# Study of cell penetrating peptide and Europium(III) and Terbium(III) Schiff base complexes interaction

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Received:18.6.2015 / Accepted:1.9.2015 / Published: 1.10.2015

Eu(III) and Tb(III) Schiff base complexes are applicable in various fields such as sensing, assays, screening protocols in vitro, imaging studies in cellulo or in vivo. Fluorescent europium and terbium complexes and their interaction with cell penetrating peptide (KKKRKC) can represent an excellent key for understanding pathway of peptide transportation though cell membrane and the application of Schiff base complexes as potential antibacterial drugs. Mass spectrometry (MALDI TOF) and spectrophotometry measurements have been used for study of complex formation between Eu(III) and Tb(III) Schiff base complex with cell penetrating peptide.

**Keywords:** Schiff base; mass spectrometry; spectrophotometry; europium; terbium; cell penetrating peptide.

## 1. Introduction

During the last decade newly developed potential therapeutic drugs such as proteins, nucleic acids, and new types of hydrophilic drugs are being reported. However, these drugs mostly have limitations due to their inability to reach the appropriate intracellular targets as a consequence of poor possibility to penetrate through cell membranes or deactivation by resistance mechanisms that transport these compounds out of the cell, both limiting their interaction with intracellular targets [1,2]. It has been also reported that poor cell specificity and normal cell cytotoxicity are common in application of standard technique for drug delivery systems, such as microinjection, electroporation, liposomal formulation and use of viral vectors [3-5]. In the search for new anticancer agents and drug delivery systems, cell penetrating peptides (CPP) attracted attention due to their possibilities for intracellular delivery of a wide range of macromolecules. CPP are short peptides consisting of less than 30 amino acids. CPP structures are mostly composed of positively charged amino acids (e.g. Arg, Lys and His) providing them possibility to translocate through the cell membrane by various mechanism, including endocytosis, and easily deliver various cell-impermeable covalently or noncovalently conjugated bioactive cargo such as proteins [6],

nucleic acid [7], siRNA [8], peptide nucleic acid [9] and quantum dots [10]. CPPs as delivery agents were in focus of many investigations with the aim to increase stability and efficiency of cargo delivery avoiding the problem of cytotoxicity effect, lack of cell specificity and unexpected side effects. However, it has been shown that side effects on normal cells during cancer therapy or antibacterial application are minimized [11-13]. Peptides as drug carriers offer some advantages over other carriers as they are relatively easy to modify with various organic or inorganic materials, especially with compounds that have fluorescent properties, enabling easy tracking of drugs after application and for better understanding of the structure and functions of biological systems [14]. Many of luminescent materials such as organic fluorophores [15], recombinant proteins [16], semiconductor nanoparticles [17], and emissive metal complexes [18] are being used for peptide labeling. Application of organic dyes has many limitations associated with poor extinction coefficient or quantum yield and low stability against bleaching. However, on the other hand, metal complexes, especially rare earth metal-based materials show excellent optical properties since the f–f emission lines of Pr(III), Sm(III), Eu(III), Tb(III), Dy(III) and Tm(III) ions are in the visible range [19,20]. Especially great attention attracts luminescent rare-earth metal complexes with Eu(III) and Tb(III) as the excited states of these ions are less sensitive to vibrational quenching by intra or intermolecular energy transfer to adjacent high--energy vibrators such as hydroxyl groups [21]. Comparing metal-rare complex with quantum dots (QDs), they have long life time fluorescence and the fluorescence wavelength of Ln(III) ions is not sensitive to particle size, where the study of the function and properties of these compounds is simplified in comparison to QDs [22]. However, Ln(III) complexes are mostly used for study of their magnetic properties and imagine purpose, only few scientific reports are dealing with application of the complexes in biological applications [23-25].

Based on this consideration, we were interested in preparation of Schiff bases 2-[(E)--2-pyridylmethyleneamino]-N-[2-[(E)-2-pyridylmethyleneamino]ethyl]ethanamine (S-5) and 2-[(E)-2-pyridylmethyleneamino]-N,N--bis[2-[(E)-2-pyridylmethyleneamino]ethyl] ethanamine (S-6) and their europium(III) and terbium(III) complexes Eu(III)-S-5 and Tb(III)--S-6 with luminescent properties in order to study their interaction with cell penetrating peptide and possible biological applications. The compounds were evaluated against several bacterial species with respect to their toxicity.

