Synthetic Pept-Ins as a generic amyloid-like aggregation-based platform for in vivo PET imaging of intracellular targets

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Supplementary Figures

Figure S1: Atomic-resolution architecture of the P2 APR in an amyloid conformation

Molecular model of the P2 APR in an amyloid conformation as predicted by the Cordax algorithm\(^1\), rendered by the Yasara visualisation software\(^2\). The atomic structure of a pair of \(\beta\)-sheets based on the amyloid-forming P2 sequence is shown in projection down the crystal axis, of which each strand has the same amino acid sequence resulting in tightly packed, in-register stacking of identical side chains. The stacked segments demonstrate a dry and steric-zipper interaction which is constituted by the interdigitation of side chains between the two \(\beta\)-sheets.
Figure S2: $^{153}$Ga-labelled NODAGA-PEG₄-vascin conserves inhibitory effect of unmodified vascin on VEGFR2

HUVECs were treated overnight with a 20 µM solution of unmodified vascin ($n=2$), $[^{153}$Ga]$^3$Ga-NODAGA-PEG₄-vascin (3 replicates, $n=6$) or $[^{153}$Ga]$^3$Ga-NODAGA-PEG₄-vascin(Pro) (2 replicates, $n=4$) overnight. Total phosphorylated ERK was determined by ERK1/2 MSD ELISA after VEGF stimulation. Positive control condition was constructed with HUVECs without peptide treatment that were stimulated with VEGF. Negative control condition consists of HUVECs without peptide treatment and without VEGF stimulation. %pERK on the y-axis is % phosphorylated ERK.
Figure S3: $^{nat}$Ga-labelled NODAGA-PEG$_2$-P2 induces toxic inclusion bodies in E. coli leading to bacterial cell death

(a) Structured illumination microscopy (SIM) image of E. coli treated with $[^{nat}$Ga]$^n$Ga-NODAGA-PEG$_2$-P2 and p-FTAA. (b) FACS analysis of E. coli treated with $[^{nat}$Ga]$^n$Ga-NODAGA-PEG$_2$-P2 measuring p-FTAA fluorescence (x-axis) and propidium iodide (PI) fluorescence (y-axis) of bacteria treated for 2 hours with $[^{nat}$Ga]$^n$Ga-NODAGA-PEG$_2$-P2. (c) Treated for 3 hours. (d) Treated for 6 hours. (e) Treated with $[^{nat}$Ga]$^n$Ga-NODAGA-PEG$_2$-P2(Pro) for 6h.
Figure S4: Specific *in vivo* accumulation of radiolabelled P2 at the *E. coli* infection site in a mouse hind leg muscle infection model
(a) Representative whole-body coronal summed μPET image from 3h dynamic scan in a hind leg muscle infection model after injection of \[^{68}\text{Ga}\]\text{Ga}-NODAGA-PEG\textsubscript{2}-P2. The image shown is obtained from the inactive bacteria-model. (b) Whole-body fluorescence imaging of GFP-expressing *E. coli* in hind leg muscle infection model. The image shown is obtained from the inactive bacteria-model. Inactivated *E. coli* have too low remaining GFP-expression to be detected by whole-body fluorescence imaging.

Figure S5: \[^{18}\text{F}\]\text{FDG} visualizes both inflamed and infected muscle tissue
(a) Representative coronal \[^{18}\text{F}\]\text{FDG} μPET image from static scan (30-40 min p.i.) in a foreleg muscle infection model with LPS-injected control muscle. (b) Representative coronal \[^{18}\text{F}\]\text{FDG} μPET image from static scan (30-40 min p.i.) in a foreleg muscle infection model with inactive bacteria-injected control muscle.
Supplementary Text
Supplementary Text 1: Synthesis and characterization of vasclin and P2 modified for radiolabelling

