ANTIVIRAL ACTIVITY OF FULLERENES MODIFIED WITH MAXIMIN H5 DERIVATIVES

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Abstract: The properties of various peptides, including their antiviral or antibacterial activity, are highly dependent on their sequence. Maximin and maximin H peptides can be abundantly found in the skin and brain of Asian frog *Bombina maxima*, enabling it to fight with the frequent exposure to various microbes. Derivatives of 20 amino acid long maximin H5 also show antiviral activity. In this work, 6 derivatives of maximin H5 were prepared, with aspartic acid at position 11 exchanged for alanine, asparagine, glycine, histidine, valine or tyrosine. Their antiviral and antimicrobial activity was measured using plaque assay or growth curves method, respectively. To increase these properties, the peptides were bound to C_{60} fullerenes, whose surface was activated using either nitric or trimesic acid. As model organisms, bacteriophage λ and its host bacteria *Escherichia coli* were used. The mutation of maximin H5 sequence significantly increased its antiviral activity. Maximin H5 derivatives with aspartic acid exchanged for asparagine, valine or tyrosine had the highest antiviral activity, further increased when bound on the surface of fullerenes activated with trimesic acid.

Key Words: antiviral peptides; carbon nanocarriers; plaque assay

INTRODUCTION

Nanomaterials can be used as nanocarriers, enabling the decrease of amount of administered drug and thus their negative side effects (Park 2013). They also allow to dissolve otherwise water-insoluble drugs, enhance their efficiency, biocompatibility or storage lifetime (Gu et al. 2007, Chomoucka et al. 2010). Materials that can be used to prepare nanocarriers are abundant – lipids, polymers, proteins, metals, metalloids, non-metals or carbon (Dunk et al. 2012, Peer et al. 2007). The advantage of inorganic nanocarriers is their easy preparation. Fullerenes, self-assembly cages of 30 to hundreds carbon atoms, are one of the possible carbon nanocarriers (Yamamoto et al. 2012). They can bind various drugs, such as antiviral (Shetti et al. 2012) or antibacterial (Tollas et al. 2012) agents or chemotherapeutics (Blazkova et al. 2014), especially after activation of their surface with carboxylic groups (Heister et al. 2009).

Viral infections are a severe problem in countries all over the world, often without any effective treatment (Turner 2014). Therefore, novel substances with possible antiviral properties are often studied. Many substances with antiviral or antibacterial activity can be found in naturally occurring plants or animals (Epand andVogel 1999), most often amphibians due to the frequent exposure of amphibian skin to very various microbes (Clarke 1997, Liu et al. 2011).

Asian toad *Bombina maxima* produces a very large number of peptides, called maximins or maximins H (Ortega et al. 2012), mostly containing cationic and hydrophobic amino acids providing these peptides with antibacterial properties by allowing them to selectively interact with bacterial membranes. Maximin H5 is a 20 amino acid-long peptide containing 3 anionic aspartic acids in its structure at positions 11, 14 and 15. Due to this, maximin H5 only exhibits antibacterial properties

towards gram-positive bacteria (Lai et al. 2002). However, basic derivatives of maximin H5 showed promising antiviral properties (Wang et al. 2010).

In this work, we proposed a novel nanocarrier with antiviral properties, based on fullerenes modified with 6 different derivatives of maximin H5 with aspartic acid at position 11 exchanged for alanine, asparagine, glycine, histidine, valine and tyrosine.

MATERIAL AND METHODS

Preparation of activated fullerenes

2 mg of fullerenes (C_{60}) was mixed with 0.5 mL of concentrated HNO_3 or 4 mg of trimesic acid in 0.5 mL of water and sonicated by using ultrasonic bath (Bandelin, Berlin, Germany) for 15 min. To dissolve the fullerenes properly, an additional mixing was carried out using a Thermo-mixer (Eppendorf, Hamburg, Germany) for 15 min at 90°C, 800 rpm. Then the sample was centrifuged at 25000 g at 20°C for 10 min using a table top centrifuge machine (Eppendorf, Hamburg, Germany). The supernatant was discarded and the fullerenes were washed 6–7 times by centrifugation (25000 g at 20°C for 10 min) with MiliQ water until the pH became 7. Finally, the volume was made up to 2 mL using MiliQ water. The final concentration of fullerenes was 1 $mg \cdot mL^{-1}$.

