Title: Self-assembling amyloid-like peptides as exogenous second harmonic probes for bioimaging applications

Ming Ni ^[+]1,2, Shuangmu Zhuo ^[+]3, Ciprian Iliescu^{4,5}, Peter T. C. So^{6,7}, Jodhbir S. Mehta⁸, Hanry Yu^{1,6,9}, Charlotte A. E. Hauser¹⁰

¹Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, The Nanos, Singapore 138669;

²School of Biological Sciences & Engineering, Yachay Tech University, Hacienda San José s/n, San Miguel de Urcuquí 100105, Ecuador

³Key Laboratory of Optoelectronic Science and Technology for Medicine of Ministry of Education, Fujian Normal University, Fuzhou, 350007, P. R. China

⁴BIGHEART, National University of Singapore, 14 Medical Drive, MD6, #14-01, 117597 Singapore

⁵Academy of Romanian Scientists, Splaiul Independentei nr. 54, Bucharest 050094, Romania

⁶Biosystems and Micromechanics IRG, Singapore-MIT Alliance for Research and Technology, 1 CREATE Way, #04-13/14 Enterprise Wing, 138602 Singapore;

⁷Department of Mechanical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

⁸Singapore Eye Institute and Singapore National Eye Center, 11 Third Hospital Avenue, 168751, Singapore

⁹Yong Loo Lin School of Medicine & Mechanobiology Institute, National University of Singapore, 14 Medical Drive, MD 9 #04-11, 117597, Singapore

¹⁰King Abdullah University of Science and Technology, Division of Biological & Environmental Science & Engineering, Laboratory for Nanomedicine, Al-Haytham Building (Bldg. 2), Office 4217, Thuwal 23955-6900, Kingdom of Saudi Arabia

[+] These authors contributed equally to this work.

* Corresponding Authors: M.N. (mingni.sg@gmail.com) or S.Z. (shuangmuzhuo@gmail.com) or H.Y. (hyu@ibn.a-star.edu.sg) or C.A.E.H. (charlotte.hauser@kaust.edu.sa).

Keywords: bioimaging, non-linear optical materials, second harmonic generation, self-assembly, ultrashort peptides.

ABSTRACT

Amyloid-like peptides are an ideal model for the mechanistic study of amyloidosis, which may lead to many human diseases, such as Alzheimer's. The paper reports a strong second harmonic generation (SHG) effect of amyloid-like peptides, having a signal equivalent to or even higher than those of endogenous collagen fibers. Several amyloid-like peptides (both synthetic and natural) were examined under SHG microscopy and shown they are SHG-active. These peptides can also be observed inside cells (*in vitro*). This interesting property can make these amyloid-like peptides second harmonic probes for bioimaging applications. Furthermore, SHG microscopy can provide a simple and label-free approach to detect amyloidosis. Lattice corneal dystrophy was chosen as a model disease of amyloidosis. Morphological difference between normal and diseased human corneal biopsy samples can be easily recognized, proving that SHG can be a useful tool for disease diagnosis.

1. Introduction

Amyloid-like peptides are responsible for amyloidosis which is an important feature for several human diseases, including Alzheimer (AD) [1-2], Parkinson [3], type II diabetes [4], cataracts [5], or lattice corneal dystrophy (LCD) [6]. The inhibition of amyloid-like peptides production is a promising strategy for the development of therapeutic agents to treat such diseases. However, the mechanism of amyloidosis is still unclear. One logical mechanistic approach is to study how these amyloid-like peptides/proteins fold or misfold [2, 7]. In this direction, ultrashort peptides (having only three to seven amino acids) provide us an ideal model for mechanistic studies. For example, GGVVIA (GA₆) and KLVFFAE (KE₇), can self-assemble into amyloid-like aggregates [8-9]. The previously mentioned peptides are also known as the core sequence of amyloid- β (A β). A β is the major component in the amyloid aggregates responsible for AD. Besides ultrashort peptides that can be found in natural peptides/proteins, several designed synthetic ultrashort peptides, such as IVD (ID₃) and LIVAGD (LD₆), have shown similar behavior [9-10]. Both the natural and synthetic peptides are amyloid-like peptides. Ultrashort amyloid-like peptides can not only be useful for screening of amyloid inhibitors [10-11], but also as a new type of biomaterials with desirable properties such as biocompatibility and biological activities [8-9, 12-13]. They all form cross- β peptide structure at a molecular level. Mechanically they are rigid, with strength comparable to steel [14]. Morphologically they are helical fibers of micrometers in length but only 7-10 nm in diameter [15], having a similar morphology to collagen fibers [10, 16]. Supramolecular assemblies of collagen in tissues have been visualized by SHG microscopy [17]. In particular, collagen I and collagen II have been proven to efficiently produce SHG signals [17-18]. SHG has been applied to investigate rat and human corneal samples [19-22]. Collagen fibrils in cornea were viewed under SHG microscopy without any labelling. The orientation,

