

Amino-methyl coumarin as a potential SERS@Ag probe for the evaluation of protease activity and inhibition

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Proteases are found deregulated in many diseases including cancer and neurodegenerative diseases. They thus represent good therapeutical targets for the development of inhibitors mainly small organic molecules. Peptide substrates containing fluorogenic groups constitute central tools for the monitoring of protease activities and inhibitor screening platforms. Amino-methyl coumarin (AMC) is a well-known fluorogenic group that functionalized a huge number of peptide substrates used for kinetics *in vitro* but also *in vivo*. However, either autofluorescence or quenching of the AMC fluorescence could compromise selection and accurate evaluation of these inhibitors. It is thus needed to explore alternative spectroscopic tools to unravel these limitations. Here, we investigate whether AMC could constitute a valuable Surface Enhanced Raman Spectroscopy probe in the presence of Creighton' silver colloids under 532-nm excitation to monitor protease activity and to evaluate inhibitors. The kallikrein-related peptidase 8 was used as model of proteolytic enzyme. Band-Target Entropy Minimization analysis was successfully used to validate the present Surface Enhanced Raman Spectroscopy approach. Copyright © 2016 John Wiley & Sons, Ltd.

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Keywords: silver colloids; coumarin; concentration; BTEM; protease

Introduction

Proteases constitute major actors in cell signalling and tissue remodelling. Their deregulation is implicated in many diseases including cancer, neurodegeneration and inflammation.^[1] The development of proteases inhibitors is a fruitful field in the biotech industry.^[2] A huge number of protease inhibitors are drugs of reference, e.g. HIV protease and renin inhibitors. Many biophysical approaches have been developed for the identification and characterization of small organic inhibitors.^[3] Among them, fluorescence spectroscopy is the most commonly used. Fluorescence assays are based on the use of peptide substrates bearing fluorogenic groups. Among the used fluorophores, coumarin derivatives such as aminomethylcoumarin are overrepresented. However, when screening libraries of organic molecules, various limitations are observed such as fluorescence quenching and autofluorescence. This could compromise the proper selection and evaluation of such inhibitors. Alternate approaches have to be considered, among them Surface Enhanced Raman Spectroscopy (SERS),^[4–6] which has gained interest in the area of enzymology these last 10 years.^[7,8] The use of SERS in biomedical applications in particular in enzymology is advantageous in many ways; it is convenient for dilute media, and SERS can allow the quenching of fluorescence backgrounds. Another striking point is that it may provide vibration fingerprints of individual molecules even in mixtures. This could help for the understanding of the related chemical reactions by monitoring of spectral changes of both substrates and products.

Various strategies have been proposed to follow enzyme kinetics by means of SERS including the decay or structural changes of the substrate, appearance of the reaction product through the analysis of vibration spectra. Based on SERS principles several hydrolytic enzymes including lipases, esterases as well as proteases were successfully investigated. Graham and his co-workers have extensively studied lipase reaction kinetics using in particular benzotriazole derivatives.^[9] With lipases, protease activities have also been exten-

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sively investigated by means of a large variety of experimental SERS devices. Among the developed strategies, some are based on hybrid peptide-nanoparticle probes.^[7,10] These latter have been implemented for the successful detection of prostate specific antigen, a major prostate cancer biomarker in seminal fluid and serum of patients.^[11] More recently, a novel 'turn-off' biosensing strategy for thrombin detection based on SERS using 4-mercaptopbenzoic acid labelled gold nanoparticles has been proposed.^[12] Another strategy has also reported the use of protein substrates like casein and gelatin immobilized on the surface of self-assembled monolayers of 5,5-dithiobis (2-nitrobenzoic acid) Raman reporter coated on the surface of gold nanoparticles.^[13]

