



Article

Antibacterial Activity of Indolicidin-Coated Silver Nanoparticles in Oral Disease

Carla Zannella ¹, Surbhi Shinde ¹, Mariateresa Vitiello ², Annarita Falanga ³ ,
Emilia Galdiero ⁴ , Amir Fahmi ⁵, Biagio Santella ¹, Ludovica Nucci ⁶, Roberta Gasparro ⁷,
Marilena Galdiero ¹, Mariarosaria Boccellino ⁸, Gianluigi Franci ^{9,*} and Marina Di Domenico ^{8,10}

¹ Department of Experimental Medicine, University of Campania Luigi Vanvitelli, 80138 Naples, Italy; carla.zannella@unicampania.it (C.Z.); surbhi.shinde@unicampania.it (S.S.); biagio.santella@studenti.unicampania.it (B.S.); marilena.galdiero@unicampania.it (M.G.)

² Department of Clinical Pathology, Virology Unit, San Giovanni di Dio e Ruggi d'Aragona Hospital, 84131 Salerno, Italy; mteresa.vitiello@gmail.com

³ Department of Agricultural Sciences, University of Naples Federico II, 80055 Portici, Italy; annarita.falanga@unina.it

⁴ Department of Biology, University of Naples Federico II, 80126 Naples, Italy; emilia.galdiero@unina.it

⁵ Faculty of Technology and Bionics, Rhine-Waal University of Applied Science, 47533 Kleve, Germany; amir.fahmi@hochschule-rhein-waal.de

⁶ Multidisciplinary Department of Medico-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, 80138 Naples, Italy; ludovica.nucci@studenti.unicampania.it

⁷ Department of Neuroscience, Reproductive Science and Odontostomatology, University of Naples Federico II, 80131 Naples, Italy; roberta.gasparro@unina.it

⁸ Department of Precision Medicine, University of Campania Luigi Vanvitelli, 80138 Naples, Italy; mariarosaria.boccellino@unicampania.it (M.B.); marina.didomenico@unicampania.it (M.D.D.)

⁹ Department of Medicine, Surgery and Dentistry "Scuola Medica Salernitana"/DIPMED, University of Salerno, 84081 Baronissi, Italy

¹⁰ Department of Biology, College of Science and Technology, Temple University, Philadelphia, PA 19122, USA

* Correspondence: gfranci@unisa.it

Received: 3 February 2020; Accepted: 4 March 2020; Published: 7 March 2020



Featured Application: Indolicidin-coated silver nanoparticles could be used as platforms in the dental field as oral disease preventive drug. Furthermore, nanomaterials could prevent oral cancers and balance oral health care.

Abstract: (1) Background: In dentistry, silver nanoparticles (AgNPs) have progressively earned great interest as antimicrobial drugs and are widely used in several biomedical fields. Recent progress in the analysis of complex bacterial communities has demonstrated the richness of the oral microbiota and the presence of numerous previously unexplained strains. Several efforts have been dedicated to the investigation of antimicrobial peptides (AMPs). Those peptides are a widespread group of small peptides against invading microbes. We report the production of a hybrid molecule composed of AgNPs and indolicidin, a well-known antibacterial peptide. (2) Methods: Spectroscopy and microscopy were used to analyze the optical features and to determine the size of the generated AgNPs. The AgNP antibacterial activity was evaluated versus oral Gram-positive and Gram-negative bacteria. (3) Results: The coated nanoparticles' antibacterial activity strongly inhibited the growth of microorganisms, with very low minimum inhibitory concentration (MIC) values in the range of 5–12.5 µg/mL. We hypothesize that this effect depended on the specific characteristics of the metal surface coated with indolicidin. The second result was that the coated nanoparticles observed cellular toxicity, was lower with respect to the toxicity of peptide and the naked AgNPs when used individually. (4) New investigations regarding antimicrobial effect of AgNPs coated with AMPs in oral infections are an urgent task.

Keywords: antimicrobial peptide; silver nanoparticles; Gram-negative bacteria; Gram-positive bacteria; oral disease

1. Introduction

Research in pharmaceutical and biomedicine faces the great challenge of increasing drug resistance, which endlessly forces the research community to implement new therapies [1]. In fact, resistant microbes with present medicines are weakly managed and constitute a major public health problem [2].

The oral cavity contains the habitat of numerous bacteria, whose synergy and interaction contributes to combat the invasion of external pathogens [3]. On the other hand, the human oral microbiome database (HOMD) reports more than 700 prokaryotic different species that coexist in the oral cavity that could negatively influence the health of human body and could also be involved in the progression of many infections and systemic pathologies [4].

Antibiotics are generally used to tackle the infectious states of the oral cavity, but recently the use of antimicrobial peptides (AMPs) is being considered. AMPs are a wide and diverse family of small amino acid peptides produced by the immune system of several species ranging from plant and animal kingdoms to all orders of life. AMPs play a protective function by establishing a defense system that may react in a fast and efficient manner [5]. They have a broad-spectrum activity against microorganisms, a low aptitude to induce resistance [6,7], and are excellent candidates for clinical exploitation [8,9]. However, only few AMPs have to date received the certification for entry into the market and for clinical use. Two lead examples are the magainin derivative (MSI-78), used as a topical cream for the treatment of foot infections in diabetes [10], and indolicidin (CP-226), used against severe acne and skin infections with *Staphylococcus aureus* (*S. aureus*) resistant to methicillin [11]. AMPs mechanism of action is the disruption of the lipid bilayer integrity, leading to leakage of the intracellular content and death of the bacterium.

AMPs are more specific and efficient than common antibiotics and are related to lower cell and tissue toxicity [12]. Nowadays, we know of more than 40 antimicrobial peptides in the oral cavity and many of these play a pivotal role in periodontal diseases as well as in orthodontics [13,14].

On the other hand, thanks to their powerful antimicrobial activity, metal nanoparticles (NPs) have gained growing interest in the treatment of resistant pathogens. In this context, the combined use of AMPs and metal NPs seem to be very versatile since they hold the promise of interacting with different targets, therefore becoming of huge interest as an attractive alternative to antibiotics [15]. Moreover, microorganisms are unlikely to develop resistance against nanosystems, which attack multiple targets in the microbe.

Together with AMPs, several metal NPs that represent a possible solution for the control of drug-resistant bacterial infections has also evolved [16,17]. Among metal NPs, silver NPs (AgNPs) have received attention thanks to their potent broad spectrum antimicrobial activity. Since ancient times, the antimicrobial characteristics of silver have been studied and used for various purposes, primarily to improve tissue repair, fight infections, preserve drinking water, and prevent food spoilage. Silver use became less pronounced since the introduction of antibiotics. However, the recent influx of multidrug resistance it has become so potentially fatal that silver NPs have produced an astonishing return as an alternative or supplementary medication [18], opening up a whole pathway to new tools to combat a wide range of microorganisms [19–23].