## 2. Results and Discussion

### *2.1 UV/VIS measurement*

Luminescence studies were conducted at room temperature, observing absorbance and fluorescence properties of KKKRKC cell penetrating peptide, Eu(III) and Tb(III) Schiff base complexes and interaction between cell penetrating peptide and Eu(III) and Tb(III) Schiff base complexes. Concentrations of cell penetrating peptide and Eu(III) and Tb(III) Schiff base complex samples were set on 1 mM, and the changes in the absorption spectra were monitored by spectrophotometry. The absorbance maxima of all samples were observed and it is found that all samples have maximum absorbance at 266 nm (Fig. 1a and 1b). However, from the results obtained after mixing of peptide with Eu(III) and Tb(III) Schiff base complexes a gradual decrease of the peptide-complex absorbance can be clearly observed if we compare it with absorbance of the complexes, concluding that interaction between peptide and complexes is confirmed. This can be explained using Lewis theory of acid and base, where europium and terbium ions represent acid part of complex, while nitrogen or oxygen bases can be bond via donor-acceptor bonding, in which peptide ligand transfers electron charge towards the metal center, forming interaction with the electron donor atoms leading to formation of peptide-complex structure [19,26,27].

An excitation wavelengths of 266 nm was taken in order to determine the maximum wavelength of emission of the mixed Eu(III) and Tb(III). Schiff base complexes and their interaction with cell penetrating peptide were measured. From the obtained results it can be

seen that maximum emission peaks of Tb(III)--S-5 and Tb(III)-S-6 were found at 355 nm, 450 nm, 480 nm, 500 nm and 545 nm (Fig. 1c). This can be explained by f-f transition coming from electron or energy transfer processes which is assigned to the 5D4-7FJ with J = 6–0, reported by Shintoyo et al. [28]. However, in the case of Tb(III)-S-5 and Tb(III)-S-6 complex with peptide the same position peaks as in case of only complex emission were found, while also high emission peak was found at 305 nm for which we can assume that they belong to peptide-complex interaction via donor-acceptor bond mentioned before. In the case of Eu(III)--S-5 and Eu(III)-S-6 maximum emission peaks have been found at 365 nm, 455 nm, 475 nm, 490 nm, 615 nm and 700 nm (Fig. 1d), due to f-f emission of electron or energy transfer processes which is assigned to the 5D0-7FJ with J = 4–0 transitions [28]. The same results have been obtained for Eu(III)-S-5 and Eu(III)-S-6 complex with peptide, with high emission peak at 305 nm, which probably belongs to peptide--complex interaction.

### *2.2 Mass spectrometry measurement*

To confirm that interactions between cell penetrating peptide with Eu(III) and Tb(III) Schiff base complex and complex itself were formed, mass spectrometry (MALDI-TOF) was applied. The main observed signals shown in (Fig. 2a and 2b) were assigned as follows: [Peptide$^{+}$H]$^{+}$ (*m/z* 790.63). It is evident that a proposed intramolecular complex, metal-peptide, was found, as shown in Figure 2c, 2d, 2e and 2f: [peptide--Eu(III)-H]+ (*m/z* 952.86), [2peptide-Eu(III)$^{\cdot}$H]$^{+}$ (m/z 1740.54); [peptide-2ClEu(III)$^{\cdot}$H]$^{+}$ (*m/z* 892.54), [peptide-Eu(III)-H]$^{+}$ (m/z 952.86), [2peptide-Eu(III)-H]$^{+}$ (*m/z* 1739.53); [peptide--2Cl-HTb(III)]+ (m/z 878.77), [peptide-Tb(III)--2Cl-H]+ (*m/z* 1041.89), [2peptide-2Tb(III)--$H_2O^{\cdot}$H]+ (m/z 1918.69) and [peptide-2Cl-H]$^{+}$ Tb(III)(*m/z* 878.73), [peptide-Tb(III)-2Cl-H]$^{+}$ (*m/z* 1041.83), [2peptide-2Tb(III)-$H_2$O-H]$^{+}$ (*m/z* 1918.54). The intensity of peptide signal without incubation with metal was lower than with metal complexes in both cases (Eu(III) and Tb(III)). The spectra Tb(III)-S-5-peptide and Tb(III)-S-6-peptide showed no significant