morphology and submicron heterogeneity of collagen fibrils were quantified by using SHG and potentially can be used for in vivo diagnosis of corneal diseases, such as LCD. Besides collagen is used as a biomarker for LCD, extracellular deposition of amyloid β within cornea is commonly thought as the main reason for LCD. Inspired by these findings, we demonstrate here the non-linear optical (NLO) properties of self-assembled ultrashort amyloid-like peptides [23] and explore their potentials as novel organic probes for SHG imaging. Besides significant advantages over the conventional fluorescence imaging techniques such as deeper optical penetration, lower photo-damage and longer observation time [24-27], SHG microscopy provides a simple and label-free approach to detect amyloid-like peptides, which can be a useful tool for disease diagnosis, especially for LCD.

2. Materials and Methods

2.1 Ultrashort Amyloid-Like Peptides

Amyloid-like peptides were purchased from the American Peptide Company (purity $\geq 95\%$). The peptide sequences were confirmed by liquid chromatography-mass spectrometry (LC-MS). Net peptide content varied between 70% and 85%. All peptides were acetylated at the N terminus. Peptide handling and hydrogel preparation were done as reported previously [10, 28].

2.2 Peptide Particles

The preparation of the peptide particles was performed by hydrodynamic focusing, method described in previous work [29-31]. The particle size was determined by scanning electron microscopy, showing a grain size of around 5 μm .

2.3 Cell Culture

Both HeLa and human dermal fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Singapore).

2.4 Human corneal biopsy samples

The human corneal biopsy samples were provided by Singapore General Hospital. The normal tissue was from a seventy-eight year-old Caucasian male. The diseased tissue was from a sixty-year-old female who has lattice corneal dystrophy (LCD). The study was approved by the institutional review board (IRB) from Singhealth.

2.5 SHG Imaging

SHG images were acquired using a commercial laser scanning microscopic imaging system (Zeiss LSM 510 META, Jena, Germany) coupled to a mode-locked femtosecond Ti: sapphire laser (Mai-Tai broadband, Spectra-Physics), tunable from 710 nm to 990 nm. To achieve spectral analysis and detect the SHG signal, we used the META detector with 32-gated photon counting module [17].

3 Results and Discussions

3.1 A hexamer peptide is collagen-like and SHG-active

To test whether these amyloid-like peptides are SHG-active, we first investigated a hexamer peptide, Ac-LIVAGK-NH₂, (LK₆). Peptide powder was dissolved in pure water and peptide microparticles were assembled on a microfluidic chip using hydrodynamic focusing techniques. The resulting peptide microparticles were examined under SHG microscopy. During the whole experiments, only the backscattered geometry was employed because it is the only suitable configuration for *in vivo* imaging [26]. We chose collagen I as a control, being shown that SHG microscopy can visualize supramolecular assembly of collagen in tissues [26]. Both collagen I and peptide particles showed SHG signals with comparable intensity. This is shown in **Figure 1a** where the SHG signal from the collagen fibers of a rat liver tissue was compared with