The key to silver NPs broad and potent antibacterial activity is the multifaceted mechanism by which they have an effect on microbes. These mechanisms involve the disruption of ATP production and DNA replication due to gradual release of silver ions, the direct damage of cell membranes, and the generation of reactive oxygen species [24–28]. The mechanisms are likely to participate in their reported wide range of antimicrobial activity.

In order to enhance their antimicrobial effects, AgNPs were used together with some antibiotics such as penicillin G, amoxicillin, erythromycin, and vancomycin [29,30]. Gade et al. [31] demonstrated that this combined use was able to strengthen the nanoparticles' antibacterial activity. The bactericidal potential of NPs synthesized from the leaf extract of *Murrayakoenigii*, an Indian curry leaf tree, alone and in combination with commercial antibiotics (gentamycin, ampicillin, tetracycline, and streptomycin) was investigated against *Escherichia coli*, *S. aureus* and *Pseudomonas aeruginosa*. The antibiotics' potential was effectively increased by combining NPs, making their use possible also against antibiotic-resistant pathogens [32].

Another possible approach to the effective treatment of microbial infections may be represented by the combination of drugs with different mechanism of action [33,34]. The combination of AMPs and AgNPs can aid in achieving enhanced antimicrobial activity specificities, efficiencies, and strengths with reduced toxicity, and may be an effective alternative to antibiotics. The aim is not only to promote the synergy of AMP and AgNPs in the bactericidal action, but also to overcome the eventual solubility problems of the peptide and to decrease NPs toxicity on healthy cells. The antimicrobial peptide selected for the present study is indolicidin (Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH₂), a 13-residue cationic peptide with extremely high tryptophan content. This short peptide was isolated from cytoplasmic granules of bovine neutrophils [35] and has been shown to possess a broad-spectrum activity against both Gram-positive and Gram-negative bacteria [36], and also against fungi [37,38] and HIV-1 [39]. Nevertheless, this peptide is endowed of residual cytotoxicity and haemolytic activity. Here, we report on the preparation of AgNPs coated with the indolicidin peptide and antibacterial experiments performed on the Gram-positive bacterium *S. aureus* and the Gram-negative bacteria *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). It has been widely reported that Gram-negative bacteria, such as *P. aeruginosa*, seem to be responsible for the Oral squamous cell carcinoma progression [40], whereas *S. aureus* bacteria are one of the major species that can be related to denture stomatitis [41]. Other pathogens such as *Chlamydia trachomat* induce the production of inflammatory cytokines in association with growth factors involved in epithelial mesenchymal transition, a potential mechanism correlated with cancer progression [42–44]. The interplay between the pathogens' steroid receptors and neoplastic progression is a crucial node in the development on inflammatory pathologies, which represent some of main focus in every day dental practice [45–50].

2. Materials and Methods

2.1. Materials

Rink amide p-methylbenzhydrylamine (MBHA) resin, connection reagents, and Fmoc-protected amino acid derivatives were acquired from Calbiochem-Novabiochem (Laufelfingen, Switzerland). The rest of chemicals were provided by Sigma–Aldrich, Fluka (Buchs, Switzerland).

2.2. Peptide Synthesis

Indolicidin was synthesized using the standard solid-phase-9-fluorenylmethoxycarbonyl (Fmoc) method as previously reported [51].

2.3. Preparation of Silver Colloids Using Hydrazine

150 μ L and 10 μ L hydrazine monohydrate (N₂H₄·H₂O, Sigma Aldrich) were added to 1 mL AgNO₃ solution (1 mM). Subsequently, the solution was topped up to 2 mL with deionized water, thoroughly mixed for 1 min and allowed to settle at room temperature for 4 h. Hereafter, silver colloids were prepared in the presence of indolicidin. For this purpose, 150 μ L and 10 μ L N₂H₄·H₂O were added to 1 mL AgNO₃ solution (1 mM). The solution was topped up to 2 mL with indolicidin solution (concentration of indolicidin in deionized water = 560 μ g/mL), mixed, and allowed to settle at room temperature as outlined above. The obtained silver NPs were named as follow (Table 1): AgNP1 or AgNP2 for naked NPs, where 1 stands for the preparation with 150 μ L of N₂H₄·H₂O and 2 stands for

the preparation with 10 μL of $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$, and IndAgNP1 or IndAgNP2 for silver colloids incorporating the peptide indolicidin and 1 or 2, as stated above (Figure 1).

Table 1. Indolicidin and AgNPs prepared using different amount of $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$.

Name	Reducing Agent
Indolicidin	-
AgNP1	Hydrazine (150 μL)
AgNP2	Hydrazine (10 μL)
IndAgNP1	Hydrazine (150 μL)
IndAgNP2	Hydrazine (10 μL)

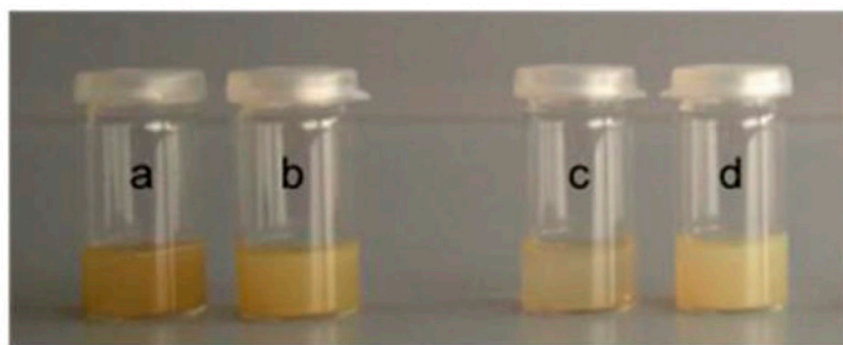


Figure 1. Silver colloids prepared using 150 μL (a,c) and 10 μL (b,d) hydrazine monohydrate in the presence of indolicidin (a,b) and without indolicidin (c,d).

2.4. Characterization UV-VIS

UV-visible spectra of the resulting nanoparticle solutions were recorded at room temperature using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, Milan, Italy). The monochromator slit width was 10 nm.

2.5. Characterization TEM

Silver NPs' morphology was analyzed using a Transmission Electron Microscope (TEM Joel 2200 fs) operated at 200 keV. Prior to TEM measurements, 0.3 mL of each sample were spotted onto a carbon-coated copper grid (300 mesh, Science Services, München, Germany). The primary particle size was measured using the ImageJ software package (Version 1.51p).

2.6. Microorganisms

For the antimicrobial assays, *E. coli* ATCC 11219, *P. aeruginosa* ATCC 13388, and *S. aureus* ATCC 6538 were used. Fresh colonies of each strain were cultured on Mueller Hinton Agar (MHA, Oxoid) and grown overnight. Then, they were inoculated in MH Broth (MHB) for another night. Subsequently, hundred-fold dilutions of the bacterial suspension were resuspended in fresh medium and further incubated at 37 °C. When the inoculum was turbid, it was resuspended in 0.9% sterile saline solution to reach an appropriate OD600 value (with a Bio-Rad Microplate Reader—Bio-Rad Laboratories, Hercules, CA, USA) corresponding to a concentration of about 1×10^8 CFU/mL. This standardized inoculum was diluted 1:10 in MHB and the inoculum size was confirmed by colony counting.

