

ULTRASHORT PEPTIDES AS EXOGENOUS SECOND HARMONIC PROBES FOR BIOIMAGING APPLICATIONS

FIELD OF INVENTION

The present invention is in the area of biodevices and diagnostics. The invention generally relates to second harmonic generation (SHG) microscopy for disease diagnosis and envisioning of tissue engineering scaffolds.

BACKGROUND

The following discussion of the background to the invention is intended to facilitate an understanding of the present invention. However, it should be appreciated that the discussion is not an acknowledgment or admission that any of the material referred to was published, known or part of the common general knowledge in any jurisdiction as at the priority date of the application.

Second harmonic generation (SHG) microscopy is a powerful imaging tool for disease diagnosis and envision of tissue engineering scaffolds. Second harmonic (SH) probes have been developed for in-vivo imaging in recent years as they possess many advantages over the classic fluorescent probes. SH probes are typically made of inorganic nanomaterials, such as barium titanate nanocrystals. Although these materials have achieved certain success in molecular imaging, they are concerned for their health and environmental issues.

In addition, second-harmonic generation (SHG) microscopy is a nonlinear optical (NLO) imaging technique that has emerged in recent years to visualize structures or functions of cells, tissues and organisms [1]. It has the advantages of being label-free, inherent three-dimensional (3D) resolution, near-infrared (NIR) excitation for superior optical penetration, lower photo-damage, and capable of providing quantitative information, which makes it an attractive tool for high resolution imaging [1-3]. It has been used to diagnose a wide range of diseases including cancer, liver fibrosis and skin damage [4]. It

has also been used in tissue engineering and regenerative medicine [5] to envision three-dimensional biomaterial scaffolds and extracellular matrices that secreted by cells [6-7].

SHG is an optical frequency doubling process: when a non-linear optical (NLO) material interacts with light, two NIR incident photons can be converted into one emerging visible photon at exactly twice the energy (or half the wavelength) [1,4,9]. This NLO material requires a noncentrosymmetric structure. Examples include potassium niobate (KNbO₃), lithium niobate (LiNbO₃), barium titanate (BaTiO₃), potassium titanyl phosphate (KTiOPO₄, KTP), and zinc oxide (ZnO), Gallium nitride (GaN), Silicon carbide (SiC), noble metal particles, and quantum dots [8,9].

Inorganic nanomaterials, such as BaTiO₃ nanocrystal has been used a second harmonic (SH) probe for *in vivo* imaging [13]. These SHG nanoprobe are superior to the existing fluorescent dyes in terms of dye bleaching, signal saturation and narrow emission spectrum (full width at half maximum (FWHM) < 5 nm) [13, 14]. Although a report showed that BaTiO₃ nanocrystals are non-toxic to zebrafish [13], inorganic nanomaterials have been questioned for their health and environmental issues which enormously limit their clinical applications [15-16].

SUMMARY

The present invention provides a peptide based biomaterial for visualization by SHG microscopy. The present invention comprises the following features:

F1) Use of short peptides as a non-linear optical (NLO) material for second harmonic generation (SHG) microscopy.

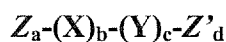
F2) A preferred peptide of F1 is LIVAGK (LK6), which contains a non-polar aliphatic tail (with decreasing hydrophobicity) and a polar head; and can self-assemble into hydrogels;

A. Wherein the peptide forms a tunable fibrous structure

B. For *in vitro* and *in vivo* imaging applications

C. In disease diagnostics such as amyloidosis, including 1) neuro-degenerative amyloidosis, e.g. Alzheimer's (AD), Parkinson's, Huntington's (PD), 2) non-neuropathic localized amyloidosis such as in Type II Diabetes, and 3) systemic amyloidosis that occurs in multiple tissues, e.g. cataracts and lattice corneal dystrophy (LCD).

In accordance with an aspect of the invention, the invention provides the use of a peptide and/or peptidomimetic, capable of self-assembling and forming a hydrogel, having the general formula I:



(I)

wherein

Z is an N-terminal protecting group;

a is 0 or 1, preferably 1;

X is, at each occurrence, independently selected from the group consisting of aliphatic amino acids and aliphatic amino acid derivatives, and wherein the overall hydrophobicity decreases from N- to C-terminus;

b is an integer selected from 1 to 7;

Y is selected from the group consisting of polar amino acids and polar amino acid derivatives;

c is 1 or 2;

Z' is a C-terminal protecting group; and

d is 0 or 1,

and **b + c** is at least 2,

as a non-linear optical (NLO) material for second harmonic generation (SHG) microscopy and/or imaging.

Preferably, said aliphatic amino acids and aliphatic amino acid derivatives, and said polar amino acids and polar amino acid derivatives are either D-amino acids or L-amino acids.

Preferably, said aliphatic amino acids are selected from the group consisting of alanine (Ala, A), homoallylglycine, homopropargylglycine, isoleucine (Ile, I), norleucine, leucine (Leu, L), valine (Val, V) and glycine (Gly, G), preferably from the group consisting of alanine (Ala, A), isoleucine (Ile, I), leucine (Leu, L), valine (Val, V) and glycine (Gly, G).

Preferably, all or a portion of said aliphatic amino acids are arranged in an order of decreasing amino acid size in the direction from N- to C-terminus, wherein the size of the aliphatic amino acids is defined as $I = L > V > A > G$.

Preferably, said aliphatic amino acids have a sequence selected from

LIVAG (SEQ ID NO: 1),

ILVAG (SEQ ID NO: 2),

ILVAA (SEQ ID NO: 3),

LIVAA (SEQ ID NO: 4),

LAVAG (SEQ ID NO: 5),

IAVAG (SEQ ID NO: 6),

AIVAG (SEQ ID NO: 7),

GIVAG (SEQ ID NO: 8),

VIVAG (SEQ ID NO: 9),

ALVAG (SEQ ID NO: 10),

GLVAG (SEQ ID NO: 11),

VLVAG (SEQ ID NO: 12),

IVAG (SEQ ID NO: 13),

LIVA (SEQ ID NO: 14),

LIVG (SEQ ID NO: 15),

ILVA (SEQ ID NO: 16),

ILVG (SEQ ID NO: 17),

LVAG (SEQ ID NO: 18),

IVA

IVG,

VIG,

IVA,

VIA,

IV and

VI.

Preferably, **b** is an integer from 2 to 7 or 2 to 6.

Preferably, said polar amino acids are selected from the group consisting of aspartic acid (Asp, D), asparagine (Asn, N), glutamic acid (Glu, E), glutamine (Gln, Q), 5-N-ethyl-glutamine (thearine), citrulline, thio-citrulline, cysteine (Cys, C), homocysteine, methionine (Met, M), ethionine, selenomethionine, telluromethionine, threonine (Thr, T), allothreonine, serine (Ser, S), homoserine, arginine (Arg, R), homoarginine, ornithine (Orn), lysine (Lys, K), N(6)-carboxymethyllysine, histidine (His, H), 2,4-diaminobutyric acid (Dab), 2,3-diaminopropionic acid (Dap), and N(6)-carboxymethyllysine,

wherein said polar amino acid is preferably selected from the group consisting of aspartic acid, asparagine, glutamic acid, glutamine, serine, threonine, methionine, lysine, ornithine (Orn), 2,4-diaminobutyric acid (Dab), and 2,3-diaminopropionic acid (Dap).

Preferably, **c** is 2 and said polar amino acids are identical amino acids, or wherein **c** is 1 and said polar polar amino acid comprises any one of aspartic acid, asparagine, glutamic

acid, glutamine, serine, threonine, cysteine, methionine, lysine, ornithine, 2,4-diaminobutyric acid (Dab) and histidine, preferably lysine, ornithine, 2,4-diaminobutyric acid (Dab) and 2,3-diaminopropionic acid (Dap).

