In situ Synthesis of Size-controlled, Stable Silver Nanoparticles within Ultrashort Peptide Hydrogels for Effective Anti-bacterial Therapy

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Abstract

We have developed a silver-releasing biomaterial with promising potential for wound healing applications. The novel material is made of ultrashort peptides which can self-assemble in water to form hydrogels. Silver nanoparticles (Ag NPs) were synthesized *in situ* within the biomaterial, using only UV irradiation and no additional chemical reducing agents. The synthetic strategy allows to precisely controlling the nanoparticle size, with the network of peptide fibres preventing aggregation of Ag NP. The biomaterial shows increased mechanical strength compared to the hydrogel control. We observed a sustained release of Ag NPs over a period of 14 days. This is a crucial prerequisite for effective antibacterial therapy. The ability to inhibit bacterial growth was tested using different bacterial strains, namely gram-negative *E. coli* and *P. aeruginosa* and gram-positive *S. aureus*. Inhibition of bacterial growth was observed for all strains. The best results were obtained for *P. aeruginosa* which is known for exhibiting multidrug resistance. Biocompatibility studies on HDFa cells, using Ag NP-containing hydrogels, did not show any significant influence on cell viability. We propose this novel silver-releasing hydrogel as an excellent biomaterial with great potential for application in wound healing due to its low silver content, sustained silver nanoparticle release and biocompatibility. (Exact 200 words of 100-200 words)

Keywords:

Silver, Nanoparticle, Peptide, Hydrogel, Self-assembly, Antibacterial

1. Introduction

We have previously introduced a novel class of ultrashort peptides, containing 3-7 amino acids, with an innate self-assembly capacity for self-assembly. The peptide monomers form helical fibers that entangle to three-dimensional networks and eventually entrap water to form hydrogels [1, 2]. These hydrogels show remarkable properties with regards to mechanical stiffness, elasticity and biocompatibility and have been successfully employed in diverse applications as biomimetic material [3]. However, earlier attempts of physically entrapping a bioactive compound within the self-assembling peptide hydrogel resulted in a burst release - a sustained release was never achieved. Hence the goal of this study was to develop an organic-inorganic hybrid system that enabled an initial burst release, followed by a sustained release of the bioactive nanoparticle for wound healing applications.

In recent years, the interest in biomaterials with inherent antimicrobial activity has significantly increased. A number of different applications have been investigated. In particular, topical applications have been explored for the prevention of infections, associated with large wounds and biofilm formation in medical devices. Due to the increase in antibiotic-resistant bacteria, a lot of effort is being put into developing biomaterial containing anti-microbial agents such as silver. Silver compounds such as silver nitrate solution have been traditionally used in the treatment of human diseases such as gonococcal eye infections,

burns and wounds [4-6]. Silver compounds possess certain advantages over classical antibiotics, such as a broad spectrum of anti-microbial activity against as many as 12 gram-positive and gram-negative bacteria [7]. Further, the existence of multiple cellular targets within the microbes lowers the propensity of inducing microbial resistance to silver-based treatments. Silver-based materials which are highly active against antibiotic-resistant bacterial strains have been developed. In particular, biomaterials impregnated with silver have been tested for clinical use and several products containing either Ag(I) or silver nanoparticles (Ag NPs) are currently available commercially [7-9]. In this context, it should be noted that a recent study compared the anti-microbial effectiveness of various silver compounds, such as silver sulfadiazine and silver nanoparticles [10]. Interestingly, it was concluded that the bactericidal properties of silver nanoparticles are superior to other silver compounds and that their potencies increase with a decrease in their sizes [11]. Martínez-Gutierrez *et al*. specifically reports that silver nanoparticles of size 20 - 25 nm were most effective in suppressing the growth of clinically relevant bacteria with moderate to high antibiotic resistance [12].

Several attempts have been made to develop hydrogels which contain silver but thus far none of these biomaterials are commercially available. Recently Messersmith *et al*., reported water-soluble polyethylene glycol (PEG) polymers containing reactive catechol moieties that can reduce Ag(I) to Ag(0) NP [13]. Varaprasad *et al*. reported the *in situ* reduction of silver in hydrogels using sodium borohydride resulting in the formation of colloidal silver in the hydrogels

[14]. In both reported methods, the reduction of silver takes place during polymer gelation. A different method was employed by Murali *et al*. who reported the controlled formation of silver nanoparticles inside a pre-formed hydrogel network [15]. Furthermore, Banerjee *et al*. reported UV light-induced reduction of silver nitrate using Fmoc protected dipeptides or single amino acids [16, 17].

In this study, we report the development of a novel silver-impregnated biomaterial made of ultrashort peptides which only contain aliphatic amino acids. This unique class of ultrashort peptides is able to self-assemble in water and in physiologically relevant buffers to form hydrogels [1,2]. The gel made from peptide Ac-LIVAGK-NH_2 (Ac-LK_6-NH_2) was used as a matrix for *in situ* Ag NP synthesis with silver nitrate as the Ag(I) source. Under UV light, the silver was reduced in a very short amount of time, without the presence of any other reducing agent. The morphological and mechanical properties of the resulting hybrid biomaterial were examined by transmission electron microscopy (TEM) and rheology and its antibacterial properties were evaluated. In addition, biocompatibility studies were performed using human primary dermal fibroblasts for evaluating suitability of the hybrid biomaterial for wound healing applications.