2.7. Antimicrobial Activity Assays

Susceptibility testing was performed using the broth microdilution method outlined by the Clinical and Laboratory Standards Institute using sterile 96-well microtiter plates (Falcon, NJ, USA). Starting from the concentration of 10^{14} NPs/L, two-fold serial dilutions of NPs were prepared together with the peptide indolicidin (IndAgNP1, IndAgNP2, AgNP1, and AgNP2) in Cation-Adjusted MHB

(CAMHB) at a volume of 100 μL /well. Each well was inoculated with 5 μL of the standardized bacterial inoculum, corresponding to the final concentration of about 5×10^5 CFU/mL. Antimicrobial effect was expressed as the Minimum Inhibitory Concentration (MIC), which is the lowest concentration of compound that completely inhibited visible growth after 24 h of incubation at 37 °C. All experiments were carried out in triplicate and standard deviations are reported.

2.8. Cytotoxicity

Vero cells were treated with increasing concentrations of compounds, and the cell viability was analyzed through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Vero cells were cultured in 96-well plates (2×10^4 cells/well), and after 24 h were treated with a series of concentrations (from 10^{12} to 10^{14} NPs/L) of filter sterilized NPs (IndAgNP1, IndAgNP2, AgNP1, and AgNP2) and peptide indolicidin for 3, 10, and 24 h. The medium was then removed, MTT solution was added, and cells were incubated for further 3 h at 37 °C. Formazan crystals were dissolved with dimethyl sulfoxide and the absorbance was measured at 570 nm using a Bio-Rad Microplate Reader. All experiments were repeated three times and standard deviations are reported.

2.9. Hemolytic Assay

The hemolytic activity of filter sterilized NPs (IndAgNP1, IndAgNP2, AgNP1, and AgNP2) and peptide indolicidin were determined using fresh human erythrocytes from healthy donors. The blood was centrifuged, and the erythrocytes were washed three times with 0.9% NaCl. The NPS and peptide were applied to the suspension of the erythrocyte (5% [vol/vol]), at the concentrations used for the MTT and for the volume of 100 μL . The samples were incubated with agitation at 37 °C for 60 min. The release of hemoglobin was monitored by measuring the absorbance (Abs) of the supernatant at 540 nm. The percentage of hemolysis was calculated using the equation $\% \text{ hemolysis} = [(Abs_{\text{sample}} - Abs_{\text{blank}})/(Abs_{\text{total lysis}} - Abs_{\text{blank}})] \times 100$. The control for zero hemolysis (blank) consisted of erythrocytes suspended in the presence $1 \times$ PBS, while the positive control (0.1% Triton) showed a 100% hemolytic effect. For a good statistical analysis and reproducibility the experiments were performed in triplicate. Results are integrated with standard deviations.

3. Results and Discussion

3.1. Structural Characterization of Nanoparticles

The Ag nanoparticles were prepared via in-situ approach by using wet chemistry method. Silver ions removal in aqueous solutions generally produces silver particles. Figure 2 highlights the UV-visible distribution of silver NPs produced through the use of hydrazine as a reducing agent and the absence and presence of indolicidin peptide as stabilizing factor.

The generated AgNPs possess unique optical properties due to the interaction with specific wavelengths of light. For instance, the conduction band and valence band of the AgNPs are localized very close to each other in which electrons are moving freely to increase the absorption band of the surface plasmon resonance (SPR), due to the constructive interfaces of the electrons oscillation of silver nanoparticles in resonance with the light wave [52,53]. The Optical absorption spectra of silver NPs is dominated by surface plasmon which depends on particle size, shape, state of aggregation, and the surface chemistry of the generated Ag NPs [54]. The UV-vis spectra in Figure 2 shows very broad band of aggregated AgNPs prepared in absence of indolicidin, nevertheless, in presence of indolicidin a narrow band appeared at 365 nm. These observations stated above could be attributed to the reduction process of silver ions to silver atom using hydrazine followed by anchoring between AgNPs at the cluster surface, while the amino acids of the oligopeptide protects the cluster from fusion with the next silver molecule to facilitate unique surface chemistry. This is contributed to the collective properties of the formed Ag nanoparticles. On the other hand, different amounts of the reducing agent was added which indicates no preferences in the wavelength 365 nm but significant increase in the intensity.

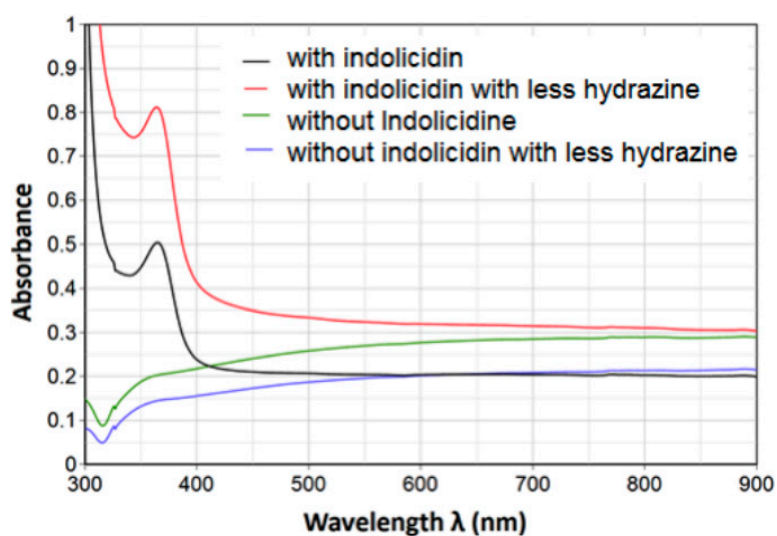


Figure 2. UV-Vis spectra of silver colloids prepared using 150 μL and 10 μL $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$ in the presence of indolicidin. UV-Vis spectra of silver colloids prepared using 150 μL and 10 μL $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$ without indolicidin indicated in light blue.

The TEM micrographs in Figure 3 demonstrate the effect of the indolicidin on the AgNPs formation. For instance, in Figure 3a,c, the AgNPs prepared within the indolicidin matrix show more control over the size and size distribution of the Ag cluster formation. To compare in Figure 3b,d, the AgNPs prepared without indolicidin elucidate aggregation of the Ag clusters in morphologies of big particles.