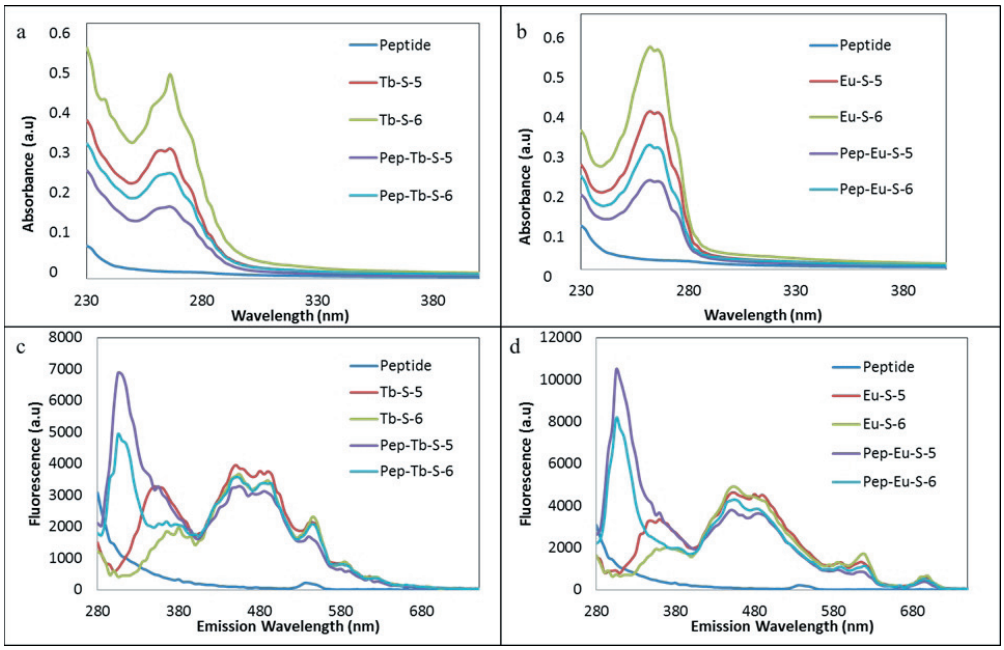


**Figure 1:** (a) Absorption and fluorescence spectra of cell penetrating peptide, Tb(III)-S-5 and Tb(III)-S-6 Schiff base complex. (b) Absorption and fluorescence spectra of cell penetrating peptide, Eu(III)-S-5 and Eu(III)-S-6 Schiff base complex.

difference in the formation of complexes. We cannot confirm the type of bonding that occurs between the metal and the peptide by MALDI-TOF, however from the results it is obvious that complexes between peptide and Eu(III) and Tb(III) are formed [29,30]. From the results we can conclude, that Schiff bases are replaced by peptides during reaction and 1:1 lanthanides complexes or dimers are formed. This conclusion can be explained by affinity of lanthanides to oxygen atoms which are present in peptides. Much stronger bonds are therefore formed in comparison with nitrogen coordination in the case of Schiff bases.