2. Materials and Methods

2.1. Materials for Peptide Synthesis

Fmoc-lys-rink resin (resin 0.42 mg/mol), Fmoc protected amino acid i.e. glycine, alanine, valine, leucine and isoleucine and O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU) were purchased from GL

Biochem (Shanghai) Ltd. Dimethylformamide (DMF) (analytical grade) was purchased from Fisher Scientific. Acetic anhydride (Ac_2O) and dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich. *N*,*N*-Diisopropylethylamine (DIPEA), dichloromethane (DCM), trifluoroacetic acid (TFA) and TIS (triisopropylsilane) were purchased from Alfa Aesar. Piperidine and silver nitrate ($AgNO_3$) were purchased from Merck Schuchardt OHG. Diethyl ether (Et_2O) was purchased from Tedia Company Inc.

Ac-LIVAGK-NH_2 (Ac-LK_6-NH_2) was purified on an Agilent 1260 Infinity preparative HPLC system equipped with a Zorbax SB-C18 column (21.2 mm × 150.0 mm Ø 7 µm). The HPLC was coupled over an active splitter to a SQ-MS for mass triggered fraction collection. MilliQ water and HPLC grade acetonitrile, both containing 0.1% formic acid, were used as eluents, and a solvent gradient was used to elute the pure peptide. ESI-MS spectra were measured in positive mode on an Agilent 6130 Quadrupole LC/MS system equipped with an ESI spray chamber and coupled to a preparative Agilent 1260 HPLC unit.

2.2 TEM Analysis

TEM analysis was performed with a Philips CM300 FEG TEM at 300 kV. TEM images were analyzed using Gatan Digital Micrograph. Samples were freeze dried prior to analysis, and then resuspended in EtOH and dropped on a carbon-film Cu grid for TEM imaging.

2.2. Peptide Synthesis and Purification

Ac-LK_6-NH_2 peptide was synthesised using solid phase peptide synthesis. Briefly, Fmoc-lys-rink resin was weighed out and the beads were allowed to swell for an hour in DMF. Subsequently, 10 equivalent of Ac_2O and DIPEA was added to prevent reaction of the side-groups, and the mixture was allowed to incubate for 45 minutes. The resin was then washed with DMF before going through a series of de-protection reactions using 20% piperidine in DMF and coupling reactions with the addition of 4 equivalents of the desired amino acid along with 4 equivalents of TBTU and 4 equivalents of DIPEA. Acetylation of the N-terminus was performed using a 4 times excess of Ac_2O and DIPEA. Following this, the resin was washed with DCM and allowed to dry, before cleaving the peptide from it using a mixture of 95% TFA, 2.5% water and 2.5% TIS. The solvents were removed under reduced pressure and Et_2O was added to precipitate the peptide. The peptide was isolated by centrifugation, washed twice with Et_2O and dried under reduced pressure. Purification was performed in a preparative high-performance liquid chromatography system coupled to an electron spray ionization mass spectroscopy equipment (HPLC-ESI-MS) (Agilent Technologies, 1260 Infinity Series). The peptides were introduced into the HPLC-MS system by completely dissolving them in a minimum amount of DMSO. Yield: 3.4 g (60 %), ESI-MS: Calculated for $C_{30}H_{57}N_8O_7$ ($[M+H^+]^+$) 641.43, Found: *m/z* 641.5.

2.3. Hydrogel preparation

All solutions for gel preparation used Tris buffer (100 mM, pH 8.5) that was prepared from Tris base and brought to a pH of 8.5 with 1 M nitric acid. The standard procedure for preparation of the hydrogel involved the addition of requisite amount of $AgNO_3$ solution prepared using the Tris buffer to weighed out samples of Ac-LK_6-NH_2 to achieve the desired concentration. Briefly, to prepare 10 mg/mL of Ac-LK_6-NH_2 hydrogel, 1 mL of $AgNO_3$ solution was added to 10 mg of peptide. After gelation in the dark, the hydrogel was subjected to UV radiation at 254 nm for approximately 5 minutes using a UV-lamp.

2.4. Oscillatory Rheometry

Viscoelasticity of the peptide hydrogels was measured using an ARESG2 rheometer (TA Instruments). A serrated stainless steel, parallel-plate geometry of 8-mm diameter was used and the gap distance was maintained between 0.8 and 2 mm. Oscillatory frequency sweep studies were performed for a range of 0.1–100 rad/s, using a 0.1% strain. Oscillatory amplitude sweep studies were conducted from 0.01 to 100% strain with an angular frequency of 1 rad/s. All measurements were done at 25°C, at 1d post gelation.

2.5. Determination of silver release by inductively coupled plasma mass spectrometry

Silver release studies were carried out in 24-well transwell plates (Corning Transwell® 6.5 mm, 8.0 µM with a polycarbonate membrane insert) using 100 µL of gel in the transwell and 400 µL of PBS solution in the peripheral well. For this

purpose, Ag NP containing gels were prepared in Tris buffer (100 mM, pH = 8.5, containing 10 mM of $AgNO_3$) and 100 µL of the resultant solution was added into the transwell. The gel was allowed to cure overnight, after which, the Ag NPs were formed by exposing the gels to UV light (254 nm for 5 mins). Then, the transwells were placed into the 24 well plate and 400 µL of PBS solution was added into the peripheral well. Samples were taken at different time points and digested with nitric acid (68% suprapur, Merck) in a 1:1 ratio by volume overnight. After dilution of the samples, the drug release was determined by ICP-MS analysis. At the end of the release study, the residual gels were dissolved in 400 µL PBS to determine the total drug concentration.

All ICP-MS analyses were performed on a Perkin Elmer Elan DRC II instrument and the ^{107}Ag isotope was used for quantification. Calibration was conducted using an external standard (Sigma-Aldrich) between 1-200 ppb. The concentrations of the samples were calculated based on the calibration curve plotted using Elan software, and was used to calculate the accumulative release profile as a percentage.