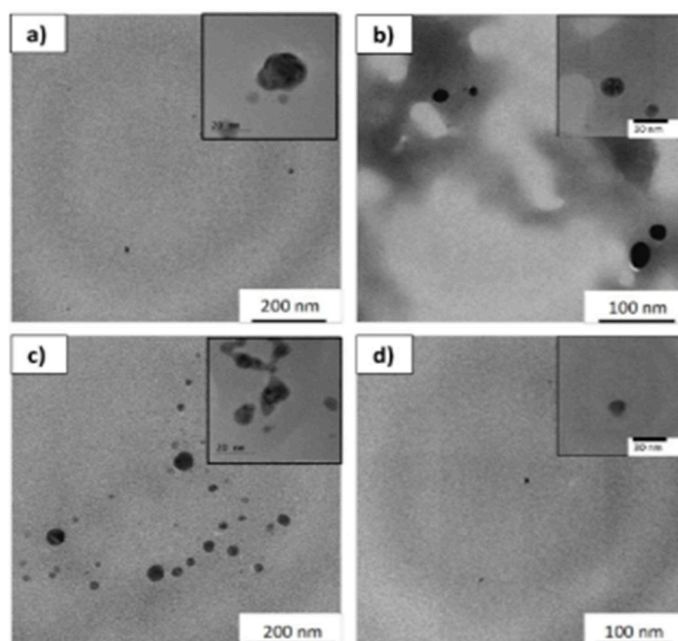


Figure 3. Morphology of silver NPs prepared using 150 μL $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$ in the presence of indolicidin (a) and without indolicidin (b); 10 μL $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$ in the presence of indolicidin (c) and without indolicidin (d).

It is worth mentioning that the amount of the reducing agent also played an important role when the Ag nanoparticles are templated with in the oligopeptide. Table 2 indicates the differences in AgNPs characteristics depending on the amount of $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$. The interactions between the amino acids within the oligopeptide facilitate networks matrix to template the coordinated Ag cations. These matrixes contribute to stabilize the formed AgNPs and to avoid the particles aggregations.

Table 2. Characteristics of Ag colloids prepared with different amounts of $N_2H_4 \cdot H_2O$, in the presence indolicidin and without the peptide.

Sample	Particle Size Distribution [nm]	Δ_{max} [nm]
Ag colloids prepared using 150 μL $N_2H_4 \cdot H_2O$ in the presence of indolicidin	12.5 ± 6.2	~ 454
Ag colloids prepared using 150 μL $N_2H_4 \cdot H_2O$ without indolicidin	32.6 ± 11.4	~ 427
Ag colloids prepared using 10 μL $N_2H_4 \cdot H_2O$ in the presence of indolicidin	5.9 ± 2.9	~ 462
Ag colloids prepared using 10 μL $N_2H_4 \cdot H_2O$ without indolicidin	21.4 ± 11.4	~ 444

Particle size was evaluated using transmission electron microscopy; Δ_{max} was determined by UV-Vis spectroscopy.

3.2. Toxicity

The MTT cytotoxicity assay is based on the activity of mitochondrial dehydrogenase. Since this enzyme is present only in the active mitochondria, the reaction can occur exclusively in viable cells. Vero cells were treated with different concentrations of NPs for 24 h showing that there was no significant reduction in the viability compared to the untreated cells. More specifically, we could observe different cytotoxicity profiles for the differently prepared silver NPs (Figure 4).

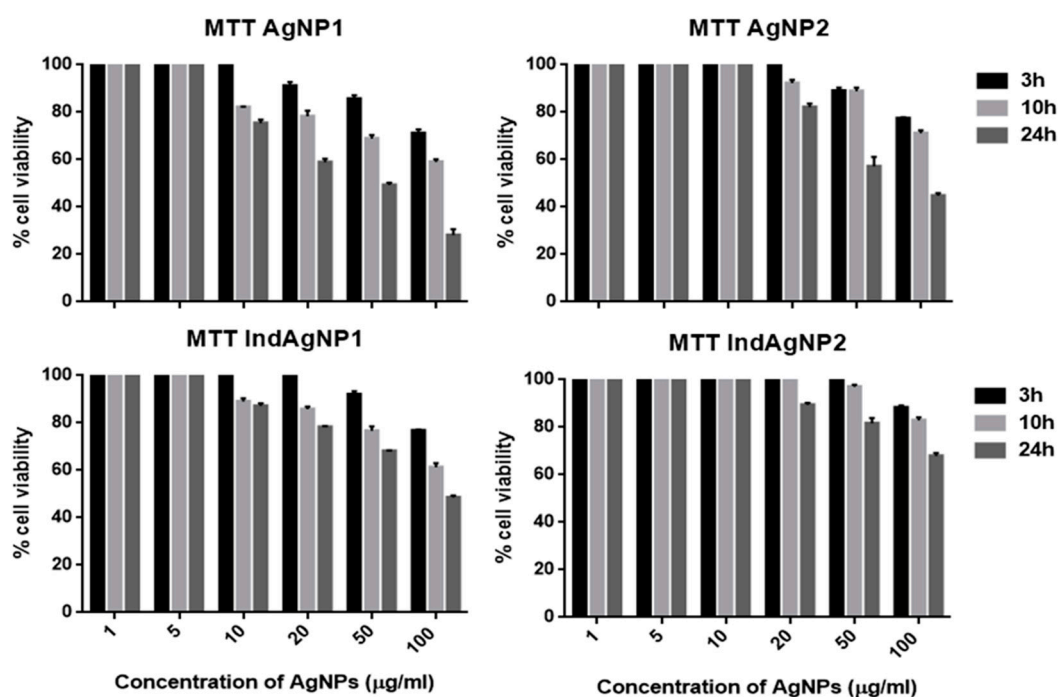


Figure 4. Cytotoxicity evaluation of AgNPs via MTT assay. Vero cells were treated for 3, 10, and 24 h with different concentrations of NPs ($\mu g/mL$).

The nanoparticles prepared with 150 μL of $N_2H_4 \cdot H_2O$ (AgNP1) were in fact revealed to be more toxic at higher concentrations (more than 50 $\mu g/mL$) and at longer time of exposure when compared to the nanoparticles prepared with 10 μL of $N_2H_4 \cdot H_2O$ (AgNP2). A clear difference could also be observed between IndAgNPs and naked AgNPs, with a lower toxicity for the functionalized silver NPs, demonstrating an advantage of shedding the AgNPs direct external surface with a peptide coating. Overall, the observed toxicity was exerted at concentrations markedly higher than those required for antibacterial test. It should be noted that the peptide indolicidin on its own also showed to be toxic at high doses (Figure 5).

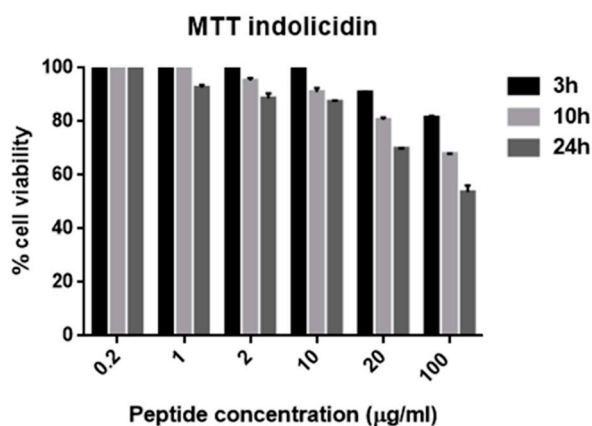


Figure 5. Cytotoxicity evaluation of indolicidin via MTT assay. Vero cells were treated for 3, 10, and 24 h with different concentrations of NPs (µg/mL).

