Control of Surface-Localized, Enzyme-Assisted Self-Assembly of Peptides through Catalyzed Oligomerization

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Supporting Information

ABSTRACT: Localized self-assembly allowing both spatial and temporal control over the assembly process is essential in many biological systems. This can be achieved through localized enzyme-assisted self-assembly (LEASA), also called enzyme-instructed self-assembly, where enzymes present on a substrate catalyze a reaction that transforms noninteracting species into self-assembling ones. Very few LEASA systems have been reported so far, and the control of the self-assembly process through the surface properties represents one essential step toward their use, for example, in artificial cell mimicry. Here, we describe a new type of LEASA system based on α-chymotrypsin adsorbed on a surface, which catalyzes the production of (KL)\textsubscript{10}OEt oligopeptides from a KLOEt (K: lysine; L: leucine; OEt ethyl ester) solution. When a critical concentration of the formed oligopeptides is reached near the surface, they self-assemble into β-sheets resulting in a fibrillar network localized at the interface that can extend over several micrometers. One significant feature of this process is the existence of a lag time before the self-assembly process starts. We investigate, in particular, the effect of the α-chymotrypsin surface density and KLOEt concentration on the self-assembly kinetics. We find that the lag time can be finely tuned through the surface density in α-chymotrypsin and KLOEt concentration. For a given surface enzyme concentration, a critical KLOEt concentration exists below which no self-assembly takes place. This concentration increases when the surface density in enzyme decreases.

INTRODUCTION

Biological processes such as cell migration or division are the result of a large number of reactions of fine spatial and temporal tuning.\textsuperscript{1,2} Among them, spatially confined, enzymatically controlled self-assembly processes play a major role. Reproducing such processes with synthetic systems represents a real challenge that constitutes an essential step toward the design of artificial life mimics. This problem can be addressed by the use of catalytically assisted self-assembly processes where the catalyst, generated at or fixed on a surface, triggers a reaction where noninteracting species are transformed into self-assembling ones. The catalyst can be a proton\textsuperscript{3} or another ion\textsuperscript{4} present at the interface, either statically in the case of a highly charged surface or generated dynamically by electrochemical means.\textsuperscript{5,6} The catalyst can also be an enzyme anchored on the surface. In this case, the process can be called localized enzyme-assisted self-assembly (LEASA). Enzyme-assisted self-assembly (EASA), also called enzyme-instructed self-assembly, was introduced to initiate gel formation in the bulk in 2004 by the group of Xu\textsuperscript{7} and intensively developed by Ulijn and co-workers.\textsuperscript{8,9} The formation of the gel is usually based on an enzymatic transformation of peptides bearing aromatic moieties to induce their self-assembly. This self-assembly is induced through a decrease of peptide solubility in water. Ulijn and co-workers were the first to show that enzymes deposited on a surface can be used to locally induce a self-assembly process of peptides leading to LEASA.\textsuperscript{10} In this case, a concentration gradient of peptides builds up near the surface,
leading to a self-assembly localized near the enzyme. Xu and co-workers showed that the overexpression of enzymes (alkaline phosphatase) on cancer cells can be used to promote peptide self-assembly localized at the cell membrane. Hartley’s group used thermolysin anchored onto a surface to create a peptide network incorporating laminin for tissue repair use. The importance of the way to immobilize enzymes on surface has been recently highlighted by Ulijn. An extensive review of enzyme-assisted self-assembly for biomedical applications initiated on particles, porous materials, and cells has been provided by Cui and co-workers. Control of the self-assembly process by carefully modulating the enzymatically active surface is essential for a rational use of LEASA. Using dephosphorylation of Fmoc-peptides by an adsorbed alkaline phosphatase layer, we showed that the amplitude and kinetics of the peptidic self-assembly process can be controlled by adsorbing a seed layer on top of the enzyme layer and by varying both the enzyme concentration and the properties of the seed layer. The seed layer plays the role of nucleation agent and favors gel formation at the interface for concentrations of assembling species much smaller than required in the bulk.

Using a system based on the oligomerization of lysine-leucine-ethyl ester dipeptides (KLOEt) catalyzed by α-chymotrypsin, we introduce here a new LEASA system resulting in the formation of a physically entangled nanofibrillar network of peptides interacting through hydrogen bonding (Scheme 1). Because the oligomerization is a multistep process, it is by far not obvious that it can lead to a LEASA buildup.

With the aim of controlling the localized self-assembly process through the enzymatic activity of the surface, we investigate the influence of the enzyme surface concentration and the peptide concentration on the self-assembly kinetic of (KL)\(_2\)OEt oligomers. This is, to our knowledge, the first LEASA system where the self-assembly process is modulated by varying the concentration of enzymes on the surface. In particular, we show the existence of the lag time before the buildup process starts, and we investigate how this lag time varies with the enzyme surface concentration. It can be pointed out that one should also be able to use LEASA for temporal control by modulating the enzymatic activity through temperature changes leading to kinetically controlling the production rate of assembling peptides and their aggregation state. This was recently shown not for LEASA but for EASA in solution.