2.6. Bacterial growth inhibition assay

2.6.1. Preparation of Ac-LK_6-NH_2/Ag NP hydrogels for bacterial suspension assays

Gels were prepared in 24-well sterile Nunc plates. 10 mM $AgNO_3$ solution in 100 mM Tris-buffer (pH = 8.5) was freshly prepared and used to dissolve the peptide

to a final concentration of 10 mg/mL. The resultant solution was sonicated for 15-20 s to ensure complete dissolution of the peptide. 400 uL of the peptide solution was then aliquoted into each well and allowed to spread evenly on the surface. The Ac-LK_6-NH_2 concentration was chosen to allow a clear, solid hydrogel to form in a reasonable time-frame while allowing uniform aliquoting of the peptide solution into the wells of the plate. Although gelation of Ac-LK_6-NH_2 occurred within minutes in Tris-buffer at 10 mg/mL, the plates were left to stand overnight in the dark to ensure complete gelation. Prior to use, the plates were exposed to short-wavelength UV at 254 nm for 5 minutes to allow Ag NP formation. For the control gels, 100 mM Tris buffer without $AgNO_3$ was used for Ac-LK_6-NH_2 hydrogel preparation.

The concentration of silver was selected so that the desired killing efficiency could be achieved using as little material as possible. Ac-LK_6-NH_2 hydrogels with different concentrations of silver i.e. 1 mM, 10 mM, 25 mM and 50 mM were evaluated for their efficacy in killing bacteria via the disc diffusion method. It was found that compared to the 10 mM gels, the 25 mM and 50 mM gels had marginally higher efficiencies while using much higher amounts of silver. Therefore, the 10 mM concentration was considered optimal and used for subsequent *in vitro* evaluations.

2.6.2. Bacterial Suspension Assays

50 µL of bacterial stock was inoculated overnight in 5 mL of Luria-Bertani (LB) medium (pH = 7.5), with constant shaking at 300 rpm at 37^0C. The log-phase bacterial solution was then diluted in LB medium to obtain an O.D. of 0.07 (at 600 nm). This was used as the starting bacterial solution (0 hr reading) and 400 µL of the solution was dispensed into each well of the 24-well plate containing the hydrogels. Tetracycline antibiotic (Sigma Aldrich, USA), at a final concentration of 10 and 20 uM was used as the positive control. Ac-LK_6-NH_2 hydrogels without Ag NPs and bacteria-only solutions served as the negative controls. The plates were wrapped in aluminium foil and placed at 37°C under 50 rpm rotation in a shaker-incubator for 24 hrs. For the O.D. readings, 100 µL of the bacterial solution was transferred into a transparent 96 well Nunc plate just before measurement, to prevent the hydrogel layer from influencing absorbance measurements. The assay was performed in triplicates, with three replicates for each independent experiment.

2.6.3. Ring-cast method for preparing hydrogels

For rheometry and disc-diffusion experiments, a ring-cast method was used for hydrogel preparation to ensure uniform gel volume and facilitate easy transfer of the intact gel. After dissolving the peptide, 200-µL aliquots of the solution were immediately transferred into plastic ring casts of 9-mm diameter placed on clean 35-mm tissue-culture dishes. The top and bottom of each ring cast was covered with a layer of parafilm, and the tissue-culture dish was tightly sealed with

parafilm to prevent evaporation. The gels were allowed to cure overnight and NP formation was achieved using UV light for about 5 minutes.

2.6.4. Disc-Diffusion Method for evaluating anti-bacterial properties

150-µL of log-phase bacterial stock was uniformly spread on LB-Agar (1.5% agar) plates and incubated until a bacterial biofilm was formed. The hydrogel discs were placed on top of the film and zones of inhibition/killing were measured at 24 hours. The diameter of the zone of inhibition was measured using a pair of vernier calipers. All experiments were performed in triplicates.

2.7. Cell Culture

Human Dermal Fibroblasts – Adult (HDFa) were purchased from Lonza. The cells were cultured in MEM with 2mM L-glutamine and Earle's salts (500 mL) supplemented with10% heat inactivated fetal bovine serum, 1% of a mixture of penicillin and streptomycin (Invitrogen, CA, U.S.A), Sodium bicarbonate (1.5 g/L final concentration), NEAA (5mL of 100X stock) and Sodium pyruvate (5 mL of 100 mM stock). The cells were maintained ether in a T175 or T75 cell culture flask (Nunc) at 37 °C in a humidified incubator with 95% air and 5% CO_2.

2.7.1. Biocompatibility Study

Biocompatibility studies were carried out in 24-well transwell plates (Corning Transwell® 6.5 mm, 8.0 µM with a polycarbonate membrane insert) using 100 µL

of gel in the transwell and 400 µL of medium in the peripheral well. Cell viability was measured after 24 h and 48 h of incubation.