Preferably, (Y)_b has a sequence selected from Asp, Asn, Glu, Gln, Ser, Thr, Cys, Met, Lys, Orn, Dab, His, Asn-Asn, Asp-Asp, Glu-Glu, Gln-Gln, Asn-Gln, Gln-Asn, Asp-Gln, Gln-Asp, Asn-Glu, Glu-Asn, Asp-Glu, Glu-Asp, Gln-Glu, Glu-Gln, Asp-Asn, Asn-Asp, Thr-Thr, Ser-Ser, Thr-Ser, Ser-Thr, Asp-Ser, Ser-Asp, Ser-Asn, Asn-Ser, Gln-Ser, Ser-Gln, Glu-Ser, Ser-Glu, Asp-Thr, Thr-Asp, Thr-Asn, Asn-Thr, Gln-Thr, Thr-Gln, Glu-Thr, Thr-Glu, Cys-Asp, Cys-Lys, Cys-Ser, Cys-Thr, Cys-Orn, Cys-Dab, Cys-Dap, Lys-Lys, Lys-Ser, Lys-Thr, Lys-Orn, Lys-Dab, Lys-Dap, Ser-Lys, Ser-Orn, Ser-Dab, Ser-Dap, Orn-Lys, Orn-Orn, Orn-Ser, Orn-Thr, Orn-Dab, Orn-Dap, Dab-Lys, Dab-Ser, Dab-Thr, Dab-Orn, Dab-Dab, Dab-Dap, Dap-Lys, Dap-Ser, Dap-Thr, Dap-Orn, Dap-Dab, Dap-Dap.

Preferably, (X)_a-(Y)_b has a sequence selected from the group consisting of

LIVAGD (SEQ ID NO: 19),

ILVAGD (SEQ ID NO: 20),

ILVAAD (SEQ ID NO: 21),

LIVAAD (SEQ ID NO: 22),

LAVAGD (SEQ ID NO: 23),

IAVAGD (SEQ ID NO: 24),

AIVAGD (SEQ ID NO: 25),

LIVAGE (SEQ ID NO: 26),

LIVAGK (SEQ ID NO: 27),

ILVAGK (SEQ ID NO: 28),

ILVAAK (SEQ ID NO: 29),

IAVAGK (SEQ ID NO: 30),

AIVAGK (SEQ ID NO: 31),
LIVAGT (SEQ ID NO: 32),
ILVAAT (SEQ ID NO: 33),
IAVAGT (SEQ ID NO: 34),
AIVAGT (SEQ ID NO: 35),
LIVAD (SEQ ID NO: 36),
LIVGD (SEQ ID NO: 37),
ILVAD (SEQ ID NO: 38),
ILVGD (SEQ ID NO: 39),
LVAGD (SEQ ID NO: 40),
IVAD (SEQ ID NO: 41),
IVAK (SEQ ID NO: 42),
IVGD (SEQ ID NO: 43),
VIGD (SEQ ID NO: 44),
IVAD (SEQ ID NO: 45),
VIAD (SEQ ID NO: 46),
IVGK (SEQ ID NO: 47),
VIGK (SEQ ID NO: 48),
IVAK (SEQ ID NO: 49),
VIAK (SEQ ID NO: 50),
IIID (SEQ ID NO: 51),
IIIK (SEQ ID NO: 52),

IVD,

IVK,

IID,

LVE,

IVE,

LVD,

VIE,

VID,

VIK,

VLD,

VLE,

LLE,

LLD,

IIE,

ID,

IE,

LIVAGOrn (SEQ ID NO: 53),

ILVAGOrn (SEQ ID NO: 54),

ILVAAOrn (SEQ ID NO: 55),

IAVAGOrn (SEQ ID NO: 56),

AIVAGOrn (SEQ ID NO: 57),

LIVAGDab (SEQ ID NO: 58),

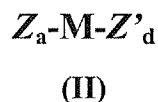
ILVAGDab (SEQ ID NO: 59),
ILVAADab (SEQ ID NO: 60),
IAVAGDab (SEQ ID NO: 61),
AIVAGDab (SEQ ID NO: 62),
LIVAGDap (SEQ ID NO: 63),
ILVAGDap (SEQ ID NO: 64),
ILVAADap (SEQ ID NO: 65),
IAVAGDap (SEQ ID NO: 66),
AIVAGDap (SEQ ID NO: 67),
LIVAOrn (SEQ ID NO: 68),
LIVGOOrn (SEQ ID NO: 69),
ILVAOrn (SEQ ID NO: 70),
ILVGOOrn (SEQ ID NO: 71),
LVAGOOrn (SEQ ID NO: 72),
LIVADab (SEQ ID NO: 73),
LIVGDab (SEQ ID NO: 74),
ILVADab (SEQ ID NO: 75),
ILVGDab (SEQ ID NO: 76),
LVAGDab (SEQ ID NO: 77),
LIVADap (SEQ ID NO: 78),
LIVGDap (SEQ ID NO: 79),
ILVADap (SEQ ID NO: 80),

ILVGDap (SEQ ID NO: 81),
LVAGDap (SEQ ID NO: 82),
IVAOm (SEQ ID NO: 83),
IVGOm (SEQ ID NO: 84),
VIGOm (SEQ ID NO: 85),
IVAOm (SEQ ID NO: 86),
VIAOm (SEQ ID NO: 87),
IVADab (SEQ ID NO: 88),
IVGDab (SEQ ID NO: 89),
VIGDab (SEQ ID NO: 90),
IVADab (SEQ ID NO: 91),
VIADab (SEQ ID NO: 92),
IVADap (SEQ ID NO: 93),
IVGDap (SEQ ID NO: 94),
VIGDap (SEQ ID NO: 95),
IVADap (SEQ ID NO: 96),
VIADap (SEQ ID NO: 97),
IVOrn,
IVDab,
IVDap,
VIOrn,
VIDab,

VIDap, and

ILVAGS (SEQ ID NO: 98).

In accordance with an aspect of the invention, the invention provides the use of a peptide having the general formula II:



wherein

Z is an N-terminal protecting group;

a is 0 or 1, preferably 1;

M is an amino acid sequence selected from SEQ ID Nos. 99 to 102;

Z' is a C-terminal protecting group; and

d is 0 or 1,

as a non-linear optical (NLO) material for second harmonic generation (SHG) microscopy and/or imaging.

The peptides with amino acid sequences selected from SEQ ID Nos. 99 to 102 are four natural amyloidogenic core sequences (KE₇, GA₆, DF₅, NL₆).

See below for further details, e.g. Table 1.

In both general formulas I and II:

Preferably, **a** is 1 and said N-terminal protecting group **Z** has the general formula –C(O)–R, wherein R is selected from the group consisting of H, unsubstituted or substituted alkyls, and unsubstituted or substituted aryls, wherein R is preferably selected from the group consisting of methyl, ethyl, propyl, isopropyl, butyl and isobutyl.

Preferably, said N-terminal protecting group **Z** is an acetyl group.

Preferably, said N-terminal protecting group **Z** is a peptidomimetic molecule, including natural and synthetic amino acid derivatives, wherein the N-terminus of said peptidomimetic molecule may be modified with a functional group selected from the group consisting of carboxylic acid, amide, alcohol, aldehyde, amine, imine, nitrile, an urea analog, phosphate, carbonate, sulfate, nitrate, maleimide, vinyl sulfone, azide, alkyne, alkene, carbohydrate, imide, peroxide, ester, aryl, ketone, sulphite, nitrite, phosphonate, and silane.

Preferably, said C-terminal protecting group **Z'** is an amide group, wherein, preferably, the C-terminus has the formula -CONHR or -CONRR', with R and R' being selected from the group consisting of H, unsubstituted or substituted alkyls, and unsubstituted or substituted aryls.

Preferably, said C-terminal protecting group **Z'** is an ester group, wherein, preferably, the C-terminus has the formula -CO₂R, with R being selected from the group consisting of H, unsubstituted or substituted alkyls, and unsubstituted or substituted aryls.

Preferably, said C-terminal group **Z'** is a peptidomimetic molecule, including natural and synthetic amino acid derivatives, wherein the C-terminus of said peptidomimetic molecule may be modified with a functional group selected from the group consisting of carboxylic acid, amide, alcohol, aldehyde, amine, imine, nitrile, an urea analog, phosphate, carbonate, sulfate, nitrate, maleimide, vinyl sulfone, azide, alkyne, alkene, carbohydrate, imide, peroxide, ester, aryl, ketone, sulphite, nitrite, phosphonate, and silane.

Preferably, **b + c** is at least 2, preferably 2 to 9, more preferably 3 to 7 or 2 to 7.

Preferably, the peptide and/or peptidomimetic is visualized under a second harmonic generation (SHG) microscopy or by SHG imaging without additional dye(s) and/or label(s).