**MATERIAL AND METHODS**

**Chemicals.** Sodium acetate, sodium phosphate dibasic, sodium phosphate monobasic, TRIS base, poly(ethylene imine) (PEI, 750 000 g/mol), α-chymotrypsin type II from bovine pancreas (25 kDa, 65 U/g) and bovine serum albumin (BSA, 66 kDa) were purchased from Sigma-Aldrich. Tannic acid (TA, 1701.2 g/mol) was purchased by Alfa Aesar. D\(_2\)O (99%) was purchased from Euriso-top. KL6 oligomer was purchased by Proteogenix. All commercial chemicals were used as received. Fluorescein-labeled α-chymotrypsin (α-chymotrypsin\(^{FITC}\)) and dipeptide ethyl ester (KLOEt) were synthesized as described in the Supporting Information (SI). Unless otherwise stated, all solutions were prepared in Milli-Q ultrapure water (Milli-Q Plus system, Millipore, Billerica, MA) that has a resistivity of 18.2 MΩ·cm. PEI, TA, α-chymotrypsin, α-chymotrypsin\(^{FITC}\), and BSA were dissolved in sodium acetate buffer (10 mM, pH = 6). α-Chymotrypsin, α-chymotrypsin\(^{FITC}\), and BSA were prepared with identical molar concentrations of 8 μM. Therefore, a proportional volume ratio of α-chymotrypsin (respectively α-chymotrypsin\(^{FITC}\)) and BSA solutions was prepared to obtain a mixture solution at a fixed molar ratio r in α-chymotrypsin (respectively α-chymotrypsin\(^{FITC}\)). KLOEt was dissolved in phosphate buffer (186 mM, pH = 8.7).

**Enzymatic Active Multilayer Buildup and Surface Hydrogel Self-Assembly.** The preparation of the enzymatic precursor film and the hydrogel self-assembly was identical for all types of surfaces used in the different experimental techniques of characterization: quartz crystal for QCM-D, ZnSe crystal for IR-ATR, and glass slide for fluorescence spectroscopy and cryo-SEM. All the solutions used to build the enzymatic precursor films, i.e., PEI, TA, and protein solutions, were prepared in sodium acetate buffer. All hydrogel self-assembly modes were realized using KLOEt solution prepared in phosphate buffer. The enzymatic precursor films were obtained by a dipping process: a first adsorption of a PEI layer (1 mg/mL) for 5 min, followed by a sodium acetate buffer rinsing step (5 min) and the adsorption of a TA layer (1.125 mg/mL) for 5 min, followed by a sodium acetate buffer rinsing step (5 min). Then, the protein solution, i.e., α-chymotrypsin (0.2 mg/mL, i.e., 8 μM), BSA (0.53 mg/mL, i.e., 8 μM) or the α-chymotrypsin\(^{FITC}\)/BSA(100 − r) mixture solution, with r being the...
molar ratio of α-chymotrypsin (the total protein concentration being 8 μM), was adsorbed for 5 min on PEI/TA film followed by a rinsing step phosphate buffer (5 min). A second rinsing step (5 min) was performed with phosphate buffer before the contact with KLOEi solution at 1 mg/mL, unless otherwise stated.

Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D). The QCM-D experiments were performed on a Q-Sense E1 apparatus (Q-Sense AB, Göteborg, Sweden) by monitoring the resonance frequencies of gold-coated crystals, as well as the dissipation factors at four frequencies, the fundamental frequency at 5 MHz (ν = 1) and the harmonics at 15, 25, and 35 MHz (ν = 3, 5 and 7, respectively). The QCM-D results provide information on the adsorption process as well as on viscoelastic properties of the adsorbed film. In a first approximation, the resonance frequency shifts are proportional to the mass of the film deposited on the crystal per unit area. However, this approximation holds only for homogeneous, quasi-rigid thin films. For a thin and rigid film, i.e., when (Δν/ν) is independent of υ, the mass m (ng/cm²) can be obtained by using the Sauerbrey equation: \[ m = -C \frac{Δν}{ν} \]

