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In *situ* synthesis of size-controlled, stable silver nanoparticles within ultrashort peptide hydrogels and their anti-bacterial properties

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ABSTRACT

We have developed a silver-releasing biomaterial with promising potential for wound healing applications. The 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 precise control of the nanoparticle size, with the network of peptide fibers preventing aggregation of Ag NPs. 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 anti-bacterial therapy. The ability to inhibit bacterial growth was tested using different bacterial strains, namely gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* and gram-positive *Staphylococcus aureus*. Inhibition of bacterial growth was observed for all strains. The best results were obtained for *Pseudomonas 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 silver-releasing hydrogel as an excellent biomaterial with great potential for applications in wound healing due to its low silver content, sustained silver nanoparticle release and biocompatibility.

1. Introduction

We have previously introduced a class of ultrashort peptides, containing 3–7 amino acids, with an innate capacity for self-assembly. The peptide monomers form helical fibers that entangle in 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 materials [3]. However, earlier attempts at physically entrapping a bioactive compound within the self-assembling peptide hydrogel resulted in a burst release – a sustained release was not achieved. Hence, the goal of this study was to develop an organic-inorganic composite system developed *in situ*, 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 anti-bacterial activity has significantly increased. In particular, topical formulations have been explored for the prevention of infections associated with large wounds and biofilm formation in biomedical devices and implants. Due to the increase in antibiotic-resistant bacteria, a lot of effort is being put into developing biomaterials containing anti-bacterial 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-bacterial activity against as many as twelve gram-positive and gram-negative bacteria [7]. Further, the existence of multiple cellular targets within the bacteria lowers the propensity of bacterial resistance evolving in response 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 commercially available [7–9]. In this context, it should be noted that a recent study compared the anti-bacterial

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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 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 composite biomaterial were examined by transmission electron microscopy (TEM) and rheology and its anti-bacterial properties were evaluated. In addition, biocompatibility studies were performed using human primary dermal fibroblasts for evaluating suitability of the composite biomaterial for wound healing applications.

2. Materials and methods

2.1. Material for peptide synthesis and purification

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) were 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. Milli-Q 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 imaging.

2.3. Peptide synthesis and purification

Ac-LK_6-NH_2 peptide was synthesized 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 equivalents of Ac_2O and DIPEA were added to prevent reaction of the side-groups, and the mixture was allowed to incubate for 45 min. The resin was then washed with DMF before going through a series of deprotection 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 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 peptide was introduced into the HPLC-MS system by completely dissolving it 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.

2.4. Hydrogel preparation

All solutions needed 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 $AgNO_3$ solutions, using Tris buffer to weigh 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 min using a UV-lamp.

2.5. Oscillatory rheometry

Viscoelasticity of the peptide hydrogel was measured using an ARES-G2 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.

2.6. Determination of silver release by inductively coupled plasma mass spectrometry (ICP-MS)

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 NPs containing gels were prepared in Tris buffer (100 mM, pH = 8.5, containing 10 mM of $AgNO_3$) and 100 μL of the resultant solutions were added into the transwells. The gels were allowed to cure overnight, after which, the Ag NPs were formed by exposing the gels to UV light (254 nm for 5 min). 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 and 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.

2.7. Bacterial growth inhibition assay

2.7.1. 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 min to allow Ag NPs 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 inhibiting bacterial growth 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.