Vascin and P2 were synthesised in-house using standard Fmoc solid-phase peptide synthesis in high purity (Figure S6), with N-terminal conjugation to a PEG spacer followed by coupling to 1-(1-carboxy-3-carboxy-propyl)-4,7-(carboxy-methyl)-1,4,7-triazacyclononane (NODAGA) and labelled with gallium-68 to obtain $[^{68}\text{Ga}]{\text{Ga-NODAGA-PEG}}_4$-vascin (MW = 3332 Da) and $[^{68}\text{Ga}]{\text{Ga-NODAGA-PEG}}_2$-P2 (MW = 3039 Da). NOTA and its derivatives, especially NODAGA, were reported to form very stable complexes with gallium-68$^{3,4,5}$ and NODAGA-peptide conjugates labelled with different isotopes of gallium were found to be stable against radiolysis, transchelation and transmetallation in vitro$^{4,5}$. The conjugation of PEG-units to peptides is known to enhance the hydrophilicity and reduce the sensitivity to proteolysis, and has been approved for use in humans$^6$. PEG was also previously successfully used as a linker for synthetic amyloid peptides to couple chemical moieties such as biotin$^7$, to prevent steric hindrance of the chemical moiety on the β-sheet forming properties and hence functionality of the peptides, while improving solubility of the resulting construct. For both vascin and P2, NODAGA-PEG$_2$-variants and NODAGA-PEG$_4$-variants were tested (data not shown). For vascin, $[^{\text{nat}}\text{Ga}]{\text{Ga-NODAGA-PEG}}_4$-vascin best preserved the vascin functional effect, while no difference was observed between $[^{\text{nat}}\text{Ga}]{\text{Ga-NODAGA-PEG}}_2$-P2 and $[^{\text{nat}}\text{Ga}]{\text{Ga-NODAGA-PEG}}_4$-P2 in MIC value and $[^{\text{nat}}\text{Ga}]{\text{Ga-NODAGA-PEG}}_2$-P2 was chosen because it requires one synthesis step less compared to the synthesis of the NODAGA-PEG$_4$-variant. Radiochemical purity of $[^{68}\text{Ga}]{\text{Ga-NODAGA-PEG}}_4$-vascin and $[^{68}\text{Ga}]{\text{Ga-NODAGA-PEG}}_2$-P2 exceeded 95 % (radioHPLC) for all batches and the identity was confirmed against $[^{\text{nat}}\text{Ga}]{\text{Ga-NODAGA-PEG}}_4$-vascin and $[^{\text{nat}}\text{Ga}]{\text{Ga-NODAGA-PEG}}_2$-P2 (Figure S6). $[^{68}\text{Ga}]{\text{Ga-NODAGA-PEG}}_4$-vascin was obtained in $33 \pm 3$ % decay-corrected yield ($n=3$) and the specific activity at the end of synthesis was $10 \pm 4$ MBq/µg ($n=9$). $[^{68}\text{Ga}]{\text{Ga-NODAGA-PEG}}_2$-P2 was obtained in $65 \pm 9$ % decay-corrected yield ($n=3$) and the specific activity at the end of synthesis was $13 \pm 4$ MBq/µg.
Supporting information

Figure S6: Characterization of vascin and P2 modified for radiolabelling

(a) RadioHPLC analysis of $[^{68}\text{Ga}]\text{Ga-NODAGA-PEG}_4$-vascin co-injected with $[^{nat}\text{Ga}]\text{Ga-NODAGA-PEG}_4$-vascin (UV signal at 220 nm). (b) RadioHPLC analysis of $[^{68}\text{Ga}]\text{Ga-NODAGA-PEG}_2$-P2 co-injected with $[^{nat}\text{Ga}]\text{Ga-NODAGA-PEG}_2$-P2 (UV signal at 220 nm).

Supplementary Text 2: Plasma radiometabolite analysis of radiolabelled vascin and P2

Plasma ex vivo radiometabolite analysis of $[^{68}\text{Ga}]\text{Ga-NODAGA-PEG}_4$-vascin in healthy C57BL/6 mice and of $[^{68}\text{Ga}]\text{Ga-NODAGA-PEG}_2$-P2 in healthy Swiss mice by radioHPLC revealed a slow metabolism (Figure S7). In $[^{68}\text{Ga}]\text{Ga-NODAGA-PEG}_4$-vascin injected mice, at 30 min p.i. $50 \pm 8\%$ ($n=4$, 2 replicate experiments) of the recovered plasma radioactivity of radiolabelled vascin was still in the intact form, decreasing to $41 \pm 14\%$ ($n=3$, 2 replicate experiments) at 60 min p.i., which is still high (Figure S7) especially considering the peptidic nature. In $[^{68}\text{Ga}]\text{Ga-NODAGA-PEG}_2$-P2 injected mice, at 30 min p.i. $58 \pm 19\%$ ($n=4$, 2 replicate experiments) of the recovered plasma radioactivity of radiolabelled P2 was still in the intact form, decreasing to $55 \pm 22\%$ ($n=3$, 2 replicate experiments) at 60 min p.i. (Figure S7). All detected radiometabolites were more polar than the intact radiolabelled peptides. In vitro
metabolite radioHPLC analysis of both radiolabelled vascin and P2 did not show any radiolysis up to 3 h incubation time in the labelling mixture. A substantial amount of detected radiometabolites eluted in the beginning of the HPLC runs, which are most likely non-peptidic metabolites because of their highly polar nature, presumably $^{68}$Ga-NODAGA-$\text{PEG}_4$ and/or transchelated $^{68}$Ga.