where C is the mass sensitivity constant (17.7 ng/cm²·Hz) of the QCM crystal, υ is the overtone number and (Δν/ν) is expressed in Hz. We verified that the normalized frequency changes, (Δν/ν) (except for υ = 1), were equal within ±12% so that the use of Sauerbrey’s equation to analyze our data was relevant.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF). Mass measurements were carried out on an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). This instrument was used at a maximum accelerating potential of 20 kV in positive mode and was operated in mode reflector at 19 kV. The delay extraction was fixed at 110 ns, and the frequency of the laser (nitrogen 337 nm) was set at 5 Hz. The acquisition mass range was set to 500–5000 m/z with a matrix suppression deflection (cut off) set to 500 m/z. The equipment was first externally calibrated with a standard peptide calibration mixture that contained 7 peptides (Bruker Peptide Calibration Standard #206196B, Bruker Daltonics GmbH, Bremen, Germany) covering the 1000–3200 m/z range. Each raw spectrum was opened with flexAnalysis 2.4 build 11 (Bruker Daltonics GmbH, Bremen, Germany) software and processed using the following parameters: signal-to-noise threshold of 1, Savitzky-Golay algorithm for smoothing, median algorithm for baseline subtraction, and SNAP algorithm for monoisotopic peak detection and labeling. In all cases, resolution was better than 9000. The analyzed (KL)₁₀OEt hydrogel, was grown for 12 h on a PEI/TA/α-chymotrypsin film with different surface density in α-chymotrypsin. The hydrogel sample was collected directly on the QCM-D transducer with a pipette of 0.5 μL of sample and 0.5 μL of matrix solution was prepared and allowed to dry at room temperature using the dried droplet method. The matrix solution was prepared from a saturated solution of α-cyano-4-hydroxycinnamic acid in water/acetone (57/43 v/v) diluted three times in water/acetone/trifluoroacetic acid: 50/49.9/0.1.

Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR). The ATR-FTIR spectra were acquired by the accumulation of 512 interferograms with a “Spectrum Two” spectrophotometer (PerkinElmer, USA) at a spectral resolution of 1 cm⁻¹ and at wavenumbers between 700 and 4000 cm⁻¹. Multilayer films were assembled on ZnSe crystals by a dipping method as described above for the film built by QCM-D. All polymers, enzyme, and peptide solutions were prepared in their respective buffer solution using deuterium oxide instead of water to avoid the signal of water in the amide I band region of peptides.

Circular Dichroism. Circular dichroism spectra were recorded using a Jasco J-810 spectropolarimeter with a data pitch of 1 nm on the light wavelength. CD spectra are obtained by shining the polarized light beam perpendicularly through a silicone sheet covered by the PEI/TA/α-chymotrypsin precursor film, immersed in a KLOEi solution for 12 h. The CD spectra show the ellipticity expressed as an angle as a function of the wavelength.

Molecular Dynamic Simulations (MD). The various systems were simulated by classical molecular dynamics (MD) using the AMBER.14 GPU software in which the potential energy U is empirically described by a sum of bond, angle, and dihedral deformation energies and a pair wise additive 1–6–12 (electrostatic + van der Waals (vdW)) interaction between nonbonded atoms.

\[ U = \sum_{\text{bonds}} k_b (r - r_0)^2 + \sum_{\text{angles}} k_\theta (\theta - \theta_0)^2 + \sum_{\text{dihedrals}} \sum_{n} V_n (1 + \cos (\nu p - \gamma)) + \sum_{i<j} \left[ q_i q_j \frac{R_{ij}}{R_{ij}^2} - 2q_i q_j \left( \frac{R_{ij}^6}{R_{ij}^6} + \frac{R_{ij}^2}{R_{ij}^2} \right) \right] \]

Bond and angle deformations are represented using a harmonic potential with force constants k_b and k_θ, respectively, and either an equilibrium distance r_0 or an equilibrium angle θ_0. The dihedral deformation energy is described using a torsional potential where V_n is the barrier height divided by 2, n is the periodicity of the torsional barrier, and γ is the phase shift angle of the torsional function. Pairwise electrostatic interactions are obtained via Coulomb’s law, where q_i and q_j are the atomic charges centered on atom i and j, respectively, whereas R_0 is the distance between these atoms. van der Waals interactions are calculated using a pairwise 6–12 Lennard-Jones potential, where R_6^n corresponds to the distance at which this potential reaches its minimum. Three different systems were simulated, each containing 10 (KL)_n peptides. As protonation states of all lysine residues in the peptide are unknown, we considered half of the lysine residues to be protonated. This is why five of the (KL)_n in our simulations have four of their lysine residues protonated (1, 3, 5, and 7), while the remaining five have only three of their lysine residues protonated (2, 4, and 6). The number of water molecules in each system differs (12570, 9277, and 6185, respectively). Force field parameters for the peptide were taken from the AMBER ff14SB force field, while the TIP3P model was used for water. Cross terms in van der Waals interactions were constructed using the Lorentz–Berthelot rules. One to four van der Waals and one to four electrostatic interactions were scaled by a factor of 2. The solutions were simulated using a truncated octahedron box and 3D periodic conditions. An atom-based cutoff of 10 Å for electrostatic and vdW interactions was applied, while long-range electrostatic interactions were calculated using the particle mesh Ewald method. The MD simulations were performed at 300 K starting with random velocities. System temperatures were set using a Langevin thermostat with a virtual collision frequency of 2 ps⁻¹. A time step of 2 fs was used to integrate the equations of motion via the Verlet leapfrog algorithm. The trajectories were analyzed using the CPPTRAJ software and snapshots along the trajectory were taken using the VMD software. Cryo-Scanning Electron Microscopy (Cryo-SEM). To observe cross-sectioned gels, a specific cryo-holder was designed and manufactured by the mechanical facility of the Charles Sadron Institute (Figure S-1 in the SI). The glass slide, covered by the enzymatic precursor film and the self-assembled gel, was inserted vertically in the jaws of the vise. The holder with the sample was then rapidly plunged into a nitrogen slush in the cryo preparation chamber of the Quorum PT 3010 machine. As the sample is free-standing over the holder, during the plunging, the sample was rapidly frozen by direct contact with the nitrogen slush. The sample was then transferred under vacuum into the chamber attached to the microscope and fractured with a razor blade. A slight etching at −80 °C was performed to render the fibers more visible followed by the deposition of a thin Pt layer. The samples were then transferred into a field-emission-gun scanning electron microscope (FEG-SEM; Hitachi SU8010) using a Nikon Eclipse Ti-S with a 10X PL Fluo (0.30 NA) objective equipped with a Nikon Digital Camera (with NIS-Elements software). The excitation
wavelengths of α-chymotrypsin<sup>PTC</sup> were set between 472 and 480 nm, and the emission wavelengths were measured between 525 and 535 nm. The images were processed with ImageJ.