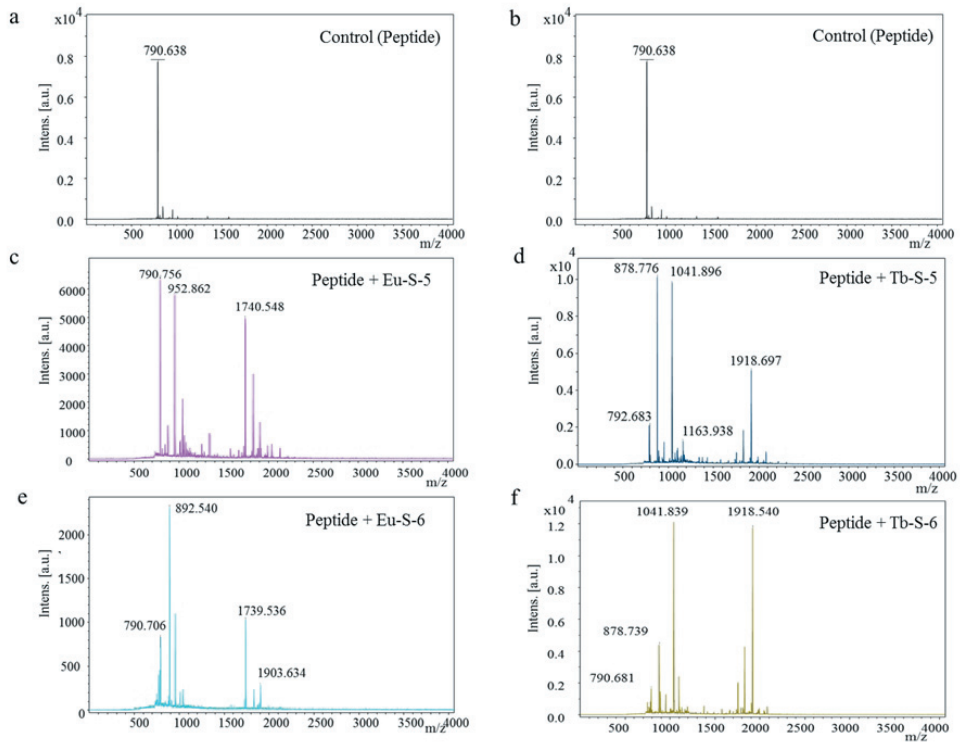


**Figure 2:** (a,b) Mass spectra of peptide. (c) Mass spectrum of peptide interaction with Eu(III)-S-5 complex, (d) Tb(III)-S-5 complex, (e) Eu(III)-S-6 complex, (f) Tb(III)-S-6 complex.

### *2.3 Influence of Eu(III) and Tb(III) Schiff base complex on **E. coli***

Today it is well known that growth of microorganisms can be limited by heavy metal ions which can be incorporated into a variety of materials and as such they perform strong antibacterial activity [31,32]. Effect of Eu(III) and Tb(III) Schiff base complex on *E. coli* was conducted by method of determination of growth dependences, where the 50% inhibitory concentration ($IC_{50}$) was determined. In this experiment antimicrobial activity of Eu(III) (S-5 and S-6) and Tb(III) (S-5 and S-6) Schiff base complex conjugates with cell penetrating peptide were determined. Obtained results show that Eu(III)-S-5 and Eu(III)-S-6 conjugate with cell penetrating peptide (Fig. 3a, 3b) have stronger antimicrobial effect. In the case of *E. coli* treated with Eu(III)-S-5 and Eu(III)-S-6, there was minimal inhibitory concentration (MIC) determined after addition of 0.125 mM and totally inhibitory concentration (TIC) after application of 5 mM. $IC_{50}$ for Eu(III)-S-5 was statistically calculated as 1.6 mM (Fig. 3a). Also, the same results were obtained for Eu(III)-S-6, only in the case of this sample $IC_{50}$ was determined as 1.3 mM (Fig. 3b). In appli-

cation of Tb(III)-S-5 and Tb(III)-S-6 conjugate with cell penetrating peptide the minimal inhibitory concentration (MIC) was 0.125 mM and the total inhibitory concentration (TIC) was 5 mM (Fig. 3c, 3d). However, treatment of *E. coli* with Tb(III)-S-5 showed that $IC_{50}$ was statistically calculated as 2.5 mM (Fig. 3c). After application of Tb(III)-S-6 on *E. coli* results showed that total inhibitory concentration (TIC) is not sufficient for inhibition, because in the case of this sample $IC_{50}$ was calculated as 10 mM (Fig. 3d), what indicates lower toxicity of Tb(III)-S-6 on bacterial strains in comparison to the other three samples, which correlates with lower concentration values required for the bacterial inhibition.