Preferably, the peptide and/or peptidomimetic comprises further compound(s), such as

- small molecules,

such as (but not limited to) sugars, alcohols, hydroxy acids, amino acids, vitamins, biotin, L-Dopa, thyroxine;

- bioactive molecules or moieties,

such as growth factors, cytokines, lipids, cell receptor ligands, hormones, prodrugs, drugs, vitamins, antigens, antibodies, antibody fragments, oligonucleotides (including but not limited to DNA, messenger RNA, short hairpin RNA, small interfering RNA, microRNA, peptide nucleic acids, aptamers), saccharides;

- label(s), dye(s),

such as imaging contrast agents;

- pathogens,

such as viruses, bacteria and parasites;

- micro- and nanoparticles

or combinations thereof

which are preferably covalently attached or coupled to the peptide and/or peptidomimetic, preferably to the C-terminal group Z' , amino acid side chain(s) and/or linker,

wherein said attachment or coupling can be carried out before, during or after self-assembly of the peptide and/or peptidomimetic.

Preferably, the at least one peptide and/or peptidomimetic forms a fibrous structure, which is preferably tunable, such as

- in the way of peptide material's preparation (fiber structures, particle structures),

- for excitation wavelength tunability of SHG signals.

Preferably, the fibrous structure is a fibrous scaffold resembling the collagen fiber in extracellular matrix.

Preferably, the at least one peptide and/or peptidomimetic as defined herein is stable in aqueous solution at physiological conditions at ambient temperature for a period of time in the range from 1 day to at least 6 months, preferably to at least 8 months more preferably to at least 12 months.

Preferably the at least one peptide and/or peptidomimetic as defined herein is stable in aqueous solution at physiological conditions, at a temperature up to 90 °C, for at least 1 hour.

Preferably, the peptide and/or peptidomimetic is present at a concentration in the range of from 0.1 % to 30 % (w/w), preferably 0.1 % to 20 % (w/w), more preferably 0.1 % to 10 % (w/w), more preferably 0.1 % to 5 % (w/w), even more preferably 0.1 % to 3 % (w/w), with respect to the total weight of said hydrogel or fibrous structure.

In accordance with another aspect of the invention there comprises a method of preparing a hydrogel or fibrous structure, the method comprising dissolving at least one peptide and/or peptidomimetic - as defined herein with general formula I - of the first aspect of the invention in an aqueous solution.

Preferably, the dissolved peptide and/or peptidomimetic in aqueous solution is further exposed to temperature, wherein the temperature is in the range from 20 °C to 90 °C, preferably from 20 °C to 70 °C.

Preferably, the at least one peptide and/or peptidomimetic is dissolved at a concentration from 0.01 µg/ml to 100 mg/ml, preferably at a concentration from 1 mg/ml to 50 mg/ml, more preferably at a concentration from about 1 mg/ml to about 20 mg/ml.

In accordance with another aspect of the invention, a method of preparing peptide particles is provided, the method comprising dissolving at least one peptide and/or peptidomimetic as defined herein with general formula I in an aqueous solution, preferably phosphate buffered saline (PBS), and solvent evaporation from said aqueous solution, such as by vacuum drying.

In another aspect of the invention, the invention provides a use according to the present invention or the use of a hydrogel, fibrous structure or peptide particle obtained by a

method according to the present invention, as probes for (bio-)imaging, preferably as exogenous SHG probes.

In another aspect of the invention, the invention provides a use according to the present invention or the use of a hydrogel, fibrous structure or peptide particle obtained by a method according to the present invention, for monitoring cells, *in vitro* and/or *in vivo* (bio-)imaging.

In another aspect of the invention, there comprises a use according to the present invention or the use of a hydrogel, fibrous structure or peptide particle obtained by a method according to the present invention for visualizing peptide-based tissue engineering scaffolds, preferably comprising studying biological processes, such as cell adhesion, migration and differentiation.

In accordance with another aspect of the invention, the invention provides the use of a hydrogel, fibrous structure or peptide particle obtained by a method according the present invention as diagnostics, preferably for the diagnosis of diseases comprising or associated with the aggregation of peptide or protein structures, such as amyloidosis, including 1) neuro-degenerative amyloidosis, e.g. Alzheimer's (AD), Parkinson's, Huntington's (PD), 2) non-neuropathic localized amyloidosis such as in Type II Diabetes, and 3) systemic amyloidosis that occurs in multiple tissues, e.g. cataracts and lattice corneal dystrophy (LCD).

In another aspect of the invention, the invention provides the use of a hydrogel, fibrous structure or peptide particle obtained by a method according to the present invention as drug or gene delivery vehicle.

In another aspect of the invention, the invention provides a peptide particle comprising the peptide and/or peptidomimetic as defined herein, preferably obtained by a method of the present invention (according to claim 28).

Preferably, the peptide particle has a particle size in the range from submicron to 10 microns.

In accordance with another aspect of the invention, the invention provides a drug or gene delivery vehicle comprising the peptide and/or peptidomimetic as defined herein, and a drug or gene to be delivered.

In accordance with another aspect of the invention, the invention provides a peptide-based tissue engineering scaffold comprising the peptide and/or peptidomimetic as defined herein, preferably obtained by a method according to the present invention.

In accordance with another aspect of the invention, the invention provides a biosensor or bioimaging device comprising the peptide and/or peptidomimetic as defined herein.

In accordance with another aspect of the invention, the invention provides a wound dressing comprising the peptide and/or peptidomimetic as defined herein.

Other aspects and features of the present invention will become apparent to those skilled in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF DRAWINGS

Embodiments of the invention will now be described by way of example with reference to the accompanying drawings.

Figure 1: *Fine structure and SHG activity of collagen and amyloid-like peptides.*

(a) Comparison of the SHG signal intensity from endogenous collagen I (inset, *in vivo* SHG image of the normal capsular region of rat liver, left) to LK₆ peptide particles (inset, SHG image of LK₆ peptide particles, right). Instrument settings for both SHG imaging were the same. Five spots from each image were chosen and the SHG signal intensity was normalized to collagen I (mean $\pm$ standard deviation, n=5); SEM images of collagen fibers (b) and peptide particles (c).

Figure 2. *LK₆ peptides are SHG active.*

(a) 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.

- (b) Spectral peaks at various excitation wavelengths spanning a broad spectral region (810-890 nm).
- (c) Emission λ -scan of the SHG signal ($\lambda_{\text{ex}} = 850 \text{ nm}$) 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.
- (d) 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 3. *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 μm .

Figure 4. *Interface of LK₆ peptide particles and animal fat tissue.*

- (a) Schematic illustration of experiment arrangement: a low intensity ultrafast infrared laser penetrating glass cover slip and rat fat tissue (thickness $\sim 50 \mu\text{m}$) and viewing LK₆ peptide particles sandwiched in between the glass slide and the glass cover slip.
- (b) Three dimensional image of LK₆ peptide particles in the entire volume of ($225 \times 225 \times 4.5 \mu\text{m}^3$). SHG z-stack was generated with 0.5 μm step. Total nine images were taken ($z = 4.5 \mu\text{m}$).

Figure 5. *SHG images of human corneal biopsy samples.*

- (a) Sample from a normal person (control; (b)) and a patient who has Lattice corneal dystrophy (LCD) disease. Collagen fibrils can be seen under SHG (green). Arrow indicates the amyloid deposit. Scale bar = 20 μm .

Figure 6: *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 $KE_7 > GA_6 > DF_5 > NL_6$. Scale bar = 50 μm .

Figure 7: *Cytotoxicity studies of LK_6 peptide treated HDF and HeLa cells.*

(a) Cell viability at 48 h of HDF and HeLa cells incubated with cell culture media containing LK_6 peptide solutions, as determined by the 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 $\mu\text{g/mL}$ LK_6 peptide solutions using non-treated cells as control.

Scale bars: 100 μm .

Other arrangements of the invention are possible and, consequently, the accompanying drawings are not to be understood as superseding the generality of the preceding description of the invention.

DETAILED DESCRIPTION

Peptide/protein based biomaterials can also be assembled into large, ordered non-centrosymmetric structures, which make them possible to produce second harmonic signals [1]. Famous example include collagen I [10], diphenylalanine (FF, a dimer peptide) [11], elastin and muscle myosin [12]. Among them, collagen I has been commonly used as an endogenous SHG probe for disease diagnosis [4].

