles, with a water core surrounded by a peculiar layered shell: several adjacent concentric bilayers are in close contact, heads to heads, without any water penetration. The scattered intensity profile of Span20-based loaded niosomes, reported in Figure 3 (panel B), presents a pronounced increase in the low-q region, sign of the presence of AgNps enclosed in the internal aqueous core of the niosomes. The increase is definitely higher than the one

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observed in Tween 20 based ~~AgNPs~~ Nio-AgNPs, suggesting that Span 20 is more efficient in entrapping metallic NPs. On the local scale, two additional peaks at q = 1 nm-1 and q = 2 nm-1 are visible, revealing a swollen multilamellar structure with characteristic distance d = 6 nm, coexisting with the tight one at d = 3.9 nm. The 6 nm distance is typical for lipid multilamellar structures and also found in Tween®20-derivatives/cholesterol niosomes [27]. The presence of AgNPs induces partial disjunction of adjacent bilayers with an increased water penetration.

Stability studies of NioTw20/NioTw20+AgNPs and NioSp20/NioSp20+AgNPs were performed in bovine serum and human serum to compare their behavior in biological compartment, as shown in Figure 4. Empty niosomes are stable both at room temperature and at 4°C, while Nio-AgNPs ~~Nio+AgNPs~~ showed a different behavior in terms of dimensional increase at RT conditions, not confirmed at 4°C storage temperature. Colloidal stability at 4°C is higher because of reduced collision events of the dispersed particles and hence coalescent phenomena.

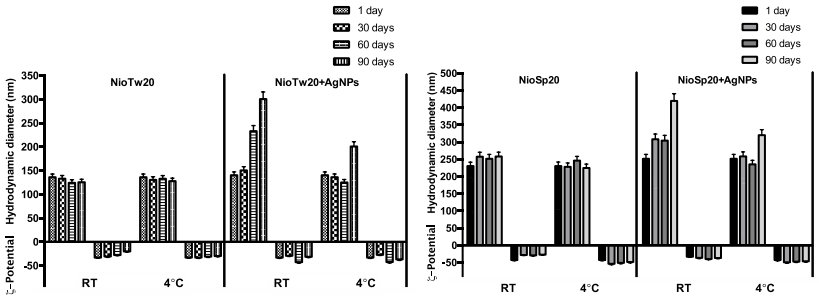

Figure 4. Stability studies of empty niosomes and Nio –AgNPs at room temperature and 4°C.

The effect of the serum (human and bovine) is another important element to evaluate in order to define the interaction between vesicles and fluids. Experiments were performed at 37° C evaluating the size and ζ~~z~~-potential (data not shown) variations by DLS analysis up to 3h (Figure 5). During the time interval analyzed, the same trend is observed for all vesicles. Vesicles in 45% bovine serum do not show a dimensional increase, while in 45% human serum the trend is different. The different protein composition of human serum is the reason of the attractive interaction between negatively charged niosomal surface and proteins. These interactions are strong enough to observe the same populations for the three hours.

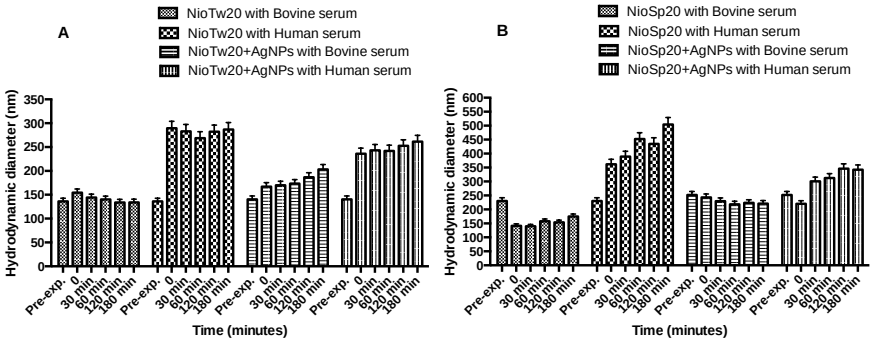

Figure 5: Niosomal biological stability at 37°C in different media. A) NioTw20/AgNPs in bovine and human serum; B) NioSp20/AgNPs in bovine and human serum.

In order to confirm the ability of Nio to release lipophilic and hydrophilic probes, also in presence of AgNPs, calcein and Nile Red release studies were carried out. ~~Qui andrebbe messa frase che spiega perchè è stato fatto questo esperimento con calceina, tanto per rendere più fluido il discorso.~~

In figures 6 and 7 release profiles ~~of the hydrophilic probe~~ calcein and Nile Red. ~~are reported a che temperature questo esperimento?.~~ In each experiment, the calcein release data from empty niosomes and NioTw20 AgNPs were compared in order to evaluate the influence of the silver nanoparticles, entrapped in the same compartment, on the hydrophilic probe release. The calcein entrapment in both samples was comparable. Experiments were carried ~~-~~out at 37°C both in ~~Hepes~~HEPES and human serum. The results obtained in human serum are not reported because the calcein release was not significant (20%) probably due to coating and masking effects of serum, which makes it difficult to quantify the calcein release in the external medium. In ~~Hepes~~HEPES buffer, the presence of AgNPs influences the release profile of calcein only in NioTw20 formulation. As demonstrated by SAXS analyses, only a double layer is present, so more susceptible to silver nanoparticles destabilization. Calcein release by this sample is around 65% in 24 hours, respect to 30% by NioSp20/AgNPs, where the presence of different bilayer can make more difficult calcein release.