However, to confirm that metal-peptide complex have main role in antibacterial activity, peptide conjugate with S-5 and S-6 Schiff base without Terbium and Europium were applied on E. coli. Results show that treatment of E. coli with S-5 Schiff base conjugate with cell penetrating peptide showed that $IC_{50}$ was statistically calculated at 19.2 mM and for S-6 Schiff base conjugate with cell penetrating peptide at 20 mM. Due to that we can conclude that main role in antibacterial activity have samples that contain Schiff base complex with Terbium and Europium. Antibacterial effect of applied samples come from Schiff base complex with Terbium and Europium and peptide, with ability to easily penetrate the bacterial cell membrane by coordination of metal ion through oxygen or nitrogen donor atom with lipopolysaccharide (LPS) causing the damage of cell membrane. Atabay et. al, report application of monometallic complexes with $PdCl_2$ and $ZnX_2$, were ligand acts as a chelating tridentate or bidentate, through two of the nitrogen atoms in the imidazole ring and the sulfur atom of the bridging group together with two chloride ions forming a rare five coordinate complex

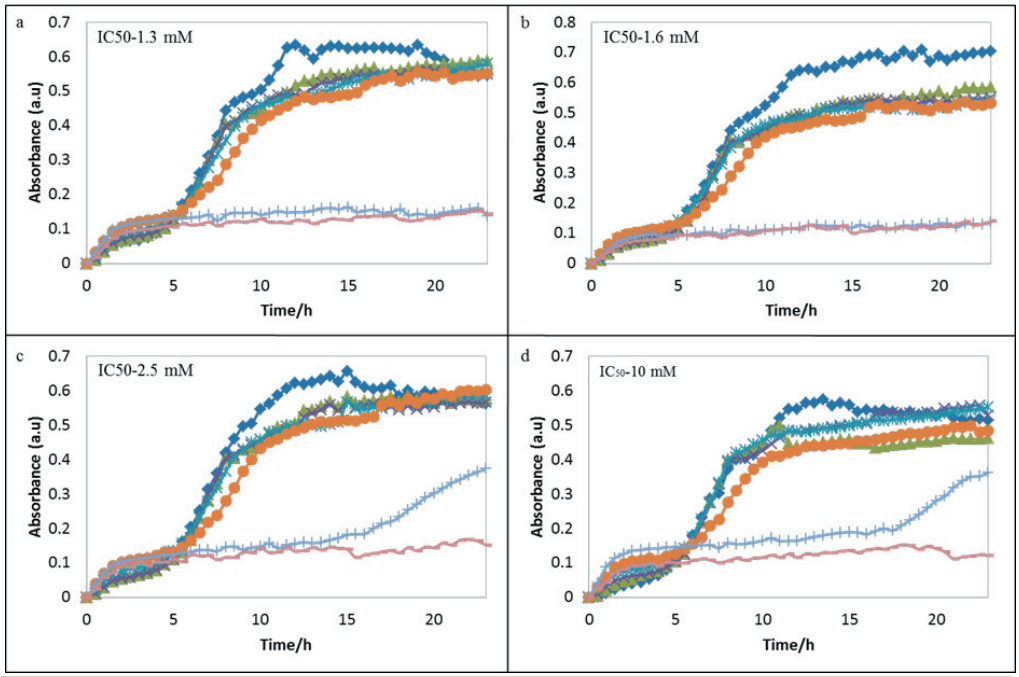


**Figure 3:** Spectrophotometric determination of growth curves obtained by treatment with 0, 0.125, 0.312, 0.625, 1, 2.5 and 5 mM concentration of Eu(III) and Tb(III) Schiff base complex conjugate with cell penetrating peptide of: (a) E. coli after application of Eu(III)-S-5 conjugate with CPP. (b) E. coli after application of Eu(III)-S-6 conjugate with CPP. (c) E. coli after application of Tb(III)-S-5 conjugate with CPP. (d) E. coli after application of Tb(III)-S-6 conjugate with CPP.

and tetrahedral complexes, which have strong antibacterial activity [33,34].

## 3. Experimental Section

### *3.1 Chemicals*

2-pyridinecarboxaldehyde, diethylenetriamine, tris(2-aminoethyl)amine, europium(III) chloride, terbium(III) chloride, methanol, glycerol, glucose, tryptophan, yeast extract were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Deionized water underwent demineralization by reverse osmosis using an Aqua Osmotic 02 (Aqua Osmotic, Czech Republic) and was subsequently purified using a Millipore RG (MiliQ water, 18 M, Millipore Corp., Billerica, MA, USA).