Peptide/protein based biomaterials appear to be a promising candidate for a SH probe due to their biocompatibility and biodegradability. A peptide-based biomaterial, i.e. a ultrashort peptide biomaterial is presented that can be directly visualized by second harmonic generation microscopy. It shows excitation wavelength tenability, similar second harmonic signal intensity as endogeneous collagen I, and cytocompatibility with two human cell lines.

Based on the above, the inventors concluded that the purely synthetic, peptide-based material holds the potential to be used as a future SH probe for bioimaging applications.

In particular the Supramolecular assembly of collagen in tissues has been visualized by SHG microscopy for more than a decade. It was discovered by the inventors that striking similarity exists between ultrashort peptides and collagen fibers. Inspired by such findings, the non-linear optical (NLO) properties of a hexamer peptide, Ac-LIVAGK-NH₂ (LK6) was investigated.

A hexamer peptide Ac-LIVAGK-NH₂ (LK6) was designed and synthesized. This hexamer peptide can self-assemble into hydrogels through hydrophobic interactions, ionic interactions, hydrogen bonding and van de Waals forces. It contains a non-polar aliphatic tail and a polar head. The non-polar aliphatic tail was designed to have a decreasing hydrophobicity. This type of arrangement favours a parallel-antiparallel stacking. The nanostructured peptide aggregates then form a fibrous scaffold, which resembles the collagen fiber in extracellular matrix [17-18]. In this work, the ultrashort peptide is submitted as a novel NLO material for SHG imaging. Results suggest this purely synthetic, peptide-based material holds the potential to be used as a future SH probe for bioimaging applications.

Supramolecular assembly of collagen in tissues has been visualized by SHG microscopy for more than a decade [4]. In particular, collagen I (in blood vessels, bone, cornea, kidney, liver, lung, ovary, skin and tendon), and collagen II (in cartilage) have been found to efficiently produce SHG [4]. Since diseased and normal tissues show different distribution patterns of collagen fiber alignment and intensity, their SHG images can be used for disease diagnosis.

Success has been achieved in many clinical applications, for example cancer delineation and liver fibrosis [4]. Collagen is a triple-helix molecule and can self-assemble into fibers with diameters up to a few micrometres. A recent study [19] showed that the origin of SHG signals from collagen fibers possibly lies in their peptide bonds. The building blocks of collagen fiber were mimicked by tri-amino acid peptides PPG and GGG (P and G are the one letter code for Proline and Glycine respectively) and 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

$\times 10\text{--}30$ esu (esu is the unit of the first hyperpolarizability) [19]. However, the first hyperpolarizability of collagen I was found to be $(1250 \pm 20) \times 10\text{--}30$ esu [19], which could be viewed as ten thousand trimers combining together.

Previous studies from our lab showed striking similarity between the ultrashort peptides and collagen fibers [20]. Inspired by these findings, we investigated the NLO properties of our hexamer peptide, LK6. Based on the non-linearity of the intrinsic peptide bonds, we hypothesizes that our hexamer peptide, like collagen (which could be viewed as GX1X2, with X1 and X2 corresponding mainly to proline and hydroxyproline), should be able to generate SHG signals.

Peptide particles were prepared by solvent evaporation. Phosphate buffered saline (PBS) was chosen as the solvent. After vacuum drying, the various sizes and shapes of peptide particles were formed (FE-SEM image, Figure 1c). The particle size was ranging from submicron to 10 microns and the particle shape was irregular. Peptide particles also tended to form larger aggregates during drying (Figure 2d, Figure 1a inset and Figure 4b). Currently, we are manipulating preparation parameters to achieve better control over the size and the shape of peptide particles.

To test our hypothesis, we directly examined LK6 peptide particles using SHG imaging. The SHG signal from LK6 peptide particles was strong and spectrally well defined (Figure 2a). Illuminating the LK6 peptide particles at 810 nm with a conventional two-photon microscope yielded an SHG signal profile with a discrete peak at 405 nm.

SHG image (green) of LK6 peptide particles was shown in Figure 1a (inset). LK6 peptide particles showed bright SHG signals. The size and shape of particles can be visualized, consistent with FE-SEM image (Figure 1a). We then examined the excitation wavelength tunability of SHG signals from these peptide particles. As shown in Figure 2b, the exciting wavelength was increased from 810 to 890 nm in a conventional two-photon laser-scanning microscopy, the wavelength of the SHG signals from the LK6 peptide particles increased from 405 to 450 nm respectively. Excitation wavelength tunability is

one of the advantages of SHG. This enables us freely tune the excitation wavelength to best match the optical properties of the sample.

Biological samples, such as cells and tissues, are known to have auto-fluorescence. With the excitation wavelength tunability, we can avoid auto-fluorescence. In addition, we can further increase penetration depth in tissues by simply increasing excitation wavelength. Figure 2c shows the emission wavelength data of the SHG signal ($\lambda_{\text{ex}}=850$ nm). The data in the graph were fitted to a Gaussian curve exhibiting a maximum at 425nm, which is exactly half the excitation wavelength of 850nm. The full width at half-maximum of Gaussian distribution (~10nm) obeys a relationship to the corresponding wavelength profile of the fundamental beam (~15nm). Moreover, the dependence of the output signal on irradiation intensity was measured by varying the 850nm excitation intensity. The linear regression was applied to the log-log plots revealed a quadratic power dependence of SHG intensity to the power intensity, as shown in the Figure 2d.

$$\log [I_{425}] = 0.45 + 2.01 * \log [I_{850}]$$

This confirms the two-photon nature of the emission from LK6 ultrashort peptide particles.

As mentioned previously, collagen fibres produce much stronger SHG signals compared to their building blocks (ten thousand times higher). It is interesting to compare our hexamers to endogenous collagen in terms of SHG signal intensity. In this case, we chose the normal capsular region of rat liver. From *in vivo* imaging, we observed bright SHG signals from its collagen (Figure 1a, left inset). Interestingly, our hexamer shows similar intensity to that of collagen (Figure 1a). This suggested that our hexamer can be used as SHG probes for bioimaging.

In order to use LK6 for bioimaging applications, such as monitoring cells and *in vivo* imaging, we assessed the cytotoxicity of LK6 by the MTS test. The MTS test was applied to two human cell lines, HeLa and primary human dermal fibroblasts (HDF). The toxicity of LK6 was measured at 0.1, 1, 10, 100 and 1000 $\mu\text{g/mL}$. Cells were exposed to cell culture media containing LK6 solution for 48 h. Cell viability is reported in Figure

7a. 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 (0.1 to 1000 $\mu\text{g/mL}$). To further confirm these results, we stained cells with and without peptide treatment with solutions containing a mixture of calcein AM and ethidium homodimer-1 (EthD-1). Calcein AM can penetrate cell membrane and be converted to cell membrane impermeable green fluorescent calcein, while red fluorescent EthD-1 is excluded by the intact cell membrane of live cells. Figure 7b shows the representative live/dead images of cells in response to LK6 solutions (1 and 1000 $\mu\text{g/mL}$). LK6 shows no toxicity to both HDF and HeLa cells, in agreement with results of MTS assays.

Biological Application

The ability to manufacture SH probes entirely out of LK6 peptides offers additional opportunities as they are biocompatible, biodegradable. A possible application for all-protein/peptide spectra elements such as LK6 is to use them within biological tissues to provide exogenous spectral signatures. These structures can be visualized as all-peptide based contract agents that do not require dyes or chemicals. This is validated by performing an *ex vivo* experiment in which LK6 peptides are placed under slices of rat fat tissues (Figure 4a). Peptide particles (green, Figure 4b) can still be seen clearly as a laser penetrates the tissue. This is a proof of the concept for using LK6 as exogenous SH probes for bioimaging.

As our ultrashort peptides show striking similarity to collagen (an extracellular matrix protein, SHG-active molecule and fibrous structures), we can study the interaction between cells and these ultrashort peptides using SHG imaging. Many fundamental biological processes including cell adhesion, migration and differentiation on these ultrashort peptide matrices can be directly visualized by SHG. The three-dimensional architecture of the tissue engineering scaffolds made of these ultrashort peptides can be viewed *in situ* and without labelling. The degradation of these scaffolds can be monitored and traced. It also opens the possibility to study the interaction between scaffolds and seeded cells *in vitro* prior implantation, as well as to monitor the interplay between the scaffolds with the surrounding native tissue *in vivo*.