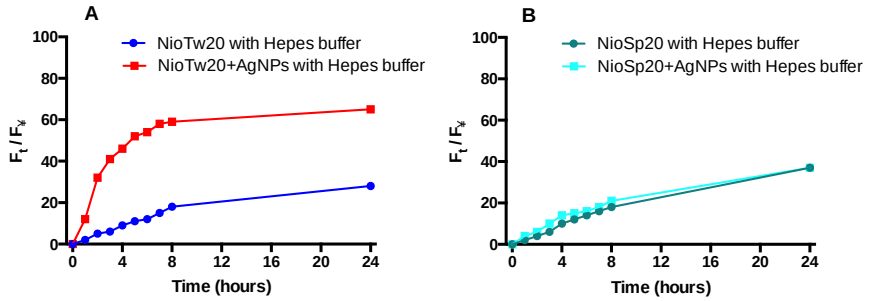

Figure 6: Calcein ~~R~~release studies in ~~Hepes~~HEPES buffer at 37°C ~~temperature ???~~ from: A) NioTw20/AgNPs ~~calcein release profiles in Hepes buffer~~; B) NioSp20/AgNPs ~~calcein release profiles in Hepes buffer~~.

On the contrary, in both samples Nile Red release profiles, obtained in ~~HEPES~~HEPES at 37°C ~~….a che temperature??~~ ~~(figure 7 A and B)~~ are comparable, because of the lipophilic nature of the probe, as shown in figure 7.

The number of bilayers to cross is the limiting step for hydrophilic probe release, while it is not for lipophilic ~~probe, that~~probe that is located in the bilayer and will be barely released for the poor affinity with the aqueous external medium.

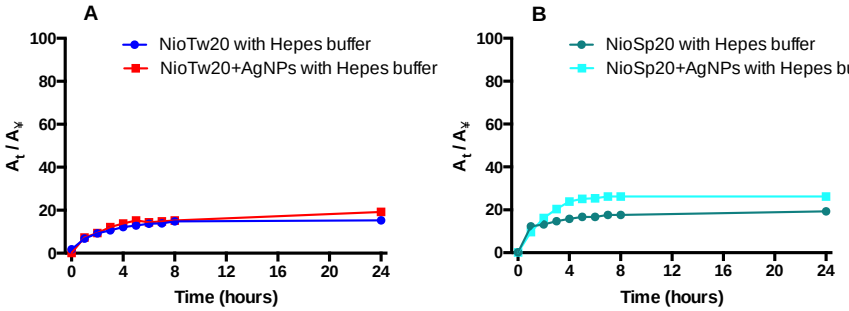

Figure 7: Nile Red release studies ~~studies~~ in ~~Hepes~~HEPES buffer at ~~temperature ???~~37°C A) NioTw20/AgNPs ~~in Hepes buffer~~; B) NioSp20/AgNPs. ~~in Hepes buffer.~~

The morphological structure of the two different niosomal formulations and the presence or not AgNPS, could to influence the release profiles of the two probes. ~~Frase finale???~~

4. Conclusions potete scrivere voi conclusioni?

This study confirms the possibility of preparing niosomal vesicles by Tween 20, or Span 20 loaded with AgNPs which, as expected, ~~enter~~are located inside the aqueous compartment of the two systems, as confirmed by the SAXS data, that also allow ~~; these have allowed to highlight~~ the different structure of the double layer of the studied samples. Our experiments ~~make us think that~~show that the samples prepared with the surfactant

Span 20 trapped particles more efficiently than Tween 20, reaching the concentration at which metallic nanoparticle effects are observed on in vitro cell cultures, as demonstrated by studies reported in the literature [16~~35~~]; in vesicles based on Tween 20 the concentration reached is lower but presumably such as to fall within the efficacy range of the nanoparticles.

The systems ~~we study are~~could be important ~~in increasing skin permeation~~for biological applications. ~~; this data, together with the ability to transport silver nanoparticles with antibacterial activity, emerged from the thesis work, makes them an innovative drug delivery system for the treatment of skin lesions and chronic skin diseases.~~

~~The studies carried out during my thesis work have revealed a different behavior of the samples prepared on the basis of their surfactant composition, the loading with the silver nanoparticles, and according to the medium in which they were placed.~~

~~In particular, it was found that the formation of the niosomal vesicles also occurs in the presence of the AgNPS, which, as expected, enter the aqueous compartment of the system, as confirmed by the SAXS data; these have allowed to highlight the different structure of the double layer of the studied samples. Our experiments make us think that the samples prepared with the surfactant~~

~~Span 20 trapped particles more efficiently than Tween 20, reaching the concentration at which metallic nanoparticle effects are observed on in vitro cell cultures, as demonstrated by studies reported in the literature [35] in vesicles based on Tween 20 the concentration reached is lower but presumably such as to fall within the efficacy range of the nanoparticles.~~

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Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

Author Contributions: Conceptualization, C.M., M.C., I.V. and I.F.; Chemical synthesis and characterizations of AgNPs: I.V. and I.F.; Chemical synthesis of niosomes and silver doped niosomes C.M., F.R., M.C.; SAXS investigations: E.d.F., J.M.; AFM investigations: D.P., M.R., L.A.; all authors reviewed and approved the entire manuscript.

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