**Hydrolysis of para-Nitrophenyl Acetate.** The relative quantity of amine-containing self-assembled (KL)<sub>3</sub>OEt oligopeptides on the surface was determined by incubation of the surfaces (prepared on 14 mm–diameter glass slides) in a para-nitrophenyl acetate (p-NPA) solution (500 μL at 2.5 mM in 10 mM Tris buffer pH 8) and by following the production of para-nitrophenol (p-NP) at 405 nm. More precisely, p-NPA was dissolved in ethanol to prepare a 0.5 M stock solution. A volume of 125 μL of freshly prepared solution was added to 5 mL of TRIS buffer (10 mM, pH 8) giving the work solution. Concentration and volume ensured a large excess of substrate. Hydrolysis of colorless p-NPA yields a yellow optical density (OD) at λ = 405 nm of p-NP followed by Microplate reader UV spectroscopy (FLX-Xenius, SAFAS, Monaco) in 24-well plates. The blank curve, which corresponds to the hydrolysis of p-NPA in Tris bu(10 mM, pH 8) giving the work solution. Hydrolysis bands at 1614 and 1622 cm<sup>−1</sup> were followed in situ. Vibration bands at 1642 and 1650 cm<sup>−1</sup> correspond to the hydrolysis of p-NPA in Tris bu. The slope of the OD curve, taking into account the blank, of the first 7 min was used for calculation of the reaction rates (OD<sub>405 nm/min</sub>).

**RESULTS AND DISCUSSION**

Introduced by Gross and co-workers, the enzymatic-triggered self-assembly chosen is based on the oligomerization of KLOEt catalyzed by α-chymotrypsin (Scheme 1a). In solution, the obtained (KL)<sub>n</sub>OEt oligomers form an hydrogel based on intermolecular backbone hydrogen bonding leading to physically entangled nanofibrillar networks.<sup>25</sup> The dipeptide ethyl ester (KLOEt) was prepared at the multigram scale in three steps from KL with an overall yield of 92% (see SI). This condensation between KL and OEt at pH 8.7 and by turning the vial upside down (Figure S-2 in SI). To obtain the LEASA process, we adsorbed the enzyme on a surface and further brought it in contact with KLOEt solution (Scheme 1b). To anchor α-chymotrypsin on the surface, a layer of poly(ethylene imine) (PEI), known to deposit in a reproducible manner on a large variety of surfaces, was first adsorbed, followed by a second layer of tannic acid (TA) allowing a strong adsorption of α-chymotrypsin through a dense hydrogen bond network. Each adsorption step was followed by a rinsing step. QCM-D was used to follow in situ the anchoring of the enzyme and the self-assembly of the gel on the surface of a gold coated quartz crystal (Figure 1). According to Sauerbreys’s equation, and assuming a density of the 1 g/cm<sup>3</sup> for the this precursor film could be estimated at 35 nm. After the buildup of the enzymatic precursor film, it was brought in contact with a KLOEt solution at 1 mg/mL leading to a huge increase of the fundamental frequency shift (Figure 1) as well as an important increase of the corresponding dissipation (Figure S-3 in SI). After 40 min of contact with the KLOEt solution, both the fundamental frequency shift and the dissipation reached a plateau at 1300 Hz and 395 × 10<sup>−6</sup>, respectively (Figure S-3 in SI). Such high values indicate an important mass deposition on top of the enzyme layer. Moreover, the high dissipation value is characteristic of a highly viscoelastic film as expected for a hydrogel.