However, the amount of the peptide on the functionalized NPs is lower than the concentration used in the MTT assay. Therefore, we can conclude that the NPs described herein do not raise safety concerns due to the low doses needed to produce an antimicrobial effect. Moreover, the less toxic nanoparticles is the IndAgNP2 produced in 10 µL of $N_2H_4 \cdot H_2O$ pointing out that the method for production of silver NPs needs to be carefully considered since, at least partially, the toxicity could be due to the chemical used for production.

Hemolysis assay was performed at the same concentrations used in the MTT assay. The hemolytic action of peptides or silver NPs is regarded as a negative function of antimicrobial compounds, posing a serious obstacle to their use for pharmaceutical application. Interestingly, in contrast to the hemolytic activity of indolicidin at high concentration (20 to 100 µg/mL) (Table 3), all compounds were found to possess little hemolytic activity (less than 25%) at the highest concentrations tested (100 µg/mL) (Table 4).

Table 3. Hemolytic activity of indolicidin peptide at various concentrations.

Concentration (µg/mL)	% Hemolysis
0.2	0
1	0
2	0
10	0
20	5
100	25

Values are expressed as mean ± SD.

Table 4. Hemolytic activity of nanosystems.

Name	Indolicidin (µg/mL)	AgNPs (µg/mL)	% Hemolysis
IndAgNP1	1.5	1	0
	3	5	0
	6	10	0
	12	20	0
	25	50	8
	50	100	21
AgNP1	-	1	0
	-	5	0
	-	10	0
	-	20	0
	-	50	12
	-	100	24

Table 4. Cont.

Name	Indolicidin ($\mu\text{g/mL}$)	AgNPs ($\mu\text{g/mL}$)	% Hemolysis
IndAgNP2	1.5	1	0
	3	5	0
	6	10	0
	12	20	0
	25	50	0
	50	100	15
AgNP2	-	1	0
	-	5	0
	-	10	0
	-	20	0
	-	50	7
	-	100	18

Values are expressed as mean \pm SD.

The level of hemolytic activity was negligible when using the concentrations close to the MIC obtained (as described below).

3.3. Antibacterial Activity

The antibacterial potential of the different NPs containing or not containing indolicidin was analyzed by MIC against both Gram-positive and Gram-negative bacteria. Determination of MIC values of indolicidin and AgNPs alone (Table 5) and their conjugates (Table 6) was carried out using three model bacteria strains: *E. coli*, *P. aeruginosa*, and *S. aureus*.

Table 5. Antibacterial activity of indolicidin and AgNPs alone against Gram-positive and negative bacteria. The antibacterial activity is expressed as MIC values.

Name	<i>E. coli</i>		<i>P. aeruginosa</i>		<i>S. aureus</i>	
	Indolicidin ($\mu\text{g/mL}$)	AgNPs ($\mu\text{g/mL}$)	Indolicidin ($\mu\text{g/mL}$)	AgNPs ($\mu\text{g/mL}$)	Indolicidin ($\mu\text{g/mL}$)	AgNPs ($\mu\text{g/mL}$)
Indolicidin	30	-	30	-	20	-
AgNP1	-	15	-	15	-	25
AgNP2	-	10	-	10	-	15

Values are expressed as mean \pm SD.

Table 6. Antibacterial activity of AgNPs conjugated to the peptide indolicidin against Gram-positive and negative bacteria. The antibacterial activity is expressed as MIC values.

Name	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
	$\mu\text{g/mL}$	$\mu\text{g/mL}$	$\mu\text{g/mL}$
IndAgNP1	5	12.5	12.5
IndAgNP2	5	7.5	10

Values are expressed as mean \pm SD.

The results showed that the effective doses were in a similar range (5–12.5 $\mu\text{g/mL}$) for the two nanoparticles containing indolicidin, regardless of the amount of $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$ that was used for their preparation. Therefore, hydrazine has a negative effect on toxicity, but a non-discernible effect on the antibacterial activity. The inhibitory activity was moderately increased with the nanoparticles containing indolicidin compared to AgNP1 and AgNP2, suggesting that the addition of indolicidin is of great importance for the general mechanism and probably for the shape and size of the nanoparticles. In fact, both IndAgNP1 and IndAgNP2 are considerably smaller than their counterparts without indolicidin, and the antibacterial activity has been often correlated to the size of nanoparticles used.

Indeed, it has been reported that smaller particles have greater surface area compared to larger particles [55–59]. Since metal ions are released from the surface, the greater the surface area, the greater the release of ions. Presumably the strong antibacterial potential of smaller nanoparticles derives from the release of a wider number of ions than the larger particles per unit mass. Furthermore, it is well known that AgNPs are generally more active against Gram-negative compared to Gram-positive bacteria, and our results are constant with this observation. It has been attributed to the differences in the bacterial cell wall and the resulting interaction with the nanoparticles. Gram-positive and Gram-negative bacteria are both characterized by a negative charge due to the presence of teichoic acids in the former and lipopolysaccharides in the latter. Furthermore, the Gram-negative outer membrane contains a group of protein-channels called porins, which are essential for the passage of small hydrophilic antibiotics and metabolites into the cell. The antibacterial activity of silver NPs has been also related to the presence of these outer membrane porin proteins. On the contrary, when indolicidin peptide was tested on its own, the MIC revealed a stronger activity of indolicidin against *S. aureus* (MIC at 20 µg/mL) and a lower inhibitory effect against both *E. coli* and *P. aeruginosa* (MIC at 30 µg/mL for both). Surprisingly, both IndAgNP1 and IndAgNP2 levelled these differences and provided a stronger antibacterial effect with similar MIC value for both Gram-positive and Gram-negative bacteria (5–12.5 µg/mL). The difference between AgNPs and IndAgNPs is not of considerable degree, but it is important to notice that the amount of peptide inserted into the nanoparticle is negligible compared to the amount of indolicidin able to exert an antibacterial effect when used on its own as antibacterial. Therefore, the addition of indolicidin, more than adding a clear and definitive boost to the known antibacterial property of silver NPs, is indeed of primary importance for the physical characteristics of the produced NPs, both in terms of size and cytotoxicity reduction.

4. Conclusions

Nowadays, the growing threat of microbial resistance against traditional antibiotics in oral disease has prompted the mandatory development of alternative therapies, especially against bacterial strains showing resistance to one or more antibiotics [60,61]. In this article, silver NPs (AgNPs) were successfully prepared using hydrazine and were further modified by a coating of indolicidin, a well-known AMP.