In conclusion, we demonstrated that our hexamer peptide, LK6 shows strong SHG signals, similar to those of endogenous normal capsule collagen of rat liver. This ultra-short peptide is biocompatible and biodegradable, which makes it attractive as a future candidate of SH probe for bioimaging.

Further preferred embodiments

Abstract

Amyloid-like peptides are an ideal model system to study the mechanisms of amyloidosis which may lead to many human diseases, such as Alzheimer's. Recently, amyloid-like peptides were also used as a new type of biomaterials as tissue engineering scaffolds and drug delivery vehicles. Here we report that these amyloid-like peptides show strong second harmonic generation (SHG) effect, signals 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 viewed under thick fat tissues (*ex vivo*) and inside cells (*in vitro*). This interesting property makes these amyloid-like peptides second harmonic probes for bioimaging applications. Furthermore, SHG microscopy provides us a simple and label-free approach to detect amyloidosis. Lattice corneal dystrophy (LCD) was chosen as a model disease of amyloidosis. Both normal and diseased human corneal biopsy samples were examined under SHG microscopy. Morphological difference between these two can be easily recognized without adequate medical knowledge. Therefore, SHG can be a useful tool for disease diagnosis.

Amyloidosis is responsible for more than fifty human diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), type II diabetes, cataracts and lattice corneal dystrophy (LCD). Inhibition of amyloidosis is a promising strategy for the development of therapeutic agents to treat such diseases. Better understanding of amyloidosis mechanisms may avoid using a trial and error method in searching for amyloid inhibitors, eventually designing or even predicting molecules with inhibitory effects to amyloids. However, the mechanism of amyloidosis is still largely unclear. One logical mechanistic

approach is to study how these amyloid-like peptides/proteins fold or misfold [29]. Ultrashort peptides with only three to seven amino acids provide us an ideal model system for mechanistic studies. For example, GGVVIA (GA₆) and KLVFFAE (KE₇), can self-assemble into amyloid-like aggregates [30, 31]. These two 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 [31, 17]. Both these natural and synthetic peptides are amyloid-like peptides. They all form cross- β peptide structure at molecular level. Morphologically they are usually micrometers in length but only 7-10 nm in diameter [32]. Mechanically they are rigid, with strength comparable to steel [33]. Ultrashort amyloid-like peptides can be not only useful for searching amyloid inhibitors [17], but also as a new type of biomaterials with desirable properties such as biocompatibility and biological activities [31, 34].

Here we report that ultrashort amyloid-like peptides exhibit strong SHG effect. SHG is a nonlinear optical (NLO) effect, first demonstrated in 1961 [35]. Recently, microscopy based on SHG effect has emerged as a powerful bioimaging technique [4]. It provides many significant advantages over the conventional fluorescence imaging techniques for its deeper optical penetration, lower photo-damage and longer observation time [4, 1]. Several amyloid-like peptides (both synthetic and natural), examined under SHG microscopy, revealed they are SHG-active. This interesting property makes these amyloid-like peptides second harmonic probes for bioimaging applications. Furthermore, 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.

For further details, see Example.

Key features of the invention

- A novel class of peptides, which only consists of 3-7 amino acids, possesses non-linear optical properties, which can be viewed under second harmonic generation (SHG) microscopy.

- An interesting mechanism of self-assembly into fibrous scaffolds, which resembles the collagen fiber in extracellular matrix. Based on the non-linearity of the intrinsic peptide bonds, such supramolecular assembly can generate second harmonic signals
- Peptide particles demonstrate the SHG excitation wavelength tunability. When the exciting wavelength was increased from 810 to 890 nm in a conventional two-photon laser-scanning microscopy, the wavelength of the SHG signals from the LK6 peptide particles increased from 405 to 450 nm respectively.
- Peptide particles demonstrate the two-photon nature of the emission. The emission was fitted nicely to a Gaussian curve and the SHG output signal obeyed a quadratic power dependence of SHG intensity to the power intensity.
- Peptide particles generate strong SHG signals, similar as that of endogenous collagen. This suggested that this class of peptides could be used as SHG probes for bioimaging.
- The peptides are biocompatible and biodegradable. No toxic effects of these peptides were detected by MTS assay and live/dead staining.
- All-peptide based contract agents can be visualized under SHG microscope without additional dyes or chemicals. Therefore, these peptides can be used for monitoring cells and *in vivo* imaging.
- Peptide-based tissue engineering scaffolds can be directly visualized under SHG microscope. Fundamental biological processes including cell adhesion, migration and differentiation on these peptides made matrices can be revealed.
- Visualization of peptide aggregation can be used for disease diagnostics. Diseases include Alzheimer's and Parkinson's disease.
- Peptides can be easily functionalized to introduce additional functions. Peptide particles can be used as drug or gene delivery vehicles. Peptide-based nanowires can be used as biosensors.

EXAMPLES

1. Materials and Methods

1.1 SHG imaging: We acquired SHG images 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.

1.2 Ultrashort amyloid-like peptides: We purchased peptides 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 [17].

1.3 Peptide particles: We prepared peptide particles by hydrodynamic focusing. Details can be found in elsewhere [21]. The particle size was determined by scanning electron microscopy, showing a grain size of around 5 μm (Figure 4S).

1.4 Cell culture: We cultured both HeLa and human dermal fibroblasts in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life technologies, Singapore).

1.5 Human corneal biopsy samples: Singapore General Hospital provided biopsy samples. 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).

2. Results and Discussion

2.1 A hexamer peptide is collagen-like and SHG-active.

To test whether the amyloid-like peptides of the present invention are SHG-active, we first investigated a hexamer peptide, Ac-LIVAGK-NH₂, (LK₆). Peptide particles deposited on a glass microscope slide were examined under an SHG imaging system. Instrument details can be found in our previous work [3]. During the whole experiments, only the backscattered geometry was employed because it is the only suitable

configuration for *in vivo* imaging [1]. We chose collagen I as a control. For more than a decade it has been shown that SHG microscopy can visualize supramolecular assembly of collagen in tissues [1]. Both collagen I and peptide particles showed SHG signals with comparable intensity (Figure 1a). The fine structures of collagen and peptide particles can be clearly seen under SHG and scanning electron microscopy (SEM, Figure 1a insert, 1b and 1c).

We further characterized LK₆ peptide. It exhibits typical SHG characteristics (Figure 2). When excited at 810 nm, LK₆ emitted a sharp SHG peak at 405 nm (Figure 2a). We then examined the excitation wavelength tunability of SHG signals from LK₆ peptide. In Figure 2b, 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. Biological samples, such as cells and tissues, are known to have auto-fluorescence. With excitation wavelength tunability, we can better avoid auto-fluorescence. In addition, we can further increase penetration depth in tissues by simply increasing excitation wavelength. Figure 2c shows the emission wavelength data of the SHG signal ($\lambda_{\text{ex}} = 850$ nm). The data in the graph were fitted to a Gaussian curve exhibiting a maximum at 425nm, which is exactly half the excitation wavelength of 850 nm. The band width (full width at half-maximum of Gaussian distribution, FWHM) was narrow (~ 10 nm) and it obeys a relationship to the corresponding wavelength profile of the fundamental beam (~ 15 nm). Moreover, we measured the dependence of the output signal on irradiation intensity by varying the 850 nm excitation intensity. The linear regression, applied to the log-log plots, revealed a quadratic power dependence of SHG intensity to power intensity, as shown in the Figure 2d.

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

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.

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

Next, we examined several other amyloid-like peptides, including both natural amyloidogenic core sequences (NL₆, DF₅, GA₆, and KE₇) and designed synthetic peptides (LD₆, IS₆ and IK₃). The peptide sequences are listed in Table 1.

Table 1. Powder SHG Efficiencies of Various Amyloid-like Peptides and Human Collagen Subtypes.