the signal from LK₆ peptide particles (instrument settings for both SHG imaging being the same). The fine structures of collagen and peptide particles can be clearly seen under SHG and SEM- **Figure 1b and c**. Moreover, LK₆ peptide exhibits typical SHG characteristics. Excited at 810 nm, LK₆ emitted a sharp SHG peak at 405 nm (**Figure 1d**). This is a main characteristic of SHG: two photons can be converted into one photon at exactly half of the wavelength. We then examined the excitation wavelength tunability of SHG signals from LK₆ peptide. In **Figure 1e**, as the excitation wavelength was increased from 810 to 890 nm, the wavelength of the SHG signals from the LK₆ peptide particles increased from 405 to 445 nm respectively. The opportunity of tuning the excitation wavelength gives the advantage of best matching with the optical properties of the sample. It is known that a biological sample (cells/tissues) presents auto-fluorescence. Tuning the excitation wavelength, this auto-fluorescence can be avoided. In our experiments, we used excitation wavelength ranging from 810 to 890 nm, which falls in the so called "therapeutic window" or "optical window". The common problems encounter by conventional fluorescent microscopy are circumvented. In addition, a further increase of the penetration depth in tissues can be achieved by simply increasing excitation wavelength. **Figure 1f** showed the emission wavelength data of the SHG signal ($\lambda_{\text{ex}} = 850 \text{ nm}$). The data in the graph was fitted to a Gaussian curve exhibiting a maximum at 425nm, which is exactly half the excitation wavelength of 850 nm. The bandwidth (full width at half-maximum of Gaussian distribution, FWHM) was narrow (~10 nm) and it obeyed a relationship to the corresponding wavelength profile of the fundamental beam (~15 nm). Moreover, the dependence of the output signal on irradiation intensity was measured by varying the 850nm excitation intensity. The linear regression, applied to the log-log plots (**Equation 1**), revealed a quadratic power dependence of SHG intensity to power intensity, as shown in the **Figure 1g**.

$$\log[I_{425}] = 0.45 + 2.01 \cdot \log[I_{850}] \quad (1)$$

The intensity of SHG signal is proportional to the square of the incident laser intensity. This confirmed the two-photon nature of the emission from LK₆ peptide.

3.2 Cytocompatibility of a hexamer peptide

In order to use LK₆ for bioimaging applications, such as *in vitro* cells monitoring or *in vivo* imaging, we assessed the cytotoxicity of LK₆ using MTS test and live/dead assay with two human cell lines, human epithelial carcinoma cells (HeLa) and primary human dermal fibroblasts (HDF) (**Figure 2**). Cells were exposed to cell culture media containing different concentrations of LK₆ solution (0.1 to 1000 µg/mL) and incubated for 48 h. Cell viability at various LK₆ concentrations was either equivalent to or even higher than that of untreated cells over the whole concentration range which was tested in this study (**Figure 2a**). To further confirm these results, we stained cells with or without peptide exposure using solutions containing a mixture of calcein AM and ethidium homodimer-1 (EthD-1). Calcein AM can penetrate cell membrane and be converted within the cell to membrane impermeable green fluorescent calcein, while red fluorescent EthD-1 is unable to permeate the intact cell membrane of living cells. **Figure 2 b** shows the representative live/dead images of cells in response to LK₆ solutions (1 and 1000 µg/mL). LK₆ displayed no toxicity to both HDF and HeLa cells, in agreement with the results of the MTS assays.

3.3 Comparison of synthetic and natural amyloid-like peptides under SHG microscopy

Several other amyloid-like peptides, including both natural amyloidogenic core sequences (NL₆, DF₅, GA₆, and KE₇) and designed synthetic peptides (LD₆, IS₆ and IK₃) were examined under SHG excitation. The peptide sequences are listed in Table

1. All these peptides are SHG-active. We used a powder technique developed by Kurtz and Perry [32] to evaluate the SHG efficiency of these second-order nonlinear optical materials. Sucrose was chosen as control to evaluate SHG efficiencies [33]. In Table 1, all tested amyloid-like peptides showed higher or equivalent SHG efficiency compared to sucrose. These can be explained through their molecular structures. LD₆ is known to form hydrogel at much lower concentrations compared to LK₆. The trimer IK₃ has larger dipole moment compared to LK₆. Human collagen subtypes I to V were also examined (**Table 1**) for their SHG activity. Only Collagen type I and II showed positive results, aspect that is in concordance with the previous findings reported in the literature [18, 34]. Moreover, some amyloid-like peptides such as IVK, LIVAGD or even ILVAGS showed even higher SHG efficiency than collagen subtypes (I and II). This suggests the opportunity of using these materials as SHG probes.