The prepared AgNPs possessed good, dose-dependent in vitro antimicrobial activity against Gram-positive and Gram-negative bacteria. The better results were achieved with indolicin-coated AgNPs, regardless having used either 10 or 150 µL for nanoparticles production. Notwithstanding the moderate antibacterial increase obtained with the coated NPs versus the naked ones, the importance of our results resides in the fact that the amount of indolicidin attached to the NPs was in a range well below the reported concentrations normally used for achieving antibacterial activity. Therefore, the major interest in attaching low concentration AMPs on AgNPs is to reduce the overall toxicity shown by both the naked AgNPs and indolicidin on their own.

The bactericidal effect of silver NPs may be translated into important therapeutic and clinical opportunities in the future, principally in view of the dearth of new antibiotics against the emerging antimicrobial resistant microorganisms, in particular against Gram-negative bacteria.

Author Contributions: C.Z.: Conceptualization; Data curation; Investigation; Writing the original draft; S.S.: Investigation; Methodology; M.V.: Data curation; Formal analysis; A.F.: Investigation; Methodology; E.G.: Conceptualization; Supervision; A.F. Conceptualization; Writing review & editing; B.S.: Investigation; Validation; L.N. Supervision, Data curation; R.G. Formal analysis; Methodology; M.G. Conceptualization; Writing review & editing; M.B. Supervision, Writing review & editing; G.F.: Conceptualization; Supervision; Writing the original draft; M.D.D. Conceptualization; Methodology; Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The Authors would like to acknowledge the financial support from the Rhine-Waal University of Applied Sciences for the seed fund project, Dr. Christine Strehmel for the valuable discussion and Cameron Hay for the English revision.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Vitiello, M.; Galdiero, M.; Finamore, E.; Galdiero, S.; Galdiero, M. NF- κ B as a potential therapeutic target in microbial diseases. *Mol. Biosyst.* **2012**, *8*, 1108–1120. [[CrossRef](#)] [[PubMed](#)]
2. Rai, M.K.; Deshmukh, S.D.; Ingle, A.P.; Gade, A.K. Silver nanoparticles: The powerful nanoweapon against multidrug-resistant bacteria. *J. Appl. Microbiol.* **2012**, *112*, 841–852. [[CrossRef](#)] [[PubMed](#)]
3. Campanella, V.; Syed, J.; Santacroce, L.; Saini, R.; Ballini, A.; Inchingolo, F. Oral probiotics influence oral and respiratory tract infections in pediatric population: A randomized double-blinded placebo-controlled pilot study. *Eur. Rev. Med. Pharmacol. Sci.* **2018**, *22*, 8034–8041. [[PubMed](#)]
4. Gal, L.; Xu, T.; Huang, G.; Jiang, S.; Gu, Y.; Chen, F. Oral microbiomes: More and more importance in oral cavity and whole body. *Protein Cell* **2018**, *9*, 488.
5. Nakatsuji, T.; Gallo, R.L. Antimicrobial peptides: Old molecules with new ideas. *J. Investig. Dermatol.* **2012**, *132*, 887–895. [[CrossRef](#)]
6. Hancock, R.E.; Sahl, H.G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006**, *24*, 1551–1557. [[CrossRef](#)]
7. Marr, A.K.; Gooderham, W.J.; Hancock, R.E. Antibacterial peptides for therapeutic use: Obstacles and realistic outlook. *Curr. Opin. Pharmacol.* **2006**, *6*, 468–472. [[CrossRef](#)]
8. Gordon, Y.J.; Romanowski, E.G.; McDermott, A.M. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr. Eye Res.* **2005**, *30*, 505–515. [[CrossRef](#)]
9. Lazarev, V.N.; Govorun, V.M. Antimicrobial peptides and their use in medicine. *Appl. Biochem. Microbiol.* **2010**, *46*, 803–814. [[CrossRef](#)]
10. Lipsky, B.A.; Holroyd, K.J.; Zasloff, M. Topical versus systemic antimicrobial therapy for treating mildly infected diabetic foot ulcers: A randomized, controlled, double-blinded, multicenter trial of pexiganan cream. *Clin. Infect. Dis.* **2008**, *47*, 1537–1545. [[CrossRef](#)]
11. Melo, M.N.; Dugourd, D.; Castanho, M.A. Omiganan pentahydrochloride in the front line of clinical applications of antimicrobial peptides. *Recent Pat. Antiinfect. Drug Discov.* **2006**, *1*, 201–207. [[CrossRef](#)] [[PubMed](#)]
12. Gorr, S.U. Antimicrobial peptides of the oral cavity. *Periodontology 2000* **2009**, *51*, 152–180. [[CrossRef](#)] [[PubMed](#)]
13. Bechinger, B.; Gorr, S.U. Antimicrobial Peptides: Mechanisms of Action and Resistance. *J. Dent. Res.* **2016**, *96*, 254–260. [[CrossRef](#)] [[PubMed](#)]
14. Isola, G.; Perillo, L.; Migliorati, M.; Matarese, M.; Dalessandri, D.; Grassia, V.; Alibrandi, A.; Matarese, G. The impact of temporomandibular joint arthritis on functional disability and global health in patients with juvenile idiopathic arthritis. *Eur. J. Orthod.* **2019**, *41*, 117–124. [[CrossRef](#)]
15. Seil, J.T.; Webster, T.J. Antimicrobial applications of nanotechnology: Methods and literature. *Int. J. Nanomed.* **2012**, *7*, 2767–2781.
16. Falanga, A.; Lombardi, L.; Franci, G.; Vitiello, M.; Iovene, M.R.; Morelli, G.; Galdiero, M.; Galdiero, S. Marine Antimicrobial Peptides: Nature Provides Templates for the Design of Novel Compounds against Pathogenic Bacteria. *Int. J. Mol. Sci.* **2016**, *17*, 785. [[CrossRef](#)]
17. Cantisani, M.; Leone, M.; Mignogna, E.; Kampanaraki, K.; Falanga, A.; Morelli, G.; Galdiero, M.; Galdiero, S. Structure-activity relations of myxinidin, an antibacterial peptide derived from the epidermal mucus of hagfish. *Antimicrob. Agents Chemother.* **2013**, *57*, 5665–5673. [[CrossRef](#)]
18. Hoyme, U.B. Clinical significance of Crede's prophylaxis in germany at present. *Infect. Dis. Obstet. Gynecol.* **1993**, *1*, 32–36. [[CrossRef](#)]
19. dos Santos, C.A.; Seckler, M.M.; Ingle, A.P.; Gupta, I.; Galdiero, S.; Galdiero, M.; Gade, A.; Rai, M. Silver nanoparticles: Therapeutical uses, toxicity, and safety issues. *J. Pharm. Sci.* **2014**, *103*, 1931–1944. [[CrossRef](#)]
20. Gaikwad, S.; Ingle, A.; Gade, A.; Rai, M.; Falanga, A.; Incoronato, N.; Russo, L.; Galdiero, S.; Galdiero, M. Antiviral activity of mycosynthesized silver nanoparticles against herpes simplex virus and human parainfluenza virus type 3. *Int. J. Nanomed.* **2013**, *8*, 4303–4314.
21. Galdiero, S.; Falanga, A.; Vitiello, M.; Cantisani, M.; Marra, V.; Galdiero, M. Silver nanoparticles as potential antiviral agents. *Molecules* **2011**, *16*, 8894–8918. [[CrossRef](#)] [[PubMed](#)]