Amyloid-like Peptides / Human Collagens	SEQ ID NO.	$I^{2\omega}/I^{2\omega}(\text{sucrose})^{\text{a)}}$
IVK (IK ₃)		3.31 ± 0.57
LIVAGD (LD ₆)	19	3.06 ± 0.65
ILVAGS (IS ₆)	98	1.96 ± 0.58
LIVAGK (LK ₆)	27	0.95 ± 0.19
KLVFFAE (KE ₇)	99	3.18 ± 0.67
GGVVIA (GA ₆)	100	1.52 ± 0.43
DFNKF (DF ₅)	101	0.89 ± 0.33
NFGAIL (NL ₆)	102	0.76 ± 0.21
Type I		1.23 ± 0.36
Type II		0.41 ± 0.13
Type III		N.D. ^{b)}
Type IV		N.D. ^{b)}
Type V		N.D. ^{b)}

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

All these peptides are SHG-active. We used a powder technique developed by Kurtz and Perry [22] to evaluate the SHG efficiency of these second-order nonlinear optical materials. Sucrose, having a relatively modest nonlinearity, was chosen as a standard to evaluate second harmonic generation efficiencies [23]. In Table 1, all amyloid-like peptides that have been tested showed higher or equivalent SHG efficiency compared to sucrose. The efficiency of LD₆ and IK₃ were even three times higher than that of sucrose. These may due to their molecular structures. LD₆ is known to form hydrogel at much

lower concentrations compared to LK_6 . The trimer IK_3 has larger dipole moment compared to LK_6 . Human collagen subtypes I to V were examined (also listed in Table 1). Only type I and II showed SHG activity, III, IV and V did not. This agrees with the previous findings [4, 24]. In Table 1, all the tested amyloid-like peptides were SHG-active with some showing even higher SHG efficiency than collagen subtypes. This provides a broad spectrum of materials as second harmonic probes.

We have previously showed that NL_6 , DF_5 , GA_6 , and KE_7 as well as LD_6 , IS_6 , LK_6 and IK_3 self-assembled into hydrogels at 12 mM [18]. However, in this study, the gelation speed does not correlate to that of SHG efficiency (data not shown). We chose three rational designed hexamers, LD_6 (negatively charged), IS_6 (neutral) and LK_6 (positively charged) to investigate the effect of the charged head groups. The SHG efficiency of these three hexamers followed the order of $LD_6 > IS_6 > LK_6$.

We also investigate the effect of the peptide chain length by choosing IK_3 , a trimer, compared to the other three hexamers. It followed the order of IK_3 (trimer) $> LD_6$ (hexamer). Both findings are helpful for designing a superior amyloid-like peptide.

We then investigated the SHG efficiency of four natural amyloidogenic core sequences. They followed the order of $KE_7 > GA_6 > DF_5 > NL_6$ (Table 1 and Figure 6). KE_7 (KLVFFAE), containing its diphenylalanine (FF) motif, showed the highest SHG efficiency among these four peptides. However, its SHG efficiency is similar as that of IK_3 . It is noteworthy that IK_3 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 [25]. However, our results indicated that aromatic residues are not necessary for higher SHG efficiency. LD_6 , IS_6 , LK_6 and IK_3 are all aliphatic and lacking aromatic residues, clearly makes them a unique type of peptides for SHG applications. These peptides are rational designed by Hauser and colleagues [17, 20]. They are amphiphilic, 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 [20].

We reason 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 [19]. 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).

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 (esu is the unit of the first hyperpolarizability) [19]. However, the first hyperpolarizability of collagen I was found to be $(1250 \pm 20) \times 10^{-30}$ esu [19], which could be viewed as ten thousand trimers combining together. 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.

2.3 Cytocompatibility of a hexamer peptide

In order to use LK₆ for bioimaging applications, such as *in vitro* monitoring cells and *in vivo* imaging the whole animal, we assessed the cytotoxicity of LK₆ by the MTS test and live/dead assay with two human cell lines, human epithelial carcinoma cells (HeLa) and primary human dermal fibroblasts (HDF). Cell viability and the representative live/dead images of cells in response to LK₆ solutions was reported in Figure 7. LK₆ shows no toxicity to either HDF or HeLa cells.

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

A possible application for LK₆ is cell imaging. To this end, HeLa cells were incubated with biotin-conjugated LK₆ for 4 h and then fixed for immunostaining. LK₆ peptides were visualized by both SHG and confocal fluorescence microscopes. In Figure 3, biotin-conjugated peptides displayed green color as we added DyLightTM 488-conjugated NeutrAvidinTM (Thermo Scientific, Singapore). Meanwhile, peptides can be detected under SHG, displaying pseudocolored color.

2.5 A hexamer peptide as a second harmonic probe for *ex vivo* deep imaging

Another possible application for LK₆ is to use them within biological tissues to provide exogenous SHG signatures. These structures can be envisioned without any dyes or chemicals, even in a highly scattering environment such as living tissue. This is validated by performing an *ex vivo* experiment in which LK₆ peptide samples are placed under a slice of fat tissue (great omentum removed from a sacrificed rat, thickness ~ 50 μ m). The results are shown in Figure 4 and confirm the ability to detect SHG signals under sub-millimeter thick tissue.

2.6 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. 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. Thus, SHG provides us a new diagnostic tool for lattice corneal dystrophy (LCD) that is superior to the existing histological staining methods: SHG is label-free (no staining such as Congo red is required).

2.7 Other applications

In our laboratory, we have explored the biomedical applications of ultrashort amyloid-like peptides in many aspects. In one study, an anti-cancer drug, oxaliplatin, was tagged to our peptides by click chemistry [26]. Peptides were then injected into mice tumor sites. *In vivo* studies showed significant tumor growth inhibition. In another study, we used ultrashort peptides as a wound dressing. It heals the burn wounds much faster in a rat model [27]. Because our peptides are SHG-active, we can visualize these processes with SHG microscopy when we apply our peptides as drug delivery vehicles or wound dressings. Click chemistry enables adding therapeutic molecules to the ultrashort peptides. Thus, ultrashort peptides can also be used as theranostic agents that provide imaging and therapy at the same time. Recently, SHG microendoscopy has been

developed [28]. Therefore, we could expect for more bioimaging applications by using amyloid-like peptides as a second harmonic probe.

Conclusion

We demonstrated for the first time that amyloid-like peptides are nonlinear optical materials showing strong SHG signals. Amyloid-like peptides can be viewed under thick fat tissues and inside cells which make them suitable as second harmonic probes. Amyloid-like peptide nanomaterials hold great potential in nanotechnology and nanomedicine. Their nonlinear optical properties hold promise for new bioimaging applications.

It is to be understood that the described embodiment(s) have been provided only by way of exemplification of this invention, and that further modifications and improvements thereto, as would be apparent to persons skilled in the relevant art, are deemed to fall within the broad scope and ambit of the present invention described herein.

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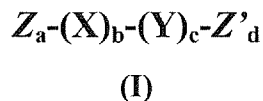
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CLAIMS

1. Use of a peptide and/or peptidomimetic, capable of self-assembling and forming a hydrogel, having the general formula I:



wherein

Z is an N-terminal protecting group;

a is 0 or 1, preferably 1;

X is, at each occurrence, independently selected from the group consisting of aliphatic amino acids and aliphatic amino acid derivatives, and wherein the overall hydrophobicity decreases from N- to C-terminus;

b is an integer selected from 1 to 7;

Y is selected from the group consisting of polar amino acids and polar amino acid derivatives;

c is 1 or 2;

Z' is a C-terminal protecting group; and

d is 0 or 1,

and **b + c** is at least 2,

as a non-linear optical (NLO) material for second harmonic generation (SHG) microscopy and/or imaging.

2. The use according to claim 1, wherein said aliphatic amino acids and aliphatic amino acid derivatives, and said polar amino acids and polar amino acid derivatives are either D-amino acids or L-amino acids.

3. The use according to claim 1 or 2, wherein said aliphatic amino acids are selected from the group consisting of alanine (Ala, A), homoallylglycine, homopropargylglycine, isoleucine (Ile, I), norleucine, leucine (Leu, L), valine (Val, V) and glycine (Gly, G), preferably from the group consisting of alanine (Ala, A), isoleucine (Ile, I), leucine (Leu, L), valine (Val, V) and glycine (Gly, G).

4. The use according to claim 3, wherein all or a portion of said aliphatic amino acids are arranged in an order of decreasing amino acid size in the direction from N- to C-terminus, wherein the size of the aliphatic amino acids is defined as $I = L > V > A > G$.