HDFa cells (12,000 cells/well) were seeded into a 24-well plate and incubated for 24 h in 400 µL medium. In the interim, 100 µL hydrogel-containing transwells were prepared. For this purpose, Ac-LK_6-NH_2 was weighed out, and tris buffer (100 mM, pH = 7.5) containing 10 mM $AgNO_3$ was added to obtain a total concentration of 10 mg/mL. 100 µL of the prepared solution was added into the transwell and allowed to gel for 4 h at 37 °C. As a control Ac-LK_6-NH_2 hydrogels were prepared the same way, but without $AgNO_3$. For the positive controls, cells without any hydrogel, cells with transwells containing 100 µL of 1% agar gels and cells with empty transwells were used. Prior to inserting the hydrogel containing transwells into the cell culture plates, Ag NPs were formed using UV light. In order to keep the samples sterile, the UV light of the laminar flow cabinet was used, and exposure time was increased to 10 minutes. Subsequently, the transwells were placed on top of the cells growing on the plates and incubated for 24 hours. Cell viability was determined by means of a colorimetric microculture assay (WST-1, Roche). The transwells were taken out of the plate, the medium was carefully removed and fresh medium containing 10% WST-1 reagent was added. After 2 h of incubation at 37 °C, 90 µL of the supernatant solution was transferred onto a 96-well plate and absorption at 450 nm was measured on a plate reader (Infinite M200, Tecan). To determine the toxicity after 48 h of incubation with the hydrogels, the WST-1 containing solution was

removed from the cells, and fresh medium was added. After 1 h incubation, the medium was replaced again and the transwells were added back. After another 24 h incubation (total 48 h incubation) the cell viability was determined as described above. With the exception of the 1% agar control and the empty transwell, all experiments were carried out in independent triplicates. Controls containing 1% agar and the empty transwell were carried out in independent duplicates with at least four samples per duplicate. The biocompatibility is expressed as a percentage of survival relative to the untreated control.

2.7.2. Live/Dead assay

HDFa cells were seeded in 24-well plates (12,000 cells/well) and incubated for 24 h. Afterwards, the cells were exposed for 48 h to Ag-Ac-LK_6-NH_2 hydrogel samples prepared in the transwell. Ac-LK_6-NH_2 hydrogel and untreated cells were used as controls. Incubation was done in transwell plates as described for the biocompatibility studies. After 48 h, the transwell was removed, the medium replaced with DPBS solution containing approximately 2 µM calcein AM and 4 µM ethidium homodimer-1 (LIVE/DEAD® Viability/Cytotoxicity Kit, Life Technologies™) and incubated for 40 minutes. Just before imaging, the media solution was removed for greater clarity. Live cells were imaged in the green channel and dead cells in the red channel. The obtained pictures were superimposed using ImageJ.

3. Results

3.1. Formation of hybrid hydrogels

Silver nanoparticle containing hydrogels (Ag-Ac-LK_6-NH_2) were formed by the addition of $AgNO_3$ solution to Ac-LIVAGK-NH_2 at a basic pH of 8.5 and exposing the hydrogels to UV radiation at 254 nm for several minutes after gelation. The rate of gelation was not significantly affected by the concentration of $AgNO_3$ solution used. Higher peptide concentrations required shorter gelation periods i.e. 15 mg/mL Ag-Ac-LK_6-NH_2 took approximately only 1 minute for gelation as compared to 30 minutes for 10 mg/mL Ag-Ac-LK_6-NH_2. Subsequent nanoparticle formation was achieved by irradiation of the samples with UV light for several minutes. A colour change (from colourless to brown) was observed for hydrogels containing higher silver nanoparticle concentration (Figure 1a). Addinitally, hydrogels containing silver nanoparticles retained the shape better (Figure 1 b) than hydrogels without silver nanoparticles.

During the process of Ag NP formation, we observed that the mechanical properties of the gels changed. To quantify this observation, differences in mechanical properties between the hydrogels with and without silver nanoparticles, and also between hydrogels of different peptide concentrations were studied using oscillatory rheometry. Frequency sweep was performed from 0.1-100 rad/sec at 0.1% strain. As depicted in Figure 2, a significant difference in

the storage modulus between samples with and without Ag NPs can be observed.

3.2. TEM images of silver nanoparticles synthesised in situ

TEM images of Ag-Ac-LK_6-NH_2 hydrogels formed using 10 mM and 20 mM $AgNO_3$ solution in Milli-Q were taken after freeze drying the samples. As seen in Figure 3a and 3c a uniform distribution of the silver nanoparticles could be achieved by reduction of silver ions with UV light. In addition, the controlled reduction with UV light leads to a uniform size distribution of the Ag NP of the particles at around 10-20 nm which can be seen in Figure 3a and 3c as well as in the high resolution immages in Figure 3b and 3d. Monodisperse NPs with a size range of 10-20 nm are ideal to achieve an efficient anti-bacterial activity [12]. In addition, monodisperse Ag NP were observed even at higher concentrations, whereby the higher concentration of Ag^+ ions only result in a higher number of silver nanoparticles but not in lager particles.

3.3. Silver release properties of composite ultrashort peptide hydrogels

Silver release from Ag-Ac-LK_6-NH_2 hydrogels was studied in transwell plates. 100 uL of Ag-Ac-LK_6-NH_2 hydrogels in tris buffer (pH = 8.4) were immersed in PBS (pH 7.4) for up to 14 days with periodic sampling of the Ag concentration in the PBS solution. Figuer 4 shows the cumulative release of silver from the hydrogels. A burst release could be observed within the first 48 h of the study

which accounts for almost 50 % of the total released Ag NPs. However, a sustained release could be observed for as long as 14 days.

3.4. Anti-microbial activity of composite ultrashort peptide hydrogels

The anti-microbial activity of Ag-Ac-LK_6-NH_2 hydrogels was investigated in three different bacteria strains, *E.coli, P. aeruginosa* and *S. aureus* respectively by the disc diffiusion method and in suspention culture. In an initial screening, Ag-Ac-LK_6-NH_2 hydrogels discs (200 µL per disc) with different concentrations of sliver ions ranging from 1 to 100 mM were placed on top of an *E.coli* covert agar plate and the increase in diameter was measured after 24 h. As expected, a concentration depending increase in diameter was observed, however, we found that 10 mM of silver was sufficient to show a significant killing effect noticeable by the increase of the diameter. Keeping in mind that that silver nanoparticles are potential toxic the use of a low concentration is preferred. Based on this initial screening all further biological studies were carried out using a silver concentration of 10 mM.