Characterization of fullerene size and composition

The average particle size and size distribution were determined by quasi-elastic laser light scattering with a Malvern Zetasizer (NANO-ZS, Malvern Instruments Ltd., Worcestershire, United Kingdom). The zeta potential was determined by laser Doppler micro-electrophoresis with a Malvern Zetasizer. Nanoparticles in 1.5 mL solution containing 0.5% peptone and 0.3% meat extract were put into a polystyrene latex cell and measured at a detector angle of 173°, a wavelength of 633 nm, temperature 25°C, refractive index of 0.30, and a real refractive index of 2.2.

The elemental analyses were carried by CHNS organic elemental analyser Flash 2000 (Thermo-Fisher Scientific Inc., Waltham, MA, USA). 2 mg of fullerenes modified with trimesic acid or oxidized with nitric acid in solid state were placed into soft tin containers and burned in furnace at 950 ºC.

The mass spectrometry experiments were performed on a MALDI-TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Bremen, Germany). For MALDI-TOF characterization of fullerenes the reflector positive mode in a mass range 400–6000 Da was used. Fullerenes were measured both with and without matrix. To prepare matrix, 2,5-dihydroxyacetophenone (15.2 $mg \cdot mL^{-1}$) was dissolved in 60% ethanol, containing 1.1 $mg \cdot mL^{-1}$ diammonium hydrogen citrate. Samples for MALDI-TOF were prepared following the dried-droplet method: the solutions of fullerenes for analysis were the mixed with matrix solution in a ratio of 1:1 v/v. After being homogeneous, 1 µL of the solution was applied on the MTP 384 polished steel target plate and dried under atmospheric pressure at 20°C. When matrix was not used, 1 µL of the fullerenes solution was applied directly on the target. Finally, the mass spectra were made from 6000 laser shots within different regions of a sample spot. The laser power was set to 35%.

Synthesis and analysis of maximin peptides

Six different derivatives of maximin H5 peptide were prepared with substitutions of aspartic acid on the position 11 for different amino acids - asparagine, glycine, histidine, alanine, valine and tyrosine. For synthesis, Liberty Blue peptide synthesizer was used (CEM, Matthews, NC, USA). The sequences and monoisotopic molecular weights of synthesized peptides were as follows: maximin H5: ILGPVLGLVSDTLDDVLGIL – 2021.17 Da; maximin H5A: ILGPVLGLVSATLDDVLGIL – 1977.18 Da; maximin H5G: ILGPVLGLVSGTLDDVLGIL – 1963.16 Da; maximin H5H: ILGPVLGLVSHTLDDVLGIL – 2043.20 Da; maximin H5N: ILGPVLGLVSNTLDDVLGIL – 2020.18 Da; maximin H5V: ILGPVLGLVSVTLDDVLGIL – 2005.21 Da; maximin H5Y: ILGPVLGLVSYTLDDVLGIL – 2069.20 Da.

Deblocking of Fmoc protecting group was performed with 20% piperidine v/v in N,N-dimethylformamide (DMF). Coupling was achieved using N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate, N,N-diisopropylethylamine and DMF. Cleavage of side chain

protecting groups was performed by treating the peptides resin with 95% trifluoroacetic acid v/v, 2.5% H_2O v/v and 2.5% triisopropylsilane v/v for 30 minutes at 38°C under microwave irradiation.

To predict the secondary structures of the peptides, software PEP-FOLD (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/) was used. DHB was used as a matrix for MALDI-TOF MS analysis of maximin peptides. The saturated matrix solution was prepared in 30% acetonitrile and 0.1% TFA. The mixture was thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin electronic, Berlin, Germany) for two minutes at 50% of intensity and 20°C. The sample deposition method was same as in the case of fullerenes. A mixture of peptide calibration standards was used to externally calibrate the instrument. All measurements were performed in the reflector positive mode in the m/z range 0–8000 Da. The mass spectra were typically acquired by averaging 2500 subspectra from a total of 2500 shots of the laser with laser power set to 5–10% above the threshold.

Modification of fullerenes with peptides

Different derivatives of maximin H5 peptide were used in this experiment to check their antiviral properties. 0.5 mg of these peptides were dissolved in 100 µL of DMF by mixing using a programmable rotator-mixer Multi RS-60 (Biosan, Riga, Latvia) at 600 rpm and 20°C for 1 h. 200 µL of the fullerenes was added to the dissolved peptides and mixed for 24 h using rotator. Then they were filtered by centrifugation (6000 g at 20°C for 15 min) using Amicon Ultra 3K Centrifugal Filters (Merck Millipore Ltd., Darmstadt, Germany). The products were washed three times with MiliQ water by centrifugation. The final volumes of these products were made up to 1 mL with MiliQ water.