5. The use according to any of claims 1 to 4, wherein said aliphatic amino acids have a sequence selected from

LIVAG (SEQ ID NO: 1),
ILVAG (SEQ ID NO: 2),
ILVAA (SEQ ID NO: 3),
LIVAA (SEQ ID NO: 4),
LAVAG (SEQ ID NO: 5),
IAVAG (SEQ ID NO: 6),
AIVAG (SEQ ID NO: 7),
GIVAG (SEQ ID NO: 8),
VIVAG (SEQ ID NO: 9),
ALVAG (SEQ ID NO: 10),
GLVAG (SEQ ID NO: 11),
VLVAG (SEQ ID NO: 12),
IVAG (SEQ ID NO: 13),
LIVA (SEQ ID NO: 14),
LIVG (SEQ ID NO: 15),
ILVA (SEQ ID NO: 16),
ILVG (SEQ ID NO: 17),
LVAG (SEQ ID NO: 18),
IVA
IVG,
VIG,
IVA,
VIA,
IV and
VI.

6. The use according to any of the foregoing claims, wherein **b** is an integer from 2 to 7 or 2 to 6.

7. The use according to any of the foregoing claims, wherein said polar amino acids are selected from the group consisting of aspartic acid (Asp, D), asparagine (Asn, N), glutamic acid (Glu, E), glutamine (Gln, Q), 5-N-ethyl-glutamine (thearnine), citrulline, thio-citrulline, cysteine (Cys, C), homocysteine, methionine (Met, M), ethionine, selenomethionine, telluromethionine, threonine (Thr, T), allothreonine, serine (Ser, S), homoserine, arginine (Arg, R), homoarginine, ornithine (Orn), lysine (Lys, K), N(6)-carboxymethyllysine, histidine (His, H), 2,4-diaminobutyric acid (Dab), 2,3-diaminopropionic acid (Dap), and N(6)-carboxymethyllysine, wherein said polar amino acid is preferably selected from the group consisting of aspartic acid, asparagine, glutamic acid, glutamine, serine, threonine, methionine, lysine, ornithine (Orn), 2,4-diaminobutyric acid (Dab), and 2,3-diaminopropionic acid (Dap).

8. The use according to any of the foregoing claims, wherein **c** is 2 and said polar amino acids are identical amino acids, or wherein **c** is 1 and said polar polar amino acid comprises any one of aspartic acid, asparagine, glutamic acid, glutamine, serine, threonine, cysteine, methionine, lysine, ornithine, 2,4-diaminobutyric acid (Dab) and histidine, preferably lysine, ornithine, 2,4-diaminobutyric acid (Dab) and 2,3-diaminopropionic acid (Dap).

9. The use according to any of the foregoing claims, wherein **(Y)_b** has a sequence selected from Asp, Asn, Glu, Gln, Ser, Thr, Cys, Met, Lys, Orn, Dab, His, Asn-Asn, Asp-Asp, Glu-Glu, Gln-Gln, Asn-Gln, Gln-Asn, Asp-Gln, Gln-Asp, Asn-Glu, Glu-Asn, Asp-Glu, Glu-Asp, Gln-Glu, Glu-Gln, Asp-Asn, Asn-Asp Thr-Thr, Ser-Ser, Thr-Ser, Ser-Thr, Asp-Ser, Ser-Asp, Ser-Asn, Asn-Ser, Gln-Ser, Ser-Gln, Glu-Ser, Ser-Glu, Asp-Thr, Thr-Asp, Thr-Asn, Asn-Thr, Gln-Thr, Thr-Gln, Glu-Thr, Thr-Glu, Cys-Asp, Cys-Lys, Cys-Ser, Cys-Thr, Cys-Orn, Cys-Dab, Cys-Dap, Lys-Lys, Lys-Ser, Lys-Thr, Lys-Orn, Lys-Dab, Lys-Dap, Ser-Lys, Ser-Orn, Ser-Dab, Ser-Dap, Orn-Lys, Orn-Orn, Orn-Ser, Orn-Thr, Orn-Dab, Orn-Dap, Dab-Lys, Dab-Ser, Dab-Thr, Dab-Orn, Dab-Dab, Dab-Dap, Dap-Lys, Dap-Ser, Dap-Thr, Dap-Orn, Dap-Dab, Dap-Dap.

10. The use according to any of the foregoing claims, wherein $(X)_a-(Y)_b$ has a sequence selected from the group consisting of

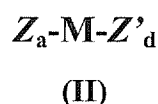
LIVAGD (SEQ ID NO: 19),
ILVAGD (SEQ ID NO: 20),
ILVAAD (SEQ ID NO: 21),
LIVAAD (SEQ ID NO: 22),
LAVAGD (SEQ ID NO: 23),
IAVAGD (SEQ ID NO: 24),
AIVAGD (SEQ ID NO: 25),
LIVAGE (SEQ ID NO: 26),
LIVAGK (SEQ ID NO: 27),
ILVAGK (SEQ ID NO: 28),
ILVAAK (SEQ ID NO: 29),
IAVAGK (SEQ ID NO: 30),
AIVAGK (SEQ ID NO: 31),
LIVAGT (SEQ ID NO: 32),
ILVAAT (SEQ ID NO: 33),
IAVAGT (SEQ ID NO: 34),
AIVAGT (SEQ ID NO: 35),
LIVAD (SEQ ID NO: 36),
LIVGD (SEQ ID NO: 37),
ILVAD (SEQ ID NO: 38),
ILVGD (SEQ ID NO: 39),
LVAGD (SEQ ID NO: 40),
IVAD (SEQ ID NO: 41),
IVAK (SEQ ID NO: 42),
IVGD (SEQ ID NO: 43),
VIGD (SEQ ID NO: 44),
IVAD (SEQ ID NO: 45),
VIAD (SEQ ID NO: 46),
IVGK (SEQ ID NO: 47),
VIGK (SEQ ID NO: 48),

IVAK (SEQ ID NO: 49),
VIAK (SEQ ID NO: 50),
IIID (SEQ ID NO: 51),
IIIK (SEQ ID NO: 52),
IVD,
IVK,
IID,
LVE,
IVE,
LVD,
VIE,
VID,
VIK,
VLD,
VLE,
LLE,
LLD,
IIE,
ID,
IE,
LIVAGOrn (SEQ ID NO: 53),
ILVAGOrn (SEQ ID NO: 54),
ILVAAOrn (SEQ ID NO: 55),
IAVAGOrn (SEQ ID NO: 56),
AIVAGOrn (SEQ ID NO: 57),
LIVAGDab (SEQ ID NO: 58),
ILVAGDab (SEQ ID NO: 59),
ILVAADab (SEQ ID NO: 60),
IAVAGDab (SEQ ID NO: 61),
AIVAGDab (SEQ ID NO: 62),
LIVAGDap (SEQ ID NO: 63),
ILVAGDap (SEQ ID NO: 64),
ILVAADap (SEQ ID NO: 65),

LAVAGDap (SEQ ID NO: 66),
AIVAGDap (SEQ ID NO: 67),
LIVAOm (SEQ ID NO: 68),
LIVGOm (SEQ ID NO: 69),
ILVAOm (SEQ ID NO: 70),
ILVGOm (SEQ ID NO: 71),
LVAGOm (SEQ ID NO: 72),
LIVADab (SEQ ID NO: 73),
LIVGDab (SEQ ID NO: 74),
ILVADab (SEQ ID NO: 75),
ILVGDab (SEQ ID NO: 76),
LVAGDab (SEQ ID NO: 77),
LIVADap (SEQ ID NO: 78),
LIVGDap (SEQ ID NO: 79),
ILVADap (SEQ ID NO: 80),
ILVGDap (SEQ ID NO: 81),
LVAGDap (SEQ ID NO: 82),
IVAOm (SEQ ID NO: 83),
IVGOm (SEQ ID NO: 84),
VIGOm (SEQ ID NO: 85),
IVAOm (SEQ ID NO: 86),
VIAOm (SEQ ID NO: 87),
IVADab (SEQ ID NO: 88),
IVGDab (SEQ ID NO: 89),
VIGDab (SEQ ID NO: 90),
IVADab (SEQ ID NO: 91),
VIADab (SEQ ID NO: 92),
IVADap (SEQ ID NO: 93),
IVGDap (SEQ ID NO: 94),
VIGDap (SEQ ID NO: 95),
IVADap (SEQ ID NO: 96),
VIADap (SEQ ID NO: 97),
IVOm,

IVDab,
 IVDap,
 VIOm,
 VIDab,
 VIDap, and
 ILVAGS (SEQ ID NO: 98).

11. Use of a peptide having the general formula II:



wherein

Z is an N-terminal protecting group;

a is 0 or 1, preferably 1;

M is an amino acid sequence selected from SEQ ID Nos. 99 to 102;

Z' is a C-terminal protecting group; and

d is 0 or 1,

as a non-linear optical (NLO) material for second harmonic generation (SHG) microscopy and/or imaging.