Disc diffusion test were conducted in independent triplicates using *E.coli, P. aeruginosa* and *S. aureus* covered agar plates. The hydrogels were placed on top of the bacterial lawn and incubated at 37 °C for 24 h. After which the increase of diameter was measured with a vernier caliper and compared to Ac-LK_6-NH_2 hydrogels disc without Ag NP (Figure 5b). As shown in Figure 5a. a clear circle

was formed around the Ag-Ac-LK_6-NH_2 hydrogels disc after 24 h whereas no killing was found for the hydrogels without Ag NP. When compared the killing efficiency of Ag NP based hydrogels between *E.coli, P. aeruginosa* and *S. aureus* it is suprising to note, that the highest efficiency, largest diameter, was observed for *P. aeruginosa* a bacteria strain know to be resistant to common antibiotics, whereas *E.coliI* showed the smallest increase in diameter. None of the bacteria strained tested was killed by the Ac-LK_6-NH_2 hydrogels controls, and thus the used Tris buffer seem to have no influence at the bacteria.

To assess the bacteria growth inhibition in suspension OD measurements were carried out. For this purpose 24 well plates were coated with 400 µL Ag-Ac-LK_6-NH_2 hydrogels and cured overnight. At the same time Ac-LK_6-NH_2 hydrogels without silver were prepared as control. After the hydrogels were subjected to UV light to form the Ag NP 400 µL bacteria solution with an initial OD value of around 0.07 was added ontop of the hydrogels. Tetracycline antibiotic, at a final concentration of 10 and 20 µg/mL was used as the positive control. Ac-LK_6-NH_2 hydrogels without Ag NPs and bacteria-only solutions served as the negative controls. After 24 h incubation the final OD value was determined and the results of the measurements can be found in Figure 6. In all three bacteria samples an OD smaller than the starting value was found for the samples treated with Ag-Ac-LK_6-NH_2 hydrogels, showing, that Ag-Ac-LK_6-NH_2 hydrogel are not only able to inhibit bacteria growth but also able to kill bacteria. In comparison, Ac-LK_6-NH_2 hydrogels alone only showed a small growth inhibition when compared to the untreated bacteria samples. Interestingly, the tetracycline controls showed similar

activity as Ag-Ac-LK_6-NH_2 hydrogels in *E. coli* but were not as effective in *S. aureus*. In *P. aeruginosa* the largest different between Ag-Ac-LK_6-NH_2 hydrogels and the tracycline control was observed, where tetracycline only showed about 50% growth inhibition compared to the untreated control, whereas Ag-Ac-LK_6-NH_2 hydrogel was able to reduce the starting OD value.

3.5. Biocompatibility

Biocompatibility studies were carried out in in 24-well transwell plates using 100 µL of gel in the transwell and 400 µL of medium in the peripheral well. Cell viability was measured after 24 h and 48 h of incubation. Ac-LK_6-NH_2 hydrogel without silver, 1 % agar, empty transwell and untreated cells were used as control and survival is presented as percentage normalized to the untreated cells (Figure 7a).

After 24 h incubation a reduction in viability of about 20 % was found for all samples containing a transwell with hydrogels, independent of gel type in comparison to untreated cells. A similar result was found after 48 h incubation where all samples containing a hydrogel in the transwell showed a 20 % reduced viability compared to the untreated cells. However, it has to be pointed out, that Ag-Ac-LK_6-NH_2 hydrogel sampled showed the similar reduced viability as all other hydrogels containing control samples. In order to verify, that reduced cell viability is due to cell death live dead images were recorded after 48 h of incubation (Figure 7b-d). The superimposing of the fluorescence images did not show an increased amount of dead cells of the hydrogels samples when

compared to the untreated control, which indicates, that the reduced viability is not due to the toxicity of the gels but rather due to a change in environment (transwell vs. no transwell).

4. Discussion

In this study, we have explored the possibility of adding antibacterial properties to ultrashort peptide hydrogels by incorporating *in situ* formed silver nanoparticles. The porous character of the fibrous peptide hydrogel and the non-reactivity of the functional groups of the peptide chains support a controlled *in situ* formation of the silver nanoparticles. Furthermore, the easy way of silver nanoparticle formation gives advantages over previous reported systems. Unlike other reported antibacterial hydrogels, such as porous chitosan blended with PEG[18] and electrospun gelatin fiber mats [19], Ag-Ac-LK_6-NH_2 hydrogels do not require additional chemical modification.

The successful synthesis of monodispersed Ag NP was confirmed by TEM microscopy showing an average size distribution of 10-20 nm (Figure 3a-d) and a homogenous distribution throughout the measured samples. The same holds true for sample measured before (Figure 3e) and after expose to bacterials (Figuer 3f) The synthesis of small, monodispersed nanoparticles is crucial for a material with antibacterial properties. Recent studies by Martínez-Gutierrez *et al*. have shown that the ideal size for antibacterial Ag NP is below 25 nm. We are well within this range. Interestingly, we observed, that the hydrogel and the Ag

NPs show a cooperative effect in terms of the mechanical properties of the hydrogel compared to a gel without Ag NP (Figure 2). Rheology studies showed that gels containing 10 mM of Ag NP have a storage modulus almost twice as large as samples of the same concentration but without Ag NP. Similar to observations with biomineralization of hydrogels with the presence of salt crystals, the presence of silver nanoparticles within the gel lead to an substantial increase of mechanical stiffness. A sustained release was observed for up to 14 days, whereby a faster release is present in the first 48 h. The release of Ag NP is crucial for the ability of the new material in order to act as an antibacterial agent. Only when Ag NP is released into the surrounding of the hydrogel, an effective bacterial treatment will be observed. Furthermore, the faster release of Ag NP in the beginning delivers a higher local concentration of the antibacterial agent, helping to quickly reduce inflammation caused by bacterial infection. Additionally, a sustained release over the following 12 days helps to prevent reinfection.