The amount of the peptide, bound on fullerenes, was determined by derivatization with fluorescamine. 50 µL of the peptide-modified fullerenes was mixed with 30 µL of 1 mM fluorescamine on a microtiter plate and incubated for 5 min at 20°C. The fluorescence of bound fluorescamine was measured using microplate reader Infinite 200 PRO (Tecan Group Ltd., Männedorf, Switzerland) with excitation at 390 nm and emission from 420 nm to 850 nm.

Assessment of antiviral activity by plaque assay

Producing *Escherichia coli* was inoculated into LB broth and cultivated for 24 h at 37°C and 600 rpm using Incubator Hood TH 15 (Edmund Bühler GmbH, Hechingen, Germany). After the cultivation, *Escherichia coli* was inoculated by 5 punctures into LB bottom agar on each Petri dish and cultivated for 24 h at 37°C in Incubator MIR-162 (Sanyo Electric Co., Ltd., Osaka, Japan). At the same time, indicator *Escherichia coli* was inoculated into LB broth and cultivated for 24 h at 37°C and 600 rpm. After cultivation, producing *Escherichia coli* on Petri dish was killed by the exposure to chloroform fumes for 30 min. Bacteriophage λ on Petri dish was covered with 3 mL of soft agar, 1 mL of indicator *Escherichia coli* and 0.25 mL of peptide-modified fullerenes. PBS served as a control. The growth of bacteriophage λ was induced with UV light to enter thy lytic replication state

The antiviral activity of peptide-modified fullerenes was checked after 16 h of cultivation by counting of observed bacteriophage λ plaques. The results were relatively compared to control, designated as 100%. They were also recalculated relatively to the amount of peptide bound on the surface of fullerenes.

RESULTS AND DISCUSSION

Characterization of components

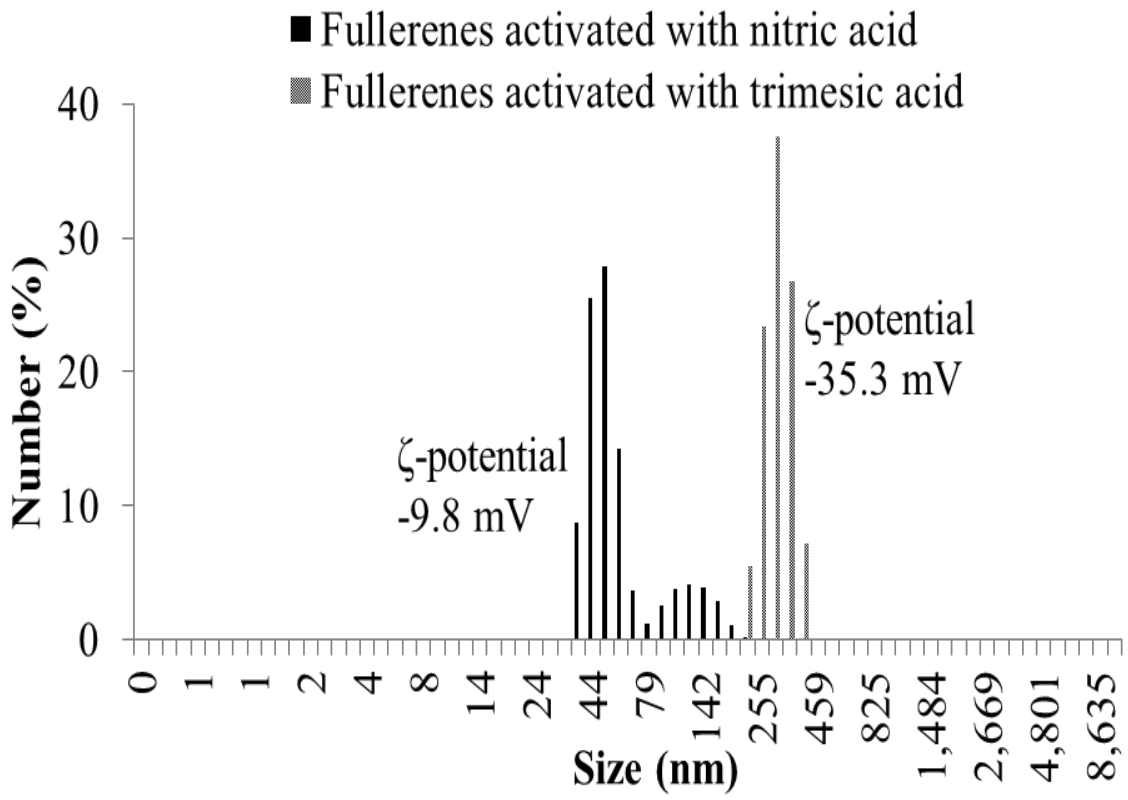
In this work, we activated the surface of fullerenes C_{60} using two approaches – nitric or trimesic acid. Nitric acid can oxidate the surface of carbon nanostructures, but the oxidation rate is usually very low (Blazkova et al. 2014, Heister et al. 2009). Molecules containing aromatic rings can also be non-covalently bound to the surface of fullerenes via π- π stacking interactions (Chen et al. 1998, Sawamura et al. 2002).