### *3.2 Synthesis of KKKRKC peptide*

For synthesis, Liberty Blue peptide synthesizer was used (CEM, Matthews, NC, USA). The sequences and monoisotopic molecular weights of synthesized peptides were as follows: KKKRKC - 790 Da. Deblock of Fmoc protecting group was performed with 20 % piperidine v/v in DMF. Coupling was achieved using N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), N,N-diisopropylethylamine (DIEA) and DMF. Cleavage of side chain protecting groups was performed by treating the peptide resin with 95 % trifluoroacetic acid v/v, 2.5 % $H_2O$ v/v and 2.5 % TIPS v/v for 30 minutes at 38 °C under microwave irradiation. Isolation of peptide was performed by centrifugation (6000 rpm, 3 min) under cold diethylether.

### *3.3 Synthesis of Eu(III)-S-5 and Tb(III)-S-5 Schiff base complexes*

2-pyridinecarboxaldehyde (2850 µl) and diethylenetriamine (1080 µl) were added in 35 mL of methanol and refluxed for 6 h. After cooling, methanol was added to 50 ml. $Tb(III)Cl_3 \cdot 6H_2O$ (0.435 g) or $Eu(III)Cl_3 \cdot 6H_2O$ (0.366 g) was dissolved in 50 ml of water. After that Schiff base (5 ml) was added to the solution with stirring. Solutions were heated at 80 °C for 2 h. After that the solutions were filtered and water was added to reach 100 ml with final concentration 10 mM.

### *3.4 Synthesis of Eu(III)-S-6 and Tb(III)-S-6 Schiff base complexes*

2-pyridinecarboxaldehyde (2850 µl) and tris(2-aminoethyl)amine (1498 µl) were added in 35 mL of methanol and refluxed for 6 h. After cooling, methanol was added to 50 ml. $Tb(III)Cl_3 \cdot 6H_2O$ (0.435 g) or $Eu(III)Cl_3 \cdot 6H_2O$ (0.366 g) were dissolved separately in 50 ml of water. After that Schiff base (5 ml) was added to the both solutions with stirring. The solutions were heated at 80 °C for 2 h. After that solutions were filtered and water was added to reach 100 ml with final concentration 10 mM.

### *3.5 Cultivation of bacterial strains*

Staphylococcus aureus (NCTC 8511), Escherichia coli (NCTC 13216) and methicillin-resistant Staphylococcus aureus (7111 2/A8) were obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic. The strains were stored as a spore suspension in 20% (v/v) glycerol at -20°C. Prior to use in this study, the strains were thawed and the glycerol was removed by washing with distilled water.

The composition of cultivation medium was as follows: glucose 10 g/l, tryptone 10 g/l and yeast extract 5 g/l, sterilized MilliQ water with 18 MΩ. pH of the cultivation medium was adjusted at 7.4 before sterilization. The sterilization of the media was carried out at 121 °C for 30 min in sterilizer (Tuttnauer 2450EL, Israel). The prepared cultivation media were inoculated with bacterial cultures or yeasts into 25 ml Erlenmeyer flasks. After inoculation, bacteria and yeasts were cultivated for 24 h on a shaker at 600 rpm and 37 °C. Strains, cultivated under these conditions, were diluted by cultivation medium to $OD_{600} = 0.1$ and used in the following experiments.

### *3.6 Determination of growth curves*

The procedure for the evaluation of the antimicrobial effect of tested compounds consisted in measuring of the absorbance using the apparatus Multiskan EX (Thermo Fisher Scientific, Germany) and subsequent analysis in the form of growth curves. Bacteria and yeasts were

cultivated in GTY medium for 24 h with shaking and were diluted with GTY medium using Specord spectrophotometer 210 (Analytik, Jena, Germany) at a wavelength of 600 nm to absorbance 0.1. On the microplate, these cultures were mixed with various concentrations of peptide and peptide-Eu(III) and Tb(III) Schiff base complex or S. aureus alone as a control for measurements. The concentrations of all samples were 0; 7.8; 15.6; 31.3; 62.5; 125; 250 and 500 μg/ml. Total volume in the microplate wells was always 300 μl. The measurements were carried out at time 0, then each half-hourly for 24 h at 37 °C and a wavelength of 600 nm. The obtained values were analyzed in graphical form as growth curves for each variant individually. Software STATISTICA (data analysis software system), version 10.0 (Tulsa, OK, USA) was used for data processing. The half-maximal concentrations (IC50) were calculated from logarithmic regression of sigmoidal dose-response curve. The general regression model was used to analyse differences between the combinations of compounds.