While sustained release of silver nanoparticles over 2 weeks has been reported by Messersmith *et al*., the water-soluble polyethylene glycol (PEG) polymers containing reactive catechol moieties were able to release approximately only 1% of the total amount of silver inside the hydrogel disks [13]. In contrast, our Ag-Ac-LK_6-NH_2 hydrogels (100 µL) are able to release up to 90 % of the *in situ* synthesised silver nanoparticles while using a much lower concentration as previous reported systems.

The ability of the new material to inhibit bacteria growth was tested using two different methods, the disc diffusion method and the suspension method. Both methods showed a similar trend with regards to growth inhibition, and thus confirmed each other's result. It is very interesting to note, that the Ag NP were most effective against *P. aeruginosa,* a bacteria strain known for its drug resistance, which can be seen by the reduced activity of tetracycline control in *P. aeruginosa* compared to *S. aureus* and *E. coli.* For both methods a killing of *P. aeruginosa* was observed, indicated by a clear circular surrounding zone when using the disc diffusion method. Also, a reduction in the starting optical density (OD) was seen using the suspension method (Figure 6). Taking into account, that *P. aeruginosa* is very hard to treat and thus can lead to severe health problems upon infection, the development of efficient antimicrobial compounds is a highly important issue. Based on the obtained bacterial inhibition data, Ag-Ac-LK_6-NH_2 could offer new valuable new opportunities in the treatment of wound infections. This would overcome the need of oral or intravenous application of traditional antibiotics and could help to reduce health cost.

To characterize, if the newly developed biomaterial showed solely bacterial killing efficacy but no mammalian cell toxicity, the biocompatibility was tested *in vitro.* For this purpose, primary HDFa cells were used and incubated with the Ag-Ac-LK_6-NH_2 in a transwell system. This experimental set-up demonstrated that the cells are exposed to the Ag NP. We propose our Ag NP hydrogel as an efficient wound dressing device, where the Ag NPs are released to the wound. The result of the biocompatibility study is shown in Figure 7, showing cell viability

after 24 and 48 h. At both time points, a reduction in the cell viability was observed by about 20 %. However, live/dead assays did not show a significant number of dead cells when compared with the untreated control (Figure 7b-d). Using 1% agar gels as a control, we observed that the agar control almost matched the values obtained by the 1% peptide hydrogels. When an empty transwell was placed used, a reduced viability was observed after 24 and 48 h. However, even the empty transwell showed about 10 % reduction in cell viability after 48 h of incubation (Figure 7a). These results let us to the conclusion, that the transwell set-up changes the cell environment and thus influences the cell proliferation rate, but not the viability which can be seen from the live dead assay analysis. Most likely, nutritional factors from the media are sustained in the gel, agar or transwell membrane matrix, leading to a reduced cell proliferation. Given these results, Ag-Ac-LK_6-NH_2 hydrogels appear to be able to efficiently kill bacteria without significantly altering the cell viability.

5. Conclusions

Anti-bacterial hydrogels containing *in situ* formed silver nanoparticles were developed using ultrashort self-assembling peptides and silver nitrate. The conversion of silver nitrate to silver nanoparticles needed only the presence of UV light. The *in situ* Ag NP formation was accomplished after hydrogel formation, enabling with the porous hydrogel structure a controlled particle size after reduction of Ag(I) to Ag NP. This method allows the formation of monodispersed Ag NP without the addition of an external reducing agent. The Ag NP formation

was characterized by TEM, demonstrating that monodispersed particles with less than 20 nm could be formed. The nanoparticles were also uniformly distributed within the measured sample. The Ag NP containing hydrogel showed increased mechanical strength when compared to the peptide hydrogel alone. We conclude that the presence of silver nanoparticles within the soft matter hydrogel results in an increase in the mechanical stiffness of the hydrogel. Anti-bacterial tests were conducted using three bacterial strains namely, *E.coli, P. aeruginosa* and *S. aureus*. Highly efficient bacterial growth inhibition was observed for all three bacteria strains, using as little as 10 mM silver nanoparticle hydrogels. This is significantly less amount than what was reported with other systems. It is important to note, that the best results were obtained for *P. aeruginosa* which is known for its multidrug resistance and thus very difficult to treat in patients. Biocompatibility studies on HDFa cells showed that the novel biomaterial does not significantly influence the cell viability. The silver concentration is obviously within the therapeutic window, but low enough to not significantly affect mammalian cell viability. We believe that this novel silver-releasing biomaterial offers great potential for application in wound healing, in particular after surgery, in treatment of chronic and large surface wounds, like diabetic ulcer or severe burns, due to its potential to reduce inflammation and its ease of application.

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Figure Caption

Figure 1. Hydrogels synthesised with $AgNO_3$ solutions. (A) Ag+ ions present in $AgNO_3$ solution were reduced to Ag with UV radiation, resulting in colour change from colourless to brown in the hydrogels. (B) Peptide was pipetted into gel cast, allowed to gel and then irradiated with UV.

Figure 2. Rheological characterization of ultrashort peptide hydrogels with and without silver nanoparticles at 10 and 15 mg/mL. Frequency sweep was performed at 0.1% strain.