In contrast when the same KLOEt solution (1 mg/mL) was put into contact with a PEI/TA-bovine serum albumin (BSA) precursor film, the fundamental frequency shift and the dissipation remain unchanged (Figure S-3 in SI). This proves that the strong increases of the QCM-D signals are due to the presence of α-chymotrypsin adsorbed on the surface and the fact that some of the deposited enzymes remain active. We did not determine the proportion of enzymes that remain active because what is of importance is that some of them remain active and that the concentration of active enzymes can be varied continuously as we will see later. To get information on the composition of the peptide network, the material obtained on the surface of the enzymatic layer was collected after 12 h with a pipet to be dried and analyzed by MALDI-TOF. A distribution of (KL)<sub>n</sub>OEt oligomers was obtained with n ranging from 2 to 7 and centered around (KL)<sub>3</sub>OEt (Figure S-4 in SI). This confirms that the adsorbed α-chymotrypsin catalyzed the condensation between KLOEt molecules. Moreover, it shows that the reaction process localized at the surface does not only form dimers but also leads to higher order oligomers at high enough rate to allow the self-assembly process to start. This is by far not obvious since the first formed (KL)<sub>2</sub>OEt dimers can diffuse into the solution, reducing the probability of further reactions. The dimers present near the surface react either with single KLOEt molecules to form (KL)<sub>2</sub>OEt trimers or together to form (KL)<sub>4</sub>OEt tetramers. The oligomerization reaction then goes on with these species until forming at least up to (KL)<sub>14</sub>OEt. In comparison to a KLOEt (12% w/w)/α-chymotrypsin (10 mg/mL) mixture solution, the distribution of (KL)<sub>n</sub>OEt oligomers extended up to n = 14. Confining α-chymotrypsin on a surface results in a self-assembled architecture based on shorter (KL)<sub>n</sub>OEt oligomers than the one obtained with the same enzyme in solution.

The self-assembly process initiated at the surface was followed by ATR-FTIR in deuterated water. The active PEI/TAGPC precipitate was adsorbed onto a ZnSe crystal and then put in contact with 1 mg/mL solution of KLOEt. IR spectra were recorded regularly over time up to 17 h to follow the evolution of the carbonyl group stretching band called amide I (Figure 2a). Vibration bands at 1614 and 1622 cm<sup>−1</sup>, corresponding to β-sheet structures,<sup>26</sup> appear after 30 min and increase during the self-assembly, reaching a plateau after 150 min (Figure 2b). In addition, characteristic bands at 1642 and 1650 cm<sup>−1</sup> grow and level off rapidly in less than 10 min (Figure 2c). These bands are assigned to the presence of helical structures (3<sub>10</sub> and α).<sup>26</sup> According to the low intensity of this band, the proportion of these structures in the self-assembled structure should be minor compared to the β-sheets structures. The main secondary structure of the self-assembly was confirmed by circular dichroism (CD). CD spectra of the self-assembly formed after 24 h on the same enzyme precursor

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**Figure 1.** Evolution of the fundamental frequency shift, measured by QCM-D, as a function of time during the buildup of PEI/TA/α-chymotrypsin precursor film (shown in the inset) followed by the contact with KLOEt solution.
film showed one strong positive peak at 198 nm and a slight broad negative peak at 215 nm (Figure 2d), characteristics of β-sheet structures in accordance with Gross et al.17 In order to gain a better understanding on the interaction between (KL)nOEt oligopeptides at the atomistic level, we performed molecular dynamics (MD) simulations.

Figure 2. (a) ATR-FTIR spectra of (KL)nOEt hydrogel formed on a surface of PEI/TA/α-chymotrypsin film in contact with KLOEt solution (1 mg/mL) measured for 17 h. From the bottom to the top, each colored curve has been recorded every 10 min starting from t = 0 min, except for the last black curve, measured at t = 17 h. Evolution of the absorbance measured at (b) 1614, (c) 1640 and 1655 cm⁻¹ as a function of time; (d) CD spectra of the hydrogel formed on PEI/TA/α-chymotrypsin film in contact with KLOEt (1 mg/mL) solution for 24 h.

Figure 3. Snapshot after 13.5 μs of the 10 (KL)7 aggregate under various concentrations. α-helices (red), 3–10 helices (green), turn (yellow), parallel β-sheet (blue), and antiparallel β-sheet (cyan). (Water has been omitted for clarity.)

Figure 4. (a) Cryo-SEM image of a cross sectioned gel showing the (KL)nOEt oligopeptide self-assembly obtained by contact of a KLOEt solution (1 mg/mL) for 12 h with the precursor enzymatic film adsorbed on a glass slide (arrowhead). The medium-size white arrow points to the dense network observed along the glass slide. Larger cavities may be due to the sublimation of ice crystals (long white arrow). (b) Well preserved network at a higher magnification showing entangled fibers all over the sample (arrowhead: glass slide, short arrow: peptide fibers).
investigated how the concentration of $(KL)_{n}OEt$ could affect the nature of peptide aggregation and, in particular, whether the increase in concentration could lead to $\beta$-sheet formation as it was observed experimentally all along the self-assembly process. Three systems of 10 $(KL)_{7}$ peptides with increasing concentration were studied by classical MD simulation. Final snapshots of the $(KL)_{7}$ aggregate taken after 13.5 $\mu$s of dynamics are given in Figure 3. From these snapshots, one can see that at low and intermediate concentration of $(KL)_{7}$, the peptides tend first to form $\alpha$-helices and then aggregate in aqueous solution, which contrasts to the highly concentrated solution where a mix of $\alpha$-helices and $\beta$-sheet formation is observed. This can also be observed by the evolution of the secondary structure of the various peptides along the dynamics (Figure S-5 in the SI). Population analysis over the last 0.5 $\mu$s of the three trajectories shows that the number of residues involved in the formation of $\alpha$-helices decreases with increasing concentration (59.9%, 54.1% and 31.8%, respectively) while the $\beta$-sheet formation increases with $(KL)_{7}$ concentration (3.7%, 5.2% and 18.0%, respectively). MD simulation hence indicates that the concentration of $(KL)_{7}$ peptides influences the formation of $\beta$-sheets.