The purification of excess nitric or trimesic acid molecules was performed by centrifugation. Next, the size distribution, zeta potential (see Figure 1) and the elemental analysis were performed. The average size and zeta potential of fullerenes activated with nitric acid were 50 nm and -18.6 mV, respectively. However, the size distribution of these fullerenes was not uniform, large number

of particles was also around the size of 122 nm (4%). The fullerenes activated with trimesic acid had an average size of 295 nm and zeta potential of -22.7 mV with uniform size distribution. The more negative zeta potential was probably caused by the carboxyl groups from trimesic acid molecules on the fullerene surface (Chen et al. 1998).

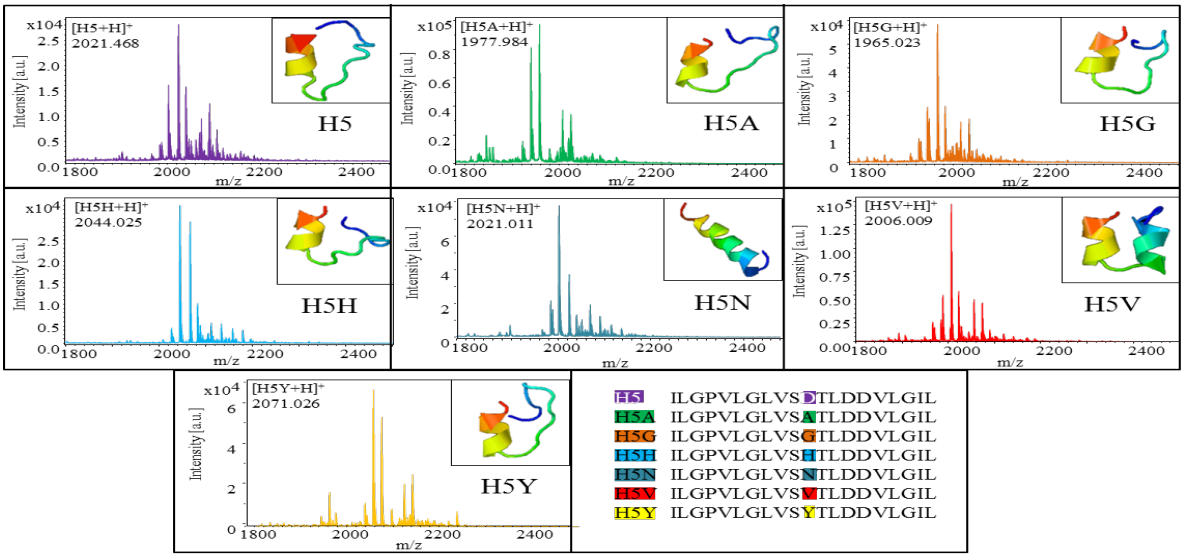
Elemental analysis of activated fullerenes with calculated oxygen ratio was performed to confirm these results (data not shown). Fullerenes activated with nitric acid (in fullerene:nitric acid ratio 1:1) contained a very low amount of elements other than carbon (0.08% of hydrogen and 99.92% of carbon). Fullerenes activated with trimesic acid in fullerene:trimesic acid ratio 1:1 contained the similar amount of hydrogen (0.05%) but the amount of carbon was lower (92.03%), which was probably caused by the presence of oxygen. With increasing applied concentration of trimesic acid (fullerene:trimesic acid 1:2) the element content changed to 93.42% of carbon, 0.20% of hydrogen and 6.38% of oxygen which corresponds to 5 trimesic acid molecules per 9 fullerenes.