Figure 3. TEM images of the silver nanoparticles distribution in Ag-Ac-LK_6-NH_2 hydrogels formed in water using 10 mM (A, B) and 20 mM (C, D) $AgNO_3$ solution and a 10 mM solution in Tris buffer at pH = 8.4 before (E) and after (F) incubation with bacteria.

Figure 4 Cumulative release of silver from 100 uL of Ag-Ac-LK_6-NH_2 hydrogels as a function of time.

Figure 5. Assessment of anti-microbial properties of Ag-Ac-LK_6-NH_2 hydrogels by disk diffusion (a) comparison of Ag-Ac-LK_6-NH_2 hydrogels and Ac-LK_6-NH_2 hydrogels on an agar plate covert with *P. aeuginosa* (b) graphical presentation of the resulting radius measurement in comparison with Ac- LK_6-NH_2 hydrogels alone.

Figure 6. Graphical presentation of the OD measurements in *E. coli*, *P. aeruginosa* and *S. aureus* at t = 0 and t = 24 h.

Figure 7. A) Graphical presentation of the biocompatibility normalized to untreated cells; live dead image of cells treated with Ag-Ac-LK_6-NH_2 (B), Ac-LK_6-NH_2 (C), or untreated cells (D) respectively.

Figure 1

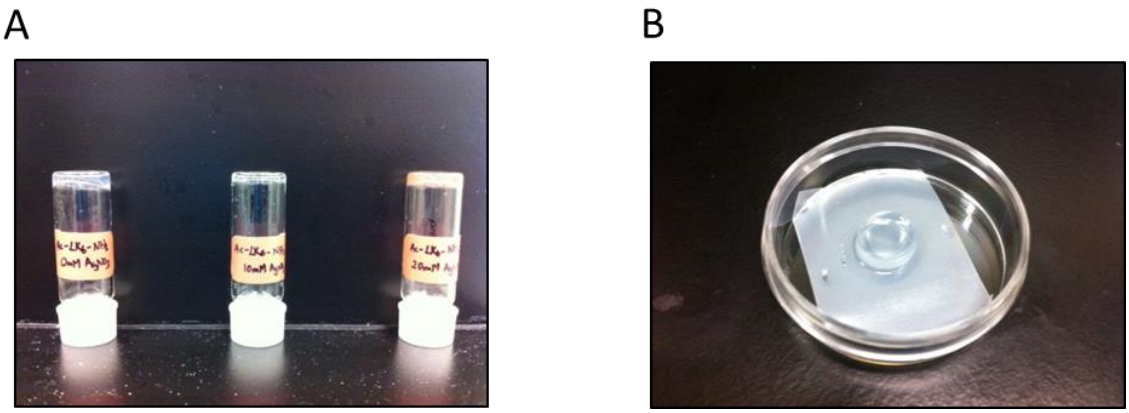

Figure 2

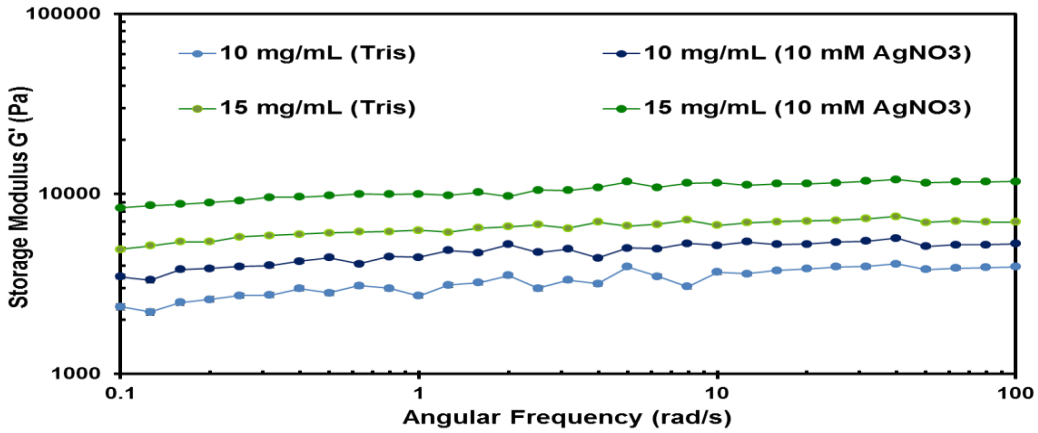

Figure 3

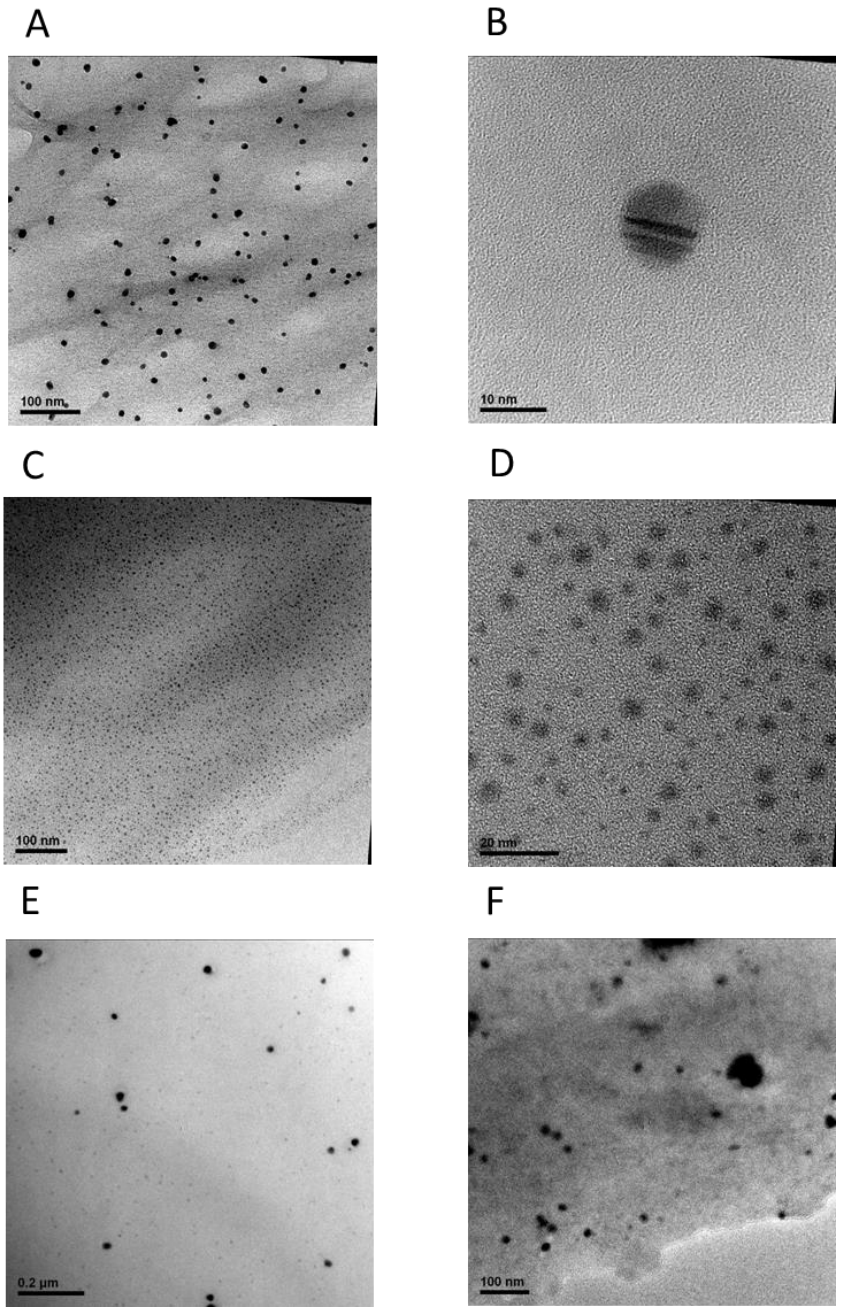

Figure 4

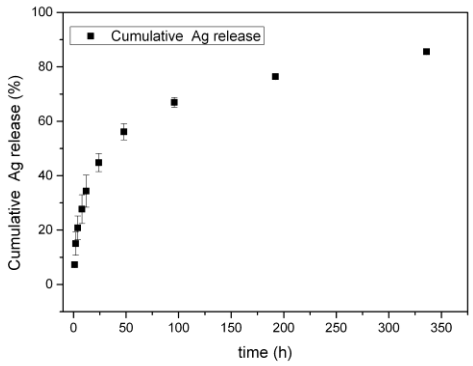

Figure 5

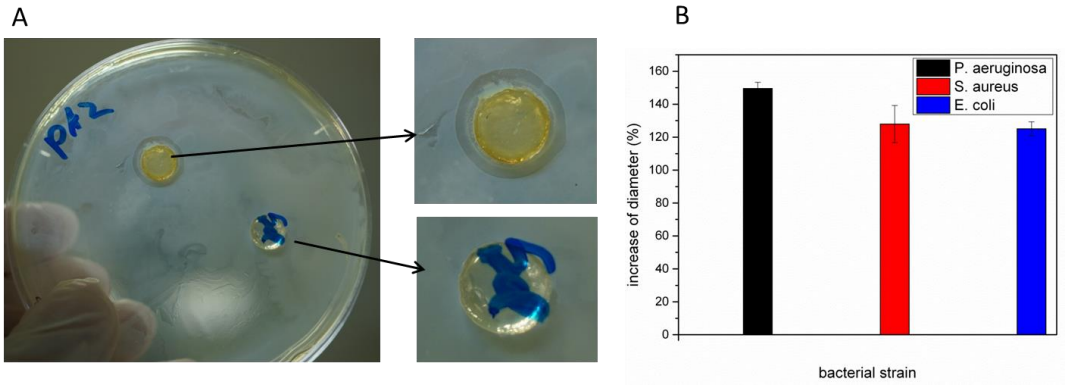

Figure 6

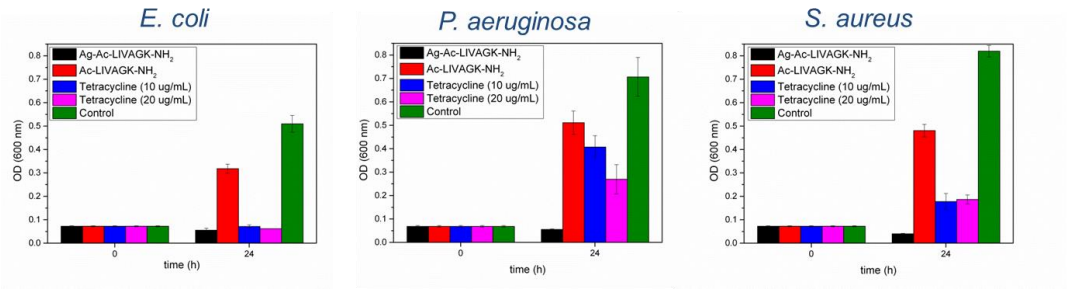

Figure 7