To observe the morphology of the obtained $(KL)_{n}OEt$ oligopeptide self-assembly, cryo-SEM was performed to obtain structural information in the presence of water, thereby limiting the impacts coming from drying steps. The self-assembly process was done on PEI/TA/$\alpha$-chymotrypsin coated glass slides and the resulting coating was frozen in a nitrogen slush to be observed in standard cryo-SEM conditions. After 12 h of contact with KLOEt solution, the entire surface was covered by a coating of 12 $\mu$m in thickness (Figure 4a). The skin observed on the surface of the coating is typically obtained by plunging samples into then nitrogen slush. The morphology of the $z$-section displays two distinct parts: a very dense network of nanofibers anchored on the surface of the glass and large oriented cavities on the upper part of the coating. A higher magnification of the glass surface shows the presence of nanofibers, of around 15 nm in diameter (Figure 4b). Large cavities on the upper part of the gel are attributed to experimental artifacts due to the formation of ice crystals during the cooling process followed by their sublimation. Because LEASA self-assembly is a bottom-up process, one can assume that the fiber density decreases as one moves away from the surface. Freezing artifacts probably appear in regions of small fiber density and are prevented in the dense organic material region in the close vicinity of the surface. We performed similar cryo-SEM experiments after 40 min of self-assembly (Figure S6). One observes a morphology similar to that observed after 12 h, but the film thickness is of the order of 5 $\mu$m compared to 12 $\mu$m after 12 h.

QCM-D and ATR-FTIR sense the surface with exponentially decaying shear and electromagnetic waves, respectively, leading to a limit of their penetration depth (1 and 1.5 $\mu$m, respectively). When the film thickness exceeds the penetration depth, the signals reach a plateau. Looking at cryo-SEM results, one can question the physical meaning of the plateaus reached in both techniques. To circumvent this issue, we used the nucleophilic property of $(KL)_{n}OEt$ oligomers to indirectly follow the growth kinetic of the hydrogel self-assembly. Indeed, as with lysine-rich peptides, $^{28}$ we found that the presence of KL$_n$ in solution triggers the transformation of p-NPA into p-NP (Figure S-7 in the SI). This reaction was used as sensor for the presence of $(KL)_{n}OEt$ peptides to investigate the network buildup process with time. For this purpose, we brought the self-assembled $(KL)_{n}OEt$ network prepared over different periods of time in contact with 1 mg/mL KLOEt solution on PEI/TA/$\alpha$-chymotrypsin and followed the evolution of the absorbance at 405 nm characteristic of the production of p-NP.

This activity increases over several hours with the contact time of KLOEt solution (Figure 5), i.e., with the growth of the self-assembled network. This result contrasts with the plateau observed after 40 min by QCM-D (Figure 5) or after 150 min by ATR-FTIR (Figure 2b). This shows that the self-assembly process goes on over at least 15 h, and that the plateaus, observed by QCM-D and ATR-FTIR, are not due to a stopping of the buildup process but reflect the properties of the self-assembled structures near the substrate when their thickness exceeds the penetration length of the sensing waves. It indicates in particular that the structure of the self-assembled network close to the substrate, in the penetration zone of the IR field, which is typically in the micrometer range, does not vary appreciably at longer times but that the self-assembled network is extending over much larger distances than the ATR-FTIR mode penetration length, in accordance with the thicknesses measured by cryo-SEM.

In the following, we mainly focus on the initial times of the self-assembly process that can be monitored by both techniques. In both cases, there is a lag time that extends over several minutes before the signal starts to grow and finally reaches a plateau. The signal then remains stable over at least several hours, indicating that the self-assembled architecture so formed remains robust. The monitoring by ATR-FTIR reveals that the lag time is roughly equal to 25 min before detection of any matter deposited on the surface (Figure 2b). Similarly, the fundamental frequency shift and dissipation, measured by QCM-D, first remain unchanged for 9 min before starting to increase, displaying finally sigmoidal growth kinetics (Figure S-3 in the SI). The time frame measured by QCM-D is shorter than the one measured by ATR-FTIR, probably because of the higher sensitivity of QCM-D (~1 ng/cm$^2$) than ATR-FTIR spectroscopy. To precisely evaluate the lag time corresponding to the period of time over which no change of the QCM-D signal is observed, we determined the intersection of the
tangent at the inflection point of the curve of the fundamental frequency with the time axis (Figure S-8 in the SI).