22. Rai, M.; Deshmukh, S.D.; Ingle, A.P.; Gupta, I.R.; Galdiero, M.; Galdiero, S. Metal nanoparticles: The protective nanoshield against virus infection. *Crit. Rev. Microbiol.* **2016**, *42*, 46–56. [[CrossRef](#)] [[PubMed](#)]
23. Rai, M.; Kon, K.; Ingle, A.; Duran, N.; Galdiero, S.; Galdiero, M. Broad-spectrum bioactivities of silver nanoparticles: The emerging trends and future prospects. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1951–1961. [[CrossRef](#)] [[PubMed](#)]
24. Morones, J.R.; Elechiguerra, J.L.; Camacho, A.; Holt, K.; Kouri, J.B.; Ramirez, J.T.; Yacaman, M.J. The bactericidal effect of silver nanoparticles. *Nanotechnology* **2005**, *16*, 2346–2353. [[CrossRef](#)]
25. Klueh, U.; Wagner, V.; Kelly, S.; Johnson, A.; Bryers, J.D. Efficacy of silver-coated fabric to prevent bacterial colonization and subsequent device-based biofilm formation. *J. Biomed. Mater. Res.* **2000**, *53*, 621–631. [[CrossRef](#)]
26. Shrivastava, S.; Bera, T.; Roy, A.; Singh, G.; Ramchandrarao, P.; Dash, D. Characterization of enhanced antibacterial effects of novel silver nanoparticles. *Nanotechnology* **2007**, *18*, 225103. [[CrossRef](#)]
27. Sondi, I.; Salopek-Sondi, B. Silver nanoparticles as antimicrobial agent: A case study on E. coli as a model for Gram-negative bacteria. *J. Colloid Interface Sci.* **2004**, *275*, 177–182. [[CrossRef](#)]
28. Kim, J.S.; Kuk, E.; Yu, K.N.; Kim, J.H.; Park, S.J.; Lee, H.J.; Kim, S.H.; Park, Y.K.; Park, Y.H.; Hwang, C.Y.; et al. Antimicrobial effects of silver nanoparticles. *Nanomedicine* **2007**, *3*, 95–101. [[CrossRef](#)]
29. Rai, M.; Yadav, A.; Gade, A. Silver nanoparticles as a new generation of antimicrobials. *Biotechnol. Adv.* **2009**, *27*, 76–83. [[CrossRef](#)]
30. Shahverdi, A.R.; Fakhimi, A.; Shahverdi, H.R.; Minaian, S. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against Staphylococcus aureus and Escherichia coli. *Nanomedicine* **2007**, *3*, 168–171. [[CrossRef](#)]
31. Gade, A.; Gaikwad, S.; Tiwari, V.; Yadav, A.; Ingle, A.; Rai, M. Biofabrication of silver nanoparticles by Opuntia ficus-indica: In vitro antibacterial activity and study of the mechanism involved in the synthesis. *Curr. Nanosci.* **2010**, *6*, 370–375. [[CrossRef](#)]
32. Bonde, S.R.; Rathod, D.P.; Ingle, A.P.; Ade, R.B.; Gade, A.K.; Rai, M.K. Murraya koenigii-mediated synthesis of silver nanoparticles and its activity against three human pathogenic bacteria. *Nanosci. Methods* **2012**, *1*, 25–36. [[CrossRef](#)]
33. Tarallo, R.; Carberry, T.P.; Falanga, A.; Vitiello, M.; Galdiero, S.; Galdiero, M.; Weck, M. Dendrimers functionalized with membrane-interacting peptides for viral inhibition. *Int. J. Nanomed.* **2013**, *8*, 521–534.
34. Galdiero, S.; Falanga, A.; Tarallo, R.; Russo, L.; Galdiero, E.; Cantisani, M.; Morelli, G.; Galdiero, M. Peptide inhibitors against herpes simplex virus infections. *J. Pept. Sci.* **2013**, *19*, 148–158. [[CrossRef](#)]
35. Selsted, M.E.; Novotny, M.J.; Morris, W.L.; Tang, Y.Q.; Smith, W.; Cullor, J.S. Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. *J. Biol. Chem.* **1992**, *267*, 4292–4295.
36. Giacometti, A.; Cirioni, O.; Greganti, G.; Quarta, M.; Scalise, G. In vitro activities of membrane-active peptides against gram-positive and gram-negative aerobic bacteria. *Antimicrob. Agents Chemother.* **1998**, *42*, 3320–3324. [[CrossRef](#)]
37. Ahmad, I.; Perkins, W.R.; Lupan, D.M.; Selsted, M.E.; Janoff, A.S. Liposomal entrapment of the neutrophil-derived peptide indolicidin endows it with in vivo antifungal activity. *Biochim. Biophys. Acta* **1995**, *1237*, 109–114. [[CrossRef](#)]
38. Lee, D.G.; Kim, H.K.; Kim, S.A.; Park, Y.; Park, S.C.; Jang, S.H.; Hahm, K.S. Fungicidal effect of indolicidin and its interaction with phospholipid membranes. *Biochem. Biophys. Res. Commun.* **2003**, *305*, 305–310. [[CrossRef](#)]
39. Robinson, W.E., Jr.; McDougall, B.; Tran, D.; Selsted, M.E. Anti-HIV-1 activity of indolicidin, an antimicrobial peptide from neutrophils. *J. Leukoc. Biol.* **1998**, *63*, 94–100. [[CrossRef](#)]
40. Al-hebshi, N.N.; Nasher, A.T.; Maryoud, M.Y.; Homeida, H.E.; Chen, T.; Idris, A.M.; Johnson, N.W. Inflammatory bacteriome featuring Fusobacterium nucleatum and Pseudomonas aeruginosa identified in association with oral squamous cell carcinoma. *Rep. Sci.* **2017**, *7*, 1834. [[CrossRef](#)]
41. Garbacz, K.; Kwapisz, E.; Wierzbowska, M. Denture stomatitis associated with small-colony variants of Staphylococcus aureus: A case report. *BMC Oral Health* **2019**, *19*, 219. [[CrossRef](#)] [[PubMed](#)]
42. Ricci, S.; Pinto, F.; Auletta, A.; Giordano, A.; Giovane, A.; Settembre, G.; Boccellino, M.; Boffo, S.; Di Carlo, A.; Di Domenico, M. The enigmatic role of matrix metalloproteinases in epithelial-to-mesenchymal transition of oral squamous cell carcinoma: Implications and nutraceutical aspects. *J. Cell. Biochem.* **2019**, *120*, 6813–6819. [[CrossRef](#)] [[PubMed](#)]
43. Rizzo, A.; Di Domenico, M.; Romano Carratelli, C.; Paolillo, R. The role of chlamydia and chlamyphila infections in reactive arthritis. *Intern. Med.* **2012**, *51*, 113–117. [[CrossRef](#)] [[PubMed](#)]