The investigation of the SHG efficiency for four natural amyloidogenic core sequences indicates the following order: KE₇ > GA₆ > DF₅ > NL₆ (**Table 1 and Figure 3**). KE₇ (KLVFFAE), containing its diphenylalanine (FF) motif, showed the highest SHG efficiency among these four peptides. However, its SHG efficiency is similar as that of IK₃. It is noteworthy that IK₃ is an aliphatic peptide. Aromatic residues such as tyrosine and tryptophan are known to be used as an endogenous molecular probe of peptides and proteins for SHG at the air-water interface [35]. However, our results indicated that aromatic residues are not necessary for higher SHG efficiency. LD₆, IS₆, LK₆ and IK₃ are all aliphatic and lacking aromatic residues, clearly makes them a unique type of peptides for SHG applications. These peptides are amphiphilic [10, 16], consisting of an aliphatic amino acid tail of decreasing hydrophobicity and a hydrophilic head. They can self-assembly via parallel-antiparallel α -helical pairs and subsequent stacking into β -turn fibrils, which show striking similarity to collagen fibers [16]. We reasoned that

the origin of these amyloid-like peptides' SHG activity comes from their nanostructures. A recent study showed that the origin of SHG signals from collagen fibers possibly lies in their peptide bonds [36]. The collagen fiber building blocks were mimicked by tri-amino acid peptides PPG and GGG (P and G are the one letter code for Proline and Glycine respectively).

3.4 A hexamer peptide as a second harmonic probe for in vitro cell imaging

Another possible application for LK₆ is to use it for high-resolution cell imaging. To this end, HeLa cells were incubated with biotin-conjugated LK₆ for 4 h and then fixed for immune staining. LK₆ peptides were visualized by both SHG and confocal fluorescence microscopy. In **Figure 4**, biotin-conjugated peptides displayed green color when DyLightTM 488-conjugated NeutrAvidinTM (Thermo Scientific, Singapore, Prod #: 22832) was added. Meanwhile, amyloid-like peptides displayed pseudo red color under SHG. These results revealed two interesting features of the investigated amyloid-like peptides: 1) they can be visualized under SHG without any label; 2) they can be up-taken by cells. We want to point out that when attaching additional labels such as chromophores to the amyloid-like peptide structure for bioimaging purposes, it may easily change the molecular properties of the native peptide molecules. Thus, additional changes of the peptide molecule are time consuming and asking for more costly synthetic approaches. Hence, using amyloid-like peptides in their native structure for a label-free SHG bioimaging technology could certainly be useful as an attractive and minimal-invasive diagnostic tool.

In addition, a molecular-level property of the nonlinearity, i.e., the first hyperpolarizability, β , was measured by Hyper Rayleigh Scattering (HRS). The first hyperpolarizability of these trimers was about 0.087×10^{-30} esu [36]. However, the first

hyperpolarizability of collagen I was found to be $(1250 \pm 20) \times 10^{-30}$ esu [36], which could be viewed as ten thousand trimers combining together. In terms of SHG signals, collagen consist a triple helix structure, which shows very strong SHG signals. Single collagen amino acid such as glycine, proline and hydroproline does not show SHG signals as strong as collagen fibrils. We hypothesize that the aggregation of these tripeptides gives the much stronger SHG signals in comparison with isolated tripeptides. Based on the non-linearity of the intrinsic peptide bonds and their aggregation capability, these amyloid-like peptides can generate SHG signals, just like collagen.