### *3.7 UV/Vis spectrophotometry*

Measurements of peptide and europium(III) and terbium(III) Schiff base complexes fluorescence were conducted by a multifunctional microplate reader Tecan Infinite 200 PRO (Tecan group Ltd., Männedorf, Switzerland). The absorbance scans were recorded in the range of 200–800 nm each 5 nm. Emission wavelengths from 260 nm to 850 nm were measured at different excitation wavelengths (280 nm, 350 nm, and 400 nm) with 200 μl of the sample placed on a Costar UV-transparent, acrylic copolymer, flat bottom, 96 well plate (Sigma–Aldrich Co. St. Louis, MO, USA). All measurements were performed at 30 °C controlled by the Tecan Infinite 200 PRO (TECAN, Switzerland).

### *3.8 Matrix-assisted laser desorption/ ionization (MALDI-TOF)*

The mass spectrometry experiments were performed on a MALDI-TOF/TOF mass spectrometer Bruker Ultraflextreme (Bruker Daltonik GmbH, Germany) equipped with a laser operating at wavelength of 355 nm with an accelerating voltage of 25 kV, cooled with nitrogen and a maximum energy of 43.2 μJ with repetition rate 2000 Hz in linear and positive mode, and with software for data acquisition and processing of mass spectra flexControl version 3.4 and flexAnalysis version 2.2. The matrix used in the MALDI method was α-cyano--4-hydroxycinnamic acid (CCA) (Bruker). The matrix was prepared in TA30 (30% acetonitrile, 0.1% trifluoroacetic acid solution). Mixture was thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin electronic GmbH, Germany) to 2 minutes 50% of intensity at ambient temperature. Working standard solutions were prepared daily by dilution of the stock solutions. The sample solutions were TA30. The solutions for analysis were mixed in ratio of 1:1 (matrix/substance). After obtaining a homogeneous solution, 1μl was applied on the target and dried under atmospheric pressure and ambient temperature. A mixture of peptide calibrations standard (Bruker) was used to externally calibrate the instrument. The MS spectra were typically acquired by averaging 20 sub spectra from a total of 500 shots of the laser (Smartbeam 2. Version: 1_0_38.5).

## 4. Conclusions

We have successfully synthesized and conjugated the Eu(III) and Tb(III) Schiff base complexes with KKKRKC cell penetrating peptide. All interactions between peptide and Eu(III) and Tb(III) Schiff complex were confirmed by spectrophotometric and mass spectrometry studies. UV/VIS spectra of Tb(III)-S-5-peptide and Tb(III)-S-6-peptide in the presence and absence of Schiff base affirmed an increase in peak intensity of complex and decrease in free Schiff base spectra indicating the formation of metal-peptide complex. The spectra of Tb(III)-S-5-peptide and Tb(III)-S-6-peptide show no significant difference in the formation of complexes by MALDI-TOF. Even though we cannot confirm the type of bonding that occurs between the metal and the peptide by MALDI-TOF, it is obvious that complexes between peptide and Eu(III) and Tb(III) Schiff base are formed. Europium and Terbium ions represent

the acid part of complex according to the Lewis acid theory where the nitrogen or oxygen bases can be bound via donor-acceptor bond, while peptide ligand transfers electron charge toward the metal center, forming interaction with the electron donor atoms. This property resulting in reduced metal ion polarity, through partial sharing of the positive charge with donor atoms of the ligand, enables higher antimicrobial activity. In other words, the chelation of peptide-Schiff base complex leads to increase in lipophilic properties allowing the complex penetration through lipid layers of the bacterial membrane [35]. Due to that reason Schiff base can easily penetrate the cell membrane by coordination of metal ions via donor-acceptor theory with lipopolysaccharide leading directly to membrane damage. Metal-peptide complex due to that in the future can shows great potential in biological application as antibacterial drug, however it is necessary conduct more experiment on metal rare complex and their application as antibacterial drug.

## Acknowledgments

The financial support from the project CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged. The authors thank Radek Chmela for his skilful technical assistance.

## Conflicts of Interest

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study. The Editorial Board declares that the manuscript met the ICMJE „uniform requirements“ for biomedical papers.

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