44. De Filippis, A.; Buommino, E.; Di Domenico, M.; Feola, A.; Brunetti-Pierri, R.; Rizzo, A. Chlamydia trachomatis induces an upregulation of molecular biomarkers podoplanin, Wilms' tumour gene 1, osteopontin and inflammatory cytokines in human mesothelial cells. *Microbiology* **2017**, *163*, 654–663. [[CrossRef](#)]
45. Pannone, G.; Santoro, A.; Feola, A.; Bufo, P.; Papagerakis, P.; Lo Muzio, L.; Staibano, S.; Ionna, F.; Longo, F.; Franco, R.; et al. The role of E-cadherin down-regulation in oral cancer: CDH1 gene expression and epigenetic blockage. *Curr. Cancer Drug Targets* **2014**, *14*, 115–127. [[CrossRef](#)]
46. Aquino, G.; Pannone, G.; Santoro, A.; Liguori, G.; Franco, R.; Serpico, R.; Florio, G.; De Rosa, A.; Mattoni, M.; Cozza, V.; et al. PEGFR-Tyr 845 expression as prognostic factors in oral squamous cell carcinoma: A tissue-microarray study with clinic-pathological correlations. *Cancer Biol. Ther.* **2012**, *13*, 967–977. [[CrossRef](#)]
47. Di Domenico, M.; Pierantoni, G.M.; Feola, A.; Esposito, F.; Laino, L.; De Rosa, A.; Rullo, R.; Mazzotta, M.; Martano, M.; Sanguedolce, F.; et al. Prognostic significance of N-cadherin expression in oral squamous cell carcinoma. *Anticancer Res.* **2011**, *31*, 4211–4218.
48. Rizzo, A.; Di Domenico, M.; Carratelli, C.R.; Mazzola, N.; Paolillo, R. Induction of proinflammatory cytokines in human osteoblastic cells by Chlamydia pneumoniae. *Cytokine* **2011**, *56*, 450–457. [[CrossRef](#)]
49. Fiorelli, A.; Ricciardi, C.; Pannone, G.; Santoro, A.; Bufo, P.; Santini, M.; Serpico, R.; Rullo, R.; Pierantoni, G.M.; Di Domenico, M. Interplay between steroid receptors and neoplastic progression in sarcoma tumors. *J. Cell. Physiol.* **2011**, *226*, 2997–3003. [[CrossRef](#)]
50. Migliaccio, A.; Castoria, G.; De Falco, A.; Di Domenico, M.; Galdiero, M.; Nola, E.; Chambon, P.; Auricchio, F. In vitro phosphorylation and hormone binding activation of the synthetic wild type human estradiol receptor. *J. Steroid Biochem. Mol. Biol.* **1991**, *38*, 407–413. [[CrossRef](#)]
51. Cantisani, M.; Finamore, E.; Mignogna, E.; Falanga, A.; Nicoletti, G.F.; Pedone, C.; Morelli, G.; Leone, M.; Galdiero, M.; Galdiero, S. Structural insights into and activity analysis of the antimicrobial Peptide myxinidin. *Antimicrob. Agents Chemother.* **2014**, *58*, 5280–5290. [[CrossRef](#)] [[PubMed](#)]
52. Sastry, M.; Patil, V.; Sainkar, S.R. Electrostatically controlled diffusion of carboxylic acid derivatized silver colloidal particles in thermally evaporated fatty amine films. *J. Phys. Chem. B* **1998**, *102*, 1404–1410. [[CrossRef](#)]
53. Tomaszewska, E.; Soliwoda, K.; Kadziola, K.; Celichowski, G.; Cichomski, M.; Szmaja, W.; Grobelny, J. Detection limits of DLS and UV-vis spectroscopy in characterization of polydisperse nanoparticles colloids. *J. Nanomater.* **2013**, *2013*, 313081. [[CrossRef](#)]
54. Leung, A.B.; Suh, K.I.; Ansari, R.R. Particle-size and velocity measurements in flowing conditions using dynamic light scattering. *Appl. Opt.* **2006**, *45*, 2186–2190. [[CrossRef](#)] [[PubMed](#)]
55. Graf, P.; Manton, A.; Foelske, A.; Shkilnyy, A.; Masić, A.; Thünemann, A.F.; Taubert, A. Peptide-coated silver nanoparticles: Synthesis, surface chemistry, and pH-triggered, reversible assembly into particle assemblies. *Chemistry* **2009**, *15*, 5831–5844. [[CrossRef](#)] [[PubMed](#)]
56. Wang, L.; Hu, C.; Shao, L. The antimicrobial activity of nanoparticles: Present situation and prospects for the future. *Int. J. Nanomed.* **2017**, *12*, 1227–1249. [[CrossRef](#)]
57. Knetsch, M.L.W.; Koole, L.H. New strategies in the development of antimicrobial coatings: The example of increasing usage of silver and silver nanoparticles. *Polymers* **2011**, *3*, 340–366. [[CrossRef](#)]
58. Huh, A.J.; Kwon, Y.J. “Nanoantibiotics”: A new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. *J. Control. Release* **2011**, *156*, 128–145. [[CrossRef](#)] [[PubMed](#)]
59. Hajipour, M.J.; Fromm, K.M.; Ashkarran, A.A.; Jimenez de Aberasturi, D.; de Larramendi, I.R.; Rojo, T.; Serpooshan, V.; Parak, W.J.; Mahmoudi, M. Antibacterial properties of nanoparticles. *Trends Biotechnol.* **2012**, *30*, 499–511. [[CrossRef](#)]
60. Franci, G.; Folliero, V.; Cammarota, M.; Zannella, C.; Sarno, F.; Schiraldi, C.; de Lera, A.R.; Altucci, L.; Galdiero, M. Epigenetic modulator UVI5008 inhibits MRSA by interfering with bacterial gyrase. *Sci. Rep.* **2018**, *8*, 13117. [[CrossRef](#)]
61. Grassia, V.; Lombardi, A.; Kawasaki, H.; Ferri, C.; Perillo, L.; Mosca, L.; Delle Cave, D.; Nucci, L.; Porcelli, M.; Caraglia, M. Salivary microRNAs as new molecular markers in cleft lip and palate: A new frontier in molecular medicine. *Oncotarget* **2018**, *9*, 18929–18938. [[CrossRef](#)] [[PubMed](#)]