3.5 Diseased and normal human corneal biopsy samples examined by SHG microscopy

More interestingly, when we examined the human corneal biopsy samples (**Figure 5a and 5b**), both amyloids (indicated by arrow) and collagen can be seen. The human corneal biopsy samples were provided by Singapore General Hospital. The normal tissue was from a seventy-eight year-old Caucasian male. The diseased tissue was from a sixty-year-old female who has lattice corneal dystrophy (LCD). Under SHG, the normal corneal sample showed collagen fibrils aligned as parallel straight lines in stroma. By contrast diseased samples showed collagen fibrils became curved and thicker. The thickened collagen fibrils could be due to amyloid deposits [37]. Thus, SHG provides us a new diagnostic tool for lattice corneal dystrophy (LCD) that is superior to the existing histological staining methods: SHG being a label-free method.

4. Conclusions

We demonstrated that amyloid-like peptides are nonlinear optical materials showing strong SHG signals and a quadratical dependence of SHG intensity to power intensity. Amyloid-like peptides show no cytotoxic effect to human cells and can be observed

inside cells without fluorescent labelling, which make them suitable as second harmonic probes. Amyloid-like peptide nanomaterials hold great potential in nanotechnology and nanomedicine. We assembled amyloid peptide microparticles via hydrodynamic focusing on a microfluidic chip. We could encapsulate small molecule drugs, DNA or RNA when we assembled them on chip. Their nonlinear optical properties hold promise for new bioimaging applications. The development of SHG microendoscopy [38] could open more opportunities for using amyloid-like peptides as a SHG probe for more bioimaging applications. The imaging capability in combination with therapeutic capability via drug encapsulation will make these amyloid-like peptides interesting theranostic agents.

Acknowledgements

This work was funded by the Institute of Bioengineering and Nanotechnology (Biomedical Research Council, Agency for Science, Technology and Research, Singapore). S.Z. thanks the SMART Scholars Programme, the National Key Basic Research Program of China (2015CB352006), the National Natural Science Foundation of China (81771881), the Natural Science Foundation of Fujian Province (2018J07004), the Special Funds of the Central Government Guiding Local Science and Technology Development (2017L3009) for financial support.

Author Information: The authors declare no competing financial interests.