The influence of the KLOEt concentration put into contact with a PEI/TA/α-chymotrypsin precursor film was first studied. Below 0.45 mg/mL in dipeptide, no self-assembly is observed, even over 12 h of contact (Figure 6). The amount of dipeptide is probably too small to reach a high enough concentration of oligomers to induce an effective localized self-assembly. When the concentration of KLOEt increases from 0.45 to 1.5 mg/mL, the enzymatic buildup process starts after a lag-time that decreases from 13 to 6 min. The more KLOEt peptides are present in solution, the more oligomers are formed by the enzyme layer in the vicinity of the surface. Physically, the lag time corresponds to the time necessary to reach near the surface the critical concentration of oligopeptides (KL)_{OEt} that is required for the fiber formation. The lag time is thus strongly dependent upon the KLOEt concentration used for the buildup process. At long contact times, the values of the plateaus reached by both QCM-D signals (frequency and dissipation) increase with the concentration of dipeptides in solution. This could be explained by the fact that using a higher KLOEt concentration leads to a higher density of self-assembled fibers near the surface in the area sensed by the shear wave.

To get more precise control over the localized self-assembly process at the surface, we varied the composition of the enzyme layer. Specifically, we wanted to investigate whether the LEASA process can be modulated by varying the surface density of the adsorbed α-chymotrypsin. For this purpose, mixtures of α-chymotrypsin and BSA, prepared at various molar ratios r while maintaining a constant protein concentration of 8 μM, were adsorbed on PEI/TA surfaces. The molar ratio r is defined as follows:

\[
r(\%) = \left(\frac{[\alpha\text{-chymotrypsin}]}{[\alpha\text{-chymotrypsin}]+[\text{BSA}]})\times 100
\]

To confirm that the α-chymotrypsin concentration on the surface can be varied in this way, mixtures of fluorescently labeled α-chymotrypsin (α-chymotrypsin^{FITC}) and nonfluorescent BSA were used to prepare the surfaces at different molar ratio r. The fluorescence intensity of the surface increases with the molar ratio r. The normalized fluorescence intensity is consequently related to the ratio in α-chymotrypsin^{FITC} adsorbed on the surface, named r_{surface}. r_{surface} represents the relative amount of α-chymotrypsin adsorbed on the surface from a mixed α-chymotrypsin/BSA solution compared to the amount of α-chymotrypsin adsorbed when using a pure enzyme solution. Higher r_{surface} thus indicates higher amounts of deposited α-chymotrypsin. The evolution of r_{surface} as a function of r is linear showing a good control of the surface density in α-chymotrypsin^{FITC} (Figure S-9 in SI). In the following study, all the results will be expressed as a function of r_{surface}. After the deposition of the protein mixture followed by a rinsing step, a fixed concentration of 1 mg/mL KLOEt solution was put into contact with the surface for 12 h to monitor the self-assembly by QCM-D. For each case, the QCM-D signals presented a similar behavior characterized by a lag time followed by an increase and a final plateau (data not shown). Figure 7 shows the lag time and the value of the fundamental frequency shift at the plateau as a function of r_{surface}.

The lag time is minimum for a 100% α-chymotrypsin layer, increases slowly up to r_{surface} = 30%, and finally increases strongly at r_{surface} = 2% in α-chymotrypsin. As expected at r_{surface} = 0% (on a PEI/TA/BSA precursor film), no self-assembly is observed, and the lag time diverges. For example, the lag time is on the order of 420 min at 2% in α-chymotrypsin, 40 min at 30% in α-chymotrypsin, and reaches the lowest value of 9 min for PEI/TA/α-chymotrypsin film. Since the synthesis of sufficient quantity of oligopeptides is required to start the self-assembly, the buildup kinetic is directly dependent on the surface density of enzymes. Concerning the plateau value, corresponding to Δf_{plateau}, it remains fairly constant when the surface density in α-chymotrypsin is decreased from 100% to

![Figure 6. Evolution of the (a) fundamental frequency shift and (b) dissipation value, measured by QCM-D, as a function of time when PEI/TA/α-chymotrypsin precursor film is brought into contact at t = 0 with 1.5 (green line), 1.0 (dark red line), 0.45 (black line), 0.4 (blue line), and 0.3 mg/mL (red line) KLOEt solution.](image)

![Figure 7. Evolution of (○) the lag time and (●) the amplitude of the fundamental frequency shift, value at the plateau, measured by QCM-D, when KLOEt (1 mg/mL) solution is put into contact for 12 h with a PEI/TA/α-chymotrypsin (r_{surface}) film, as a function of r_{surface} ratio in α-chymotrypsin adsorbed on the surface. The lines are only drawn to guide the eye.](image)
30%. Further decrease of the enzymatic surface density from 30% to 0% induces a decrease of the plateau value (Figure 7).