Figure S7: Plasma radiometabolite analysis of synthetic amyloidogenic peptides

(a) *Ex vivo* radiometabolite analysis of healthy C57BL/6 mouse plasma. Representative radioHPLC chromatograms of $^{68}$Ga-NODAGA-$\text{PEG}_4$-vascin and its metabolites in plasma 30 min p.i. ($n=4$ from 2 replicate experiments) and 60 min p.i. ($n=3$ from 2 replicate experiments). (b) *Ex vivo* radiometabolite analysis in healthy Swiss mice. Example of reconstructed radiochromatograms of plasma analysis of a mouse euthanized 30 min after injection of $^{68}$Ga-NODAGA-$\text{PEG}_2$-$\text{P}_2$ ($n=4$ from 2 replicate experiments and 60 min after injection of $^{68}$Ga-NODAGA-$\text{PEG}_2$-$\text{P}_2$ ($n=3$ from 2 replicate experiments). $n^\circ = \text{number.}$
Quality control of vascin and P2 modified for radionabelling

Quality control of radiolabelled peptides was performed by radioHPLC with an Agilent SB-C3 1.8 µm 3.0 x 100 mm column (Agilent, USA) for vascin and an Acquity UPLC BEH C18 1.7 µm 2.1 mm x 50 mm column (Waters, USA) for P2. The mobile phase consisted of a gradient of NH₄HCO₃ 10 mM buffers in milliQ (pH 7) and ACN for vascin and a gradient of 0.1% HCOOH in milliQ water and ACN for P2. Radioactivity in the eluate was analysed with a 3” NaI(Tl) detector and the UV absorbance of the eluate at 220 nm. The identity of the observed peaks in the radioactive channel was confirmed by comparison of the retention time of [natGa]Ga-NODAGA-PEG₄-vascin and [natGa]Ga-NODAGA-PEG₂-P2 at 220 nm.

Vascin ex vivo plasma radiometabolite analysis

Female C57BL/6 mice (6-8 weeks old) were anesthetized with 2.5% isoflurane in O₂ at a flow rate of 1 l/min and injected with 4 MBq of [⁶⁸Ga]Ga-NODAGA-PEG₄-vascin via a tail vein. Mice were euthanized by decapitation at 30 or 60 min post injection (p.i.). Blood was collected in K₂EDTA-containing, tubes (BD vacutainer, BD, Franklin Lakes, NJ, U.S.A.) and stored on ice. The blood was centrifuged for 10 min at 2330 x g to separate the plasma, followed by dilution with an equal volume of ACN to precipitate plasma proteins and centrifuging for 5 min at 2330 x g. The supernatant was diluted with water, filtered through a 0.22 µm filter (Millipore, Bedford, USA) and counted in a gamma counter. Subsequently the plasma samples were analysed by radio-high-performance liquid chromatography (HPLC) employing a POSI-RAM radioHPLC detector with coincidence Flow-Through Counter, an Agilent SB-C3 (1.8 µm, 3.0 x 100 mm) column (Agilent, USA) and eluted with gradient mixtures of A (10 mM NH₄HCO₃ in milliQ, pH 7) and B (10 mM NH₄HCO₃ in ACN) at 70 °C column temperature and flow rate 0.6 ml/min: first 2 min of run at 5% B, then gradient from 95% A and 5% B to 5% A and 95% B in 9 min, followed by 4 min at 95% B and then ending by 6 min at 5% B. Stability against radiolysis in the labelling mixture was tested by reinjecting after incubation of the sample for quality control for 1h, 2h and 3h.
P2 *ex vivo* plasma radiometabolite analysis

Healthy female Swiss mice from 4-6 weeks of age were anesthetized with 2.5% isoflurane in O\textsubscript{2} at a flow rate of 1 l/min and injected with 4 MBq of \textsuperscript{68}Ga-Ga-NODAGA-PEG\textsubscript{2}-P2 via a tail vein. Mice were euthanized by decapitation at 30 or 60 min p.i. Blood was collected in K\textsubscript{2}EDTA-tubes (BD vacutainer, BD, Franklin Lakes, NJ, U.S.A.) and stored on ice. The blood was centrifuged for 10 min at 2330 x g to separate the plasma, and the plasma was counted in a gamma counter. Subsequently the plasma samples were analysed by radioHPLC on a Chromolith RP C18 column (3 mm x 100 mm, Merck, Darmstadt, Germany), eluted with gradient mixtures of A (0.1% HCOOH in H\textsubscript{2}O pH 2.7) and B (0.1% HCOOH in ACN) at room temperature and flow rate at 1.2 ml/min: 0-4 min 1% B, 4-16 min from 99% A to 5%, 16-20 min 95% B, 20-26 min 1% B. After passing through a 3-inch NaI(Tl) scintillation detector connected to a single channel analyser, the HPLC eluate was collected in 20-sec fractions and radioactivity in each fraction was measured in an automated gamma counter. Stability against radiolysis in the labelling mixture was tested by reinjecting the sample for quality control after incubation for 1h, 2h and 3h.
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