References

- [1] Soto C, Brañes MC, Alvarez J, Inestrosa NC. Structural Determinants of the Alzheimer's Amyloid β -Peptide. *J. Neurochem.* 1994; 63: 1191-8.
- [2] Chiti F, Dobson CM. Protein Misfolding, Functional Amyloid, And Human Disease. *Annu. Rev. Biochem.* 2006; 75: 333-66.
- [3] Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R. Mutation In The A-Synuclein Gene Identified In Families With Parkinson's Disease. *Science* 1997; 276: 2045-7.
- [4] Clark A, Lewis C, Willis A, Cooper G, Morris J, Reid K, Turner R. Islet Amyloid Formed From Diabetes-Associated Peptide May Be Pathogenic In Type-2 Diabetes. *Lancet* 1987; 330: 231-4.
- [5] Goldstein LE, Muffat JA, Cherny RA, Moir RD, Ericsson MH, Huang X, Mavros C, Coccia JA, Faget KY, Fitch KA. Cytosolic B-Amyloid Deposition And Supranuclear Cataracts In Lenses From People With Alzheimer's Disease. *Lancet* 2003; 361: 1258-65.
- [6] Gorevic PD, Munoz PC, Gorgone G, Purcell JJ, Rodrigues M, Ghiso J, Levy E, Haltia M, Frangione B. Amyloidosis Due To A Mutation Of The Gelsolin Gene In An American Family With Lattice Corneal Dystrophy Type II. *N. Eng. J. Medicine* 1991; 325: 1780-5.
- [7] Dobson CM. Protein Folding And Misfolding. *Nature* 2003; 426: 884-90.
- [8] Kirschner DA, Inouye H, Duffy LK, Sinclair A, Lind M, Selkoe DJ. Synthetic Peptide Homologous To Beta Protein From Alzheimer Disease Forms Amyloid-Like Fibrils In Vitro. *P. Natl. Acad. Sci. USA* 1987; 84: 6953-7.
- [9] Hauser CA, Maurer-Stroh S, Martins IC. Amyloid-Based Nanosensors And Nanodevices. *Chem. Soc. Rev.* 2014; 43: 5326-45.
- [10] Hauser CA, Deng R, Mishra A, Loo Y, Khoe U, Zhuang F, Cheong DW, Accardo A, Sullivan MB, Riekel C. Natural Tri-To Hexapeptides Self-Assemble In Water To Amyloid B-Type Fiber Aggregates By Unexpected A-Helical Intermediate Structures. *P. Natl. Acad. Sci. USA* 2011; 108: 1361-6.
- [11] Azriel R, Gazit E. Analysis Of The Minimal Amyloid-Forming Fragment Of The Islet Amyloid Polypeptide An Experimental Support For The Key Role Of The Phenylalanine Residue In Amyloid Formation. *J. Biol. Chem.* 2001; 276: 34156-61.
- [12] Ni M, Zhuo S. Applications of self-assembling ultrashort peptides in bionanotechnology. *RSC Adv.* 2019; 9: 844-52.
- [13] Cherny I, Gazit E. Amyloids: not only pathological agents but also ordered nanomaterials. *Angew. Chem. Int. Edit.* 2008; 47: 4062-9.
- [14] Smith JF, Knowles TP, Dobson CM, MacPhee CE, Welland ME. Characterization Of The Nanoscale Properties Of Individual Amyloid Fibrils. *P. Natl. Acad. Sci. USA* 2006; 103:15806-11.
- [15] Shirahama T, Cohen AS. High-Resolution Electron Microscopic Analysis Of The Amyloid Fibril. *J. Cell. Biol.* 1967; 33: 679-708.
- [16] Mishra A, Loo Y, Deng R, Chuah YJ, Hee HT, Ying JY, Hauser CA. Ultrasmall Natural Peptides Self-Assemble To Strong Temperature-Resistant Helical Fibers In Scaffolds Suitable For Tissue Engineering. *Nano Today* 2011; 6: 232-9.
- [17] Zhuo S, Chen J, Wu G, Xie S, Zheng L, Jiang X, Zhu X. Quantitatively Linking Collagen Alteration And Epithelial Tumor Progression By Second Harmonic Generation Microscopy. *Appl. Phys. Lett.* 2010; 96: 213704.
- [18] Chen X, Nadiarynkh O, Plotnikov S, Campagnola PJ. Second Harmonic Generation Microscopy For Quantitative Analysis Of Collagen Fibrillar Structure. *Nat. Protoc.* 2012; 7: 654-69.