For these low α-chymotrypsin surface concentrations, the reached plateaus could be either due to the fact that the film thickness exceeds the sensing length of QCM-D or because the self-assembly process stops. To discriminate between these two possibilities, the nucleophilic activity of the (KL)₇-OEt fiber network obtained on PEI/TA/α-chymotrypsin (r_{surface} = 5.4%) was measured after different contact times of KLOEt. A continuous increase of the p-NP production triggered by the self-assembled network is observed over 15 h, indicating that the self-assembly takes place at least over this period of time when the plateau measured by QCM-D is reached after 7 h (Figure 8). The thickness of the self-assembly structure thus exceeds the penetration depth of QCM-D. A smaller value at the plateau can be related to a smaller density in self-assembled structures when the plateau measured by QCM-D is reached after 7 h. The self-assembly takes place at least over this period of time, the self-assembly increases (Figure 9a). Being on the order of 0.45 mg/mL for both 47% and 100% in α-chymotrypsin, the critical concentration becomes equal to 0.8 mg/mL for 5.5% in α-chymotrypsin. A smaller surface density in enzyme leads to a smaller amount of (KL)₇-OEt oligopeptides formed per cm², preventing the self-assembly of enough oligomers. For a given α-chymotrypsin concentration on the surface, the lag time increases when the KLOEt concentration decreases (Figure 9b). Such a behavior clearly shows that LEASA can be tuned by controlling the enzymatically active surface used as a catalyst for the film buildup.

**CONCLUSION**

The development of chemical systems localized at an interface is an essential step toward the design of powerful nanoarchitectonic coatings. In this respect, the control in space and time of the self-assembly process appears as a useful approach. Here, we have demonstrated that LEASA strategy can be applied using α-chymotrypsin adsorbed layer on a substrate to build fibrillar structures by condensation of KLOEt into oligomers, which self-assemble. At a critical concentration in KLOEt, the film buildup takes place at least over several hours leading to several micrometer thick structures with the existence of a lag time. We further demonstrated that LEASA processes can be controlled by varying the surface concentration of adsorbed α-chymotrypsin. In particular, the lag time can be finely tuned through the surface density in α-chymotrypsin and KLOEt concentration. Finally, for a given surface enzyme concentration, a critical KLOEt concentration exists below which no self-assembly takes place. This concentration increases when the surface enzyme concentration decreases.

**ASSOCIATED CONTENT**

Supporting Information

List of chemicals, detailed synthesis of KLOEt and α-chymotrypsin, image of the specific homemade cryo-holder, gelation test of KLOEt and α-chymotrypsin mixture in solution, QCM-D experiments using adsorbed BSA in comparison with adsorbed α-chymotrypsin, MALDI-TOF spectra of (KL)₇-OEt hydrogel obtained on PEI/TA/α-chymotrypsin, second structure analysis of (KL)₇-OEt oligomers by MD, Cryo-SEM.

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**Figure 8.** Evolution of (●) the p-NP production rate triggered by the (KL)₇-OEt self-assembled network and (—) the fundamental frequency as a function of time of contact with 1 mg/mL KLOEt solution with a PEI/TA/α-chymotrypsin (r_{surface} = 5.4%) surface. The p-NP production rate corresponds to the slope of the optical density (OD) at 405 nm versus time due to the release of p-NP when p-NPA was brought in contact with the (KL)₇-OEt self-assembled architecture.

**Figure 9.** Evolution of (a) the amplitude of the fundamental frequency shift, value at the plateau and (b) the lag time, determined by QCM-D, as a function of KLOEt concentration used in contact for 12 h with PEI/TA/α-chymotrypsin (r_{surface} = 5.5%) precursor film at a surface ratio r_{surface} = 5.5% (□), 15% (●), 47% (○), and 100% (●) in α-chymotrypsin.
image of a peptide network after 40 min of self-assembly. Evolution of the optical density of pNPA in solution with (KL)₆OEt, schematic representation of the lag time estimation, evolution of α-chymotrypsinFITC density as a function of the molar ratio in enzyme of the solution deposited, nucleophilic activity of (KL)₆OEt self-assembled hydrogel measured at different times for r_surface = 100% and 5%. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.7b01532.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

C.V.C. was supported by a fellowship from CNRS and Région Alsace. P.L. was supported by a postdoctoral fellowship from the University of Strasbourg Institute for Advanced Study (USIAS). Mélodie Galerne, Pauline Barrois, and Morgane Rabineau are acknowledged for their technical support. The authors acknowledge the electron microscopy platform of ICS. The authors gratefully acknowledge financial support from Labex “Chimie des Systèmes Complexes” (Labex CSC) and from the International Center for Frontier Research in Chemistry (iFRIC).

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DOI: 10.1021/acs.langmuir.7b01532 Langmuir 2017, 33, 8267–8276