Figure 1 Characterization of the average particle size, size distribution and zeta potential of fullerenes activated with nitric acid or trimesic acid. For conditions see experimental



In this work, 6 different derivatives of maximin H5 were synthesized, with aspartic acid at position 11 mutated for alanine, asparagine, glycine, histidine, valine or tyrosine. The successful synthesis was confirmed using the mass spectrometry (MS), chromatographic analysis and software prediction of their secondary structure (see Figure 2). The purity of synthesized peptides was 70% according to mass spectrometry data. The prediction of secondary structures shows that the replacement of amino acid at position 11 may have influence on the peptide secondary structure. This was evident especially in peptides H5N (where the secondary structure of the whole peptide is alpha helix due to central amino acid asparagine) and H5V (with two distinct alpha helices due to central amino acid valine).

Figure 2 MALDI-TOF and chromatographic analysis of prepared maximin H5 peptides with the prediction of peptides secondary structures obtained using the PEP-FOLD software and the sequences of maximin H5 peptides with highlighted amino acid changes. For conditions see experimental



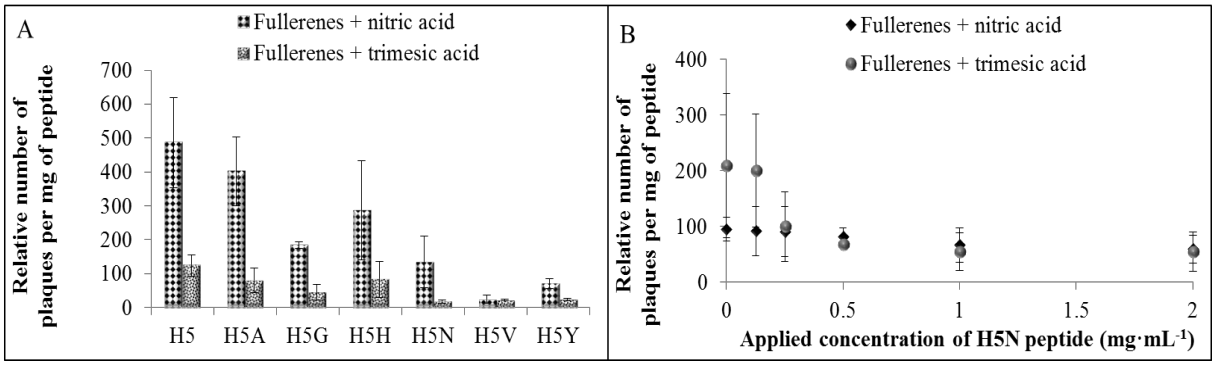
The influence of peptide-modified fullerenes on bacteriophage λ

The surface of activated fullerenes was further modified with maximin H5 derivatives. The excess molecules of peptides were removed by filtration through Amicon 3K centrifugal columns. The binding of each peptide to the fullerenes varied, as well as binding to two tested fullerenes. The combined antiviral activity of peptide-modified fullerenes was evaluated using the plaque assay during which the phage λ was induced with UV light to enter the lytic state. The relative number of plaques was compared to control and recalculated using the amount of peptide bound on fullerenes and thus applied on bacteriophage λ (see Figure 3A).

The lower relative number of plaques shows lower amount of mature, virulent bacteriophage λ and thus higher antiviral activity of the peptide-modified fullerenes. The antiviral activity of all designed peptides was significant in comparison with the H5 maximin. The highest antiviral activity was observed using the fullerenes activated with trimesic acid and H5N peptide. This antiviral activity was increased with the higher amount of peptide up to 0.5 mg·mL^{-1} of applied peptide (see Figure 3B).

The exchange of acidic amino acids in peptide was shown to enhance the antiviral activity of H5 peptide to the HIV virus. Wang et al. exchanged the aspartic acid in H5 peptide to basic amino acid arginine (Wang et al. 2010). In this work, we exchanged the aspartic acid on the position 11 to basic amino acid histidine, which helped to enhance the antiviral activity of the peptide although some other modifications of the peptide showed even higher antiviral activity.

Figure 3 The influence of peptide-modified fullerenes on bacteriophage λ. (A) Plaque assay for assessment of antiviral activity of fullerenes modified with maximin H5 derivatives. (B) Plaque assay for assessment of antiviral activity of fullerenes modified with various concentrations of maximin H5N.



CONCLUSION

A novel nanocarrier was proposed in this work, based on fullerenes modified with 6 different maximin H5 derivatives. All of the tested peptides showed higher antiviral activity, compared to non-mutated maximin H5. The highest antiviral activity was observed using the maximin H5 derivatives where the aspartic acid at position 11 was exchanged for asparagine, valine or tyrosine.

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