- [19] Latour G, Gusachenko I, Kowalczyk L, Lamarre I, Schanne-Klein MC. In vivo structural imaging of the cornea by polarization-resolved second harmonic microscopy. *Biomed. Opt. Express* 2012; 3: 1-15.
- [20] Bancelin S, Aime C, Gusachenko I, Kowalczyk L, Latour G, Coradin G, Schanne-Klein MC, Determination of collagen fibril size via absolute measurements of second-harmonic generation signals. *Nat. Commun.* 2014; 5: 4920.
- [21] Mercatelli R, Ratto F, Rossi F, Tatini F, Menabuoni L, Malandrini A, Nicoletti R, Pini R, Pavone FS, Cicchi RR. Three-dimensional mapping of the orientation of collagen corneal lamellae in healthy and keratoconic human corneas using SHG microscopy. *J. Biophoton.* 2017; 10: 75-83.
- [22] Mercatelli R, Mattana S, Capozzoli L, Ratto F, Rossi F, Pini R, Fioretto D, Pavone FS, Caponi S, Cicchi R. Morpho-mechanics of human collagen superstructures revealed by all-optical correlative micro-spectroscopies. *Communications Biology* 2019; 2: 117.
- [23] Franken PA, Hill AE, Peters CE, Weinreich G. Generation Of Optical Harmonics. *Phys. Rev. Lett.*, 1961; 7: 118-9.
- [24] Zipfel WR, Williams RM, Christie R, Nikitin AY, Hyman BT, Webb WW. Live Tissue Intrinsic Emission Microscopy Using Multiphoton-Excited Native Fluorescence And Second Harmonic Generation. *P. Natl. Acad. Sci. USA* 2003; 100: 7075-80.
- [25] Pantazis P, Maloney J, Wu D, Fraser SE. Second Harmonic Generating (SHG) Nanoprobes For In Vivo Imaging. *P. Natl. Acad. Sci. USA* 2010; 107: 14535-40.
- [26] Campagnola PJ, Loew LM. Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms. *Nat. Biotech.* 2003; 21: 1356-60.
- [27] Yu F, Zhuo S, Qu Y, Choudhury D, Wang Z, Iliescu C, Yu H. On Chip Two-Photon Metabolic Imaging For Drug Toxicity Testing. *Biomicrofluidics* 2017; 11: 034108.
- [28] Chan KH, Lee WH, Ni M, Loo Y, Hauser CAE. C-Terminal Residue of Ultrashort Peptides Impacts on Molecular Self-Assembly, Hydrogelation, and Interaction with Small-Molecule Drugs. *Sci. Rep.* 2018; 8: 17127.
- [29] Ni M, Tresset G, Iliescu C. Self-Assembled Polysulfone Nanoparticles Using Microfluidic Chip. *Sensor. Actuat. B-Chem.* 2017; 252: 458-62.
- [30] Iliescu C, Tresset G. Microfluidics-Driven Strategy for Size-Controlled DNA Compaction by Slow Diffusion through Water Stream. *Chem. Mater.* 2015; 27: 8193-7.
- [31] Tresset G, Marculescu C, Salonen A, Ni M, Iliescu C. Fine Control Over The Size Of Surfactant–Polyelectrolyte Nanoparticles By Hydrodynamic Flow Focusing. *Anal. Chem.*, 2013; 85: 5850-6.
- [32] Kurtz S, Perry T. A Powder Technique For The Evaluation Of Nonlinear Optical Materials. *J. Appl. Phys.*, 1968; 39: 3798-813.
- [33] Bourhill G, Mansour K, Perry KJ, Khundkar L, Sleva ET, Kern R, Perry JW, Williams ID, Kurtz SK. Powder Second Harmonic Generation Efficiencies Of Saccharide Materials. *Chem. Mater.* 1993; 5: 802-8.
- [34] Su PJ, Chen WL, Li TH, Chou CK, Chen TH, Ho YY, Huang CH, Chang SJ, Huang YY, Lee HS. The Discrimination Of Type I And Type II Collagen And The Label-Free Imaging Of Engineered Cartilage Tissue. *Biomaterials* 2010; 31: 9415-21.
- [35] Nasir MN, Benichou E, Loison C, Russier-Antoine I, Besson F, Brevet PF. Influence Of The Tyrosine Environment On The Second Harmonic Generation Of Iturinic Antimicrobial Lipopeptides At The Air–Water Interface. *Phys. Chem. Chem. Phys.*, 2013; 15: 19919-24.
- [36] Duboisset J, Deniset-Besseau A, Benichou E, Russier-Antoine I, Lascoux N, Jonin C, Hache F, Schanne-Klein MC, Brevet PF. A Bottom-Up Approach To Build The Hyperpolarizability Of Peptides And Proteins From Their Amino Acids. *J. Phys. Chem. B* 2013; 117: 9877-81.

- [37] Anandalakshmi V, Murugan E, Leng EGT, Ting LW, Chaurasia SS, Yamazaki T, Nagashima T, George BL, Peh GSL, Pervushin K, Lakshminarayanan R, Mehta JS. Effect of position-specific single-point mutations and biophysical characterization of amyloidogenic peptide fragments identified from lattice corneal dystrophy patients. *Biochem J.* 2017; 474:1705-25.
- [38] Zhang Y, Akins ML, Murari K, Xi J, Li MJ, Luby-Phelps K, Mahendroo M, Li X. A Compact Fiber-Optic SHG Scanning Endomicroscope And Its Application To Visualize Cervical Remodeling During Pregnancy. *P. Natl. Acad. Sci. USA* 2012; 109: 12878-83.