12. The use according to any of the foregoing claims, wherein a is 1 and said N-terminal protecting group Z has the general formula $-C(O)-R$, wherein R is selected from the group consisting of H, unsubstituted or substituted alkyls, and unsubstituted or substituted aryls,

wherein R is preferably selected from the group consisting of methyl, ethyl, propyl, isopropyl, butyl and isobutyl.

13. The use according to any of the foregoing claims, wherein said N-terminal protecting group Z is an acetyl group,

or wherein said N-terminal protecting group Z is a peptidomimetic molecule, including natural and synthetic amino acid derivatives, wherein the N-terminus of said peptidomimetic molecule may be modified with a functional group selected from the

group consisting of carboxylic acid, amide, alcohol, aldehyde, amine, imine, nitrile, an urea analog, phosphate, carbonate, sulfate, nitrate, maleimide, vinyl sulfone, azide, alkyne, alkene, carbohydrate, imide, peroxide, ester, aryl, ketone, sulphite, nitrite, phosphonate, and silane.

14. The use according to any of the foregoing claims, wherein said C-terminal protecting group **Z'** is an amide group,

wherein, preferably, the C-terminus has the formula -CONHR or -CONRR', with R and R' being selected from the group consisting of H, unsubstituted or substituted alkyls, and unsubstituted or substituted aryls.

15. The use according to any of the foregoing claims, wherein said C-terminal protecting group **Z'** is an ester group,

wherein, preferably, the C-terminus has the formula -CO₂R, with R being selected from the group consisting of H, unsubstituted or substituted alkyls, and unsubstituted or substituted aryls.

16. The use according to any of the foregoing claims, wherein said C-terminal group **Z'** is a peptidomimetic molecule, including natural and synthetic amino acid derivatives, wherein the C-terminus of said peptidomimetic molecule may be modified with a functional group selected from the group consisting of carboxylic acid, amide, alcohol, aldehyde, amine, imine, nitrile, an urea analog, phosphate, carbonate, sulfate, nitrate, maleimide, vinyl sulfone, azide, alkyne, alkene, carbohydrate, imide, peroxide, ester, aryl, ketone, sulphite, nitrite, phosphonate, and silane.

17. The use according to any of the foregoing claims, wherein **b + c** is at least 2, preferably 2 to 9, more preferably 3 to 7 or 2 to 7.

18. The use according to any of the foregoing claims, wherein the peptide and/or peptidomimetic is visualized under a second harmonic generation (SHG) microscopy or by SHG imaging without additional dye(s) and/or label(s).

19. The use according to any of the foregoing claims, wherein the peptide and/or peptidomimetic comprises further compound(s), such as

- small molecules,

such as (but not limited to) sugars, alcohols, hydroxy acids, amino acids, vitamins, biotin, L-Dopa, thyroxine;

- bioactive molecules or moieties,

such as growth factors, cytokines, lipids, cell receptor ligands, hormones, prodrugs, drugs, vitamins, antigens, antibodies, antibody fragments, oligonucleotides (including but not limited to DNA, messenger RNA, short hairpin RNA, small interfering RNA, microRNA, peptide nucleic acids, aptamers), saccharides;

- label(s), dye(s),

such as imaging contrast agents;

- pathogens,

such as viruses, bacteria and parasites;

- micro- and nanoparticles

or combinations thereof

which are preferably covalently attached or coupled to the peptide and/or peptidomimetic, preferably to the C-terminal group **Z'**, amino acid side chain(s) and/or linker,

wherein said attachment or coupling can be carried out before, during or after self-assembly of the peptide and/or peptidomimetic.

20. The use according to any of the foregoing claims, wherein the at least one peptide and/or peptidomimetic as defined in any one of claims 1 to 19 forms a fibrous structure, which is preferably tunable, such as

- in the way of peptide material's preparation (fiber structures, particle structures),
- for excitation wavelength tunability of SHG signals.

21. The use according to claim 20, wherein the fibrous structure is a fibrous scaffold resembling the collagen fiber in extracellular matrix.

22. The use according to any of the foregoing claims, wherein the at least one peptide and/or peptidomimetic as defined in any one of claims 1 to 19 is stable in aqueous

solution at physiological conditions at ambient temperature for a period of time in the range from 1 day to at least 6 months, preferably to at least 8 months more preferably to at least 12 months.

23. The use according to any of the foregoing claims, wherein the at least one peptide and/or peptidomimetic as defined in any one of claims 1 to 19 is stable in aqueous solution at physiological conditions, at a temperature up to 90 °C, for at least 1 hour.

24. The use according to any of the foregoing claims, wherein the peptide and/or peptidomimetic is present at a concentration in the range of from 0.1 % to 30 % (w/w), preferably 0.1 % to 20 % (w/w), more preferably 0.1 % to 10 % (w/w), more preferably 0.1 % to 5 % (w/w), even more preferably 0.1 % to 3 % (w/w), with respect to the total weight of said hydrogel or fibrous structure.

25. A method of preparing a hydrogel or fibrous structure, the method comprising dissolving at least one peptide and/or peptidomimetic as defined in any one of claims 1 to 10 and 12 to 19 in an aqueous solution.

26. The method method according to claim 25, wherein the dissolved peptide and/or peptidomimetic in aqueous solution is further exposed to temperature, wherein the temperature is in the range from 20 °C to 90 °C, preferably from 20 °C to 70 °C.

27. The method according to claim 25 or 26, wherein the at least one peptide and/or peptidomimetic is dissolved at a concentration from 0.01 µg/ml to 100 mg/ml, preferably at a concentration from 1 mg/ml to 50 mg/ml, more preferably at a concentration from about 1 mg/ml to about 20 mg/ml.

28. A method of preparing peptide particles, the method comprising dissolving at least one peptide and/or peptidomimetic as defined in any one of claims 1 to 10 and 12 to 19 in an aqueous solution, preferably phosphate buffered saline (PBS), and solvent evaporation from said aqueous solution, such as by vacuum drying.

29. The use according to any of claims 1 to 24 or the use of a hydrogel, fibrous structure or peptide particle obtained by a method according to any of claims 25 to 28, as probes for (bio-)imaging, preferably as exogenous SHG probes.

30. The use according to any of claims 1 to 24 or the use of a hydrogel, fibrous structure or peptide particle obtained by a method according to any of claims 25 to 28, for monitoring cells, *in vitro* and/or *in vivo* (bio-)imaging.

31. The use according to any of claims 1 to 24 or the use of a hydrogel, fibrous structure or peptide particle obtained by a method according to any of claims 25 to 28 for visualizing peptide-based tissue engineering scaffolds, preferably comprising studying biological processes, such as cell adhesion, migration and differentiation.

32. The use according to any of claims 1 to 24 or the use of a hydrogel, fibrous structure or peptide particle obtained by a method according to any of claims 25 to 28 as diagnostics, preferably for the diagnosis of diseases comprising or associated with the aggregation of peptide or protein structures, such as amyloidosis, including

- neuro-degenerative amyloidosis, e.g. Alzheimer's (AD), Parkinson's, Huntington's (PD),
- non-neuropathic localized amyloidosis such as in Type II Diabetes, and
- systemic amyloidosis that occurs in multiple tissues, e.g. cataracts and lattice corneal dystrophy (LCD).

33. The use according to any of claims 1 to 24 or the use of a hydrogel, fibrous structure or peptide particle obtained by a method according to any of claims 25 to 28 as drug or gene delivery vehicle.

34. A peptide particle comprising the peptide and/or peptidomimetic as defined in any one of claims 1 to 19,

preferably obtained by a method according to claim 28.

35. The peptide particle of claim 34, having a particle size in the range from submicron to 10 microns.

36. A drug or gene delivery vehicle comprising the peptide and/or peptidomimetic as defined in any one of claims 1 to 19,
and a drug or gene to be delivered.

37. A peptide-based tissue engineering scaffold comprising the peptide and/or peptidomimetic as defined in any one of claims 1 to 19,
preferably obtained by a method according to any of claims 25 to 27.

38. A biosensor or bioimaging device comprising the peptide and/or peptidomimetic as defined in any one of claims 1 to 19.

39. A wound dressing comprising the peptide and/or peptidomimetic as defined in any one of claims 1 to 19.