Figure 1. (a) Comparison of the SHG signal intensity from endogenous collagen I to LK6 peptide particles. Five spots from each image were chosen and the SHG signal intensity was normalized to collagen I (mean $\pm$ standard deviation, n=5); (b) SEM image of collagen fibers; (c) SEM image peptide particles. d) SHG signal spectrum of LK₆ peptide. Signal was ranging from 380 to 550 nm with excitation wavelength of 810 nm and peak emission wavelength was shown at 405 nm; (e) Spectral peaks at various excitation wavelengths spanning a broad spectral region (810-890 nm); (f) Emission λ -scan of the SHG signal ($\lambda_{\text{ex}} = 850$ nm) acquired from SHG imaging of LK₆ peptide particles. The solid spheres represent back scattering SHG data and the solid line represents a Gaussian fit. The full width at half-maximum of the fitted curve bears a $1/\sqrt{2}$ relation to the spectral profile of the corresponding beam; (g) Log-log plot of the above SHG signal measurements demonstrating a $\log[I_{425}] = 0.45 + 2.01 \times \log[I_{850}]$ dependence, quadratic to a good approximation, consistent with nonlinear second order optical upconversion.

Figure 2. Cytotoxicity studies of LK6 peptide treated HDF and HeLa cells. (a) Cell viability at 48 h of HDF and HeLa cells incubated with cell culture media containing LK₆ peptide solutions, as determined using MTS assay (mean $\pm$ standard deviation, n=9). Concentrations ranged from 0.1 to 1000 $\mu\text{g/mL}$. (b) Cytotoxicity determined by Calcein AM/EthD-1 (live/dead, green/red) staining method after 48 h treated with 1 and 1000 $\mu\text{g/mL}$ LK₆ peptide solutions using non-treated cells as control. Scale bars: 100 μm .

Figure 3. SHG images of four pristine peptides with natural amyloidogenic core sequences. The whole window was covered with pristine peptides. However, only part of the peptides can be visualized under SHG microscopy. The SHG intensity follows the order of KE7 > GA6 > DF5 > NL6. Scale bar = 50 μm .

Figure 4. Cellular uptake of biotin-conjugated LK₆. Upper panel: HeLa cells exposed to biotin-conjugated LK₆ peptide containing medium. Peptides in HeLa cells displayed pseudo red color (SHG) and green color (fluorescence). Lower panel: HeLa cells exposed to normal medium without peptide (control). Scale bars: 50 μ m

Figure 5. SHG images of human corneal biopsy samples. (a) Sample from a normal cornea (control); (b) Sample from a patient who has Lattice corneal dystrophy (LCD) disease. Collagen fibrils can be seen under SHG (green). Arrow indicates the formation of an amyloid. Scale bar = 20 μm .

Table 1. Powder SHG efficiencies of various amyloid-like peptides and human collagen subtypes.

Amyloid-like peptides / human collagens	$I^{2\omega}/I^{2\omega}(\text{sucrose})^{\text{a)}}$
IVK (IK ₃)	3.31 ± 0.57
LIVAGD (LD ₆)	3.06 ± 0.65
ILVAGS (IS ₆)	1.96 ± 0.58
LIVAGK (LK ₆)	0.95 ± 0.19
KLVFFAE (KE ₇)	3.18 ± 0.67
GGVVIA (GA ₆)	1.52 ± 0.43
DFNKF (DF ₅)	0.89 ± 0.33
NFGAIL (NL ₆)	0.76 ± 0.21
Collagen Type I	1.23 ± 0.36
Collagen Type II	0.41 ± 0.13
Collagen Type III	N.D. ^{b)}
Collagen Type IV	N.D. ^{b)}
Collagen Type V	N.D. ^{b)}

^{a)} Value = Average ± standard deviation; ^{b)} N.D. = not detected.