Herein, we take advantage of SERS@Ag activity for the detection of coumarin probe and the potential application to the qualitative inhibition characterization of human kallikreins. Kallikrein-related peptidases are a family of 15 serine proteases that constitute the larger cluster of proteases in the human genome. They are implicated in numerous physiological processes including skin homeostasis, tissue renewal and neural plasticity.^[14,15] They are often overexpressed in pathological conditions such as cancer, skin inflammation and neurodegeneration. We focus here on kallikrein-related peptidase 8 (KLK8) also called neuropsin. Kallikrein 8 is an emerging therapeutic target, implicated in skin diseases such as hyperkeratosis and anxiety.^[15] In a first part, we have evaluated the robustness of amino-methyl coumarin (AMC) as a SERS probe using Creighton silver colloid in comparison to fluorescence assay through quantitative analysis. The kinetics of AMC release upon proteolytic processing has been characterized by SERS and compared to classical fluorescence analysis. The effect of the several components present in the reaction medium as salts, buffer and solvent that may interfere with the Raman signal was also studied. Band-Target Entropy Minimization (BTEM) algorithm^[16] was also used to recover the pure component spectra in enzyme reaction mixtures and validate the use of SERS approach for AMC cleavage quantitation. Finally, the procedure will be tested on KLK8 inhibition by an irreversible inhibitor previously identified in our group.^[17]

Experimental section

Materials

KLK8 was purchased from R&D Systems Inc (Minneapolis, USA). 7-AMC and the fluorogenic substrate Valine-Proline-Arginine-AMC (VPR-AMC) (Fig. 1a and b) were purchased, respectively, from SIGMA (France) and Bachem (France). Enzymatic studies using fluorescence signal were performed using a BMG Fluostar microplate reader (black 96-well microplates). Coumarin inhibitor (Compound 4d Fig. 1c) was synthesized and purified as described in.^[17] The purity of the compound was equal to or greater than 95%. Stock solutions of substrate and inhibitor (10 mM) were prepared by dissolving the corresponding pure solid compounds in DMSO and stored at -20°C . DMSO, anhydrous, $\geq 99.9\%$ was purchased from SIGMA (France).

Colloid preparation and characterization

Several silver colloid batches were synthesized using the classical Creighton method.^[18] Samples presenting a thin absorption peak with a maximum at 398 nm correspond to almost monodisperse and poorly aggregated colloids and were selected for this study (Fig. S1a, Supporting Information). UV-vis measurements were

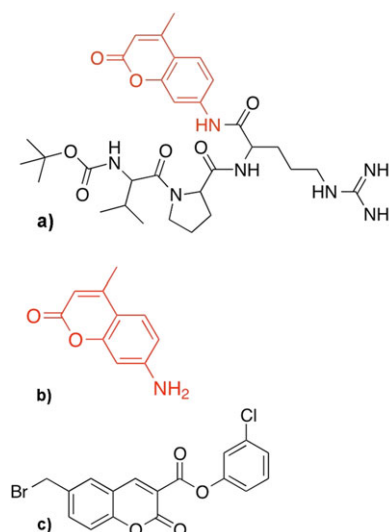


Figure 1. Structures of the investigated molecules: a) Peptide substrate VPR-AMC of human kallikrein 8 (KLK8); b) Amino-methyl coumarin (AMC) c) KLK8 selected inhibitor: compound 4d.

performed on a Cary 3 UV-visible spectrometer to check colloid stability. Colloid stability was investigated in the presence of the various components of the activity buffer incorporated individually and gathered: DMSO, Tris buffer, NaCl and KLK8 (Fig. S1b, Supporting Information).

Raman and SERS measurements

The Raman spectra were recorded on a Senterra Bruker Optics instrument (Ettlingen, Germany) using a 532-nm diode laser source and a 1200 lines/mm grating (resolution $< 4\text{ cm}^{-1}$). The Rayleigh filtering is achieved using Notch Filters. The laser power was generally 20 mW at the output of a $\times 50$ long working distance Olympus objective, the $\sim 20\text{-}\mu\text{m}^2$ spot being focused in the liquid; the acquisition time was 20 s (up to 10 accumulations). The spectra of the various samples were recorded in at least three independent experiments. Data were analysed using Opus[®] software (Bruker, Germany) and figures generated using Origin 6[®] (Microcalc. Inc., USA). The intensities of all the registered spectra were normalized to water band intensity ($3600\text{--}3000\text{ cm}^{-1}$) and DMSO. For optimal reproducibility of SERS measurements, the samples were inserted in glass tubes using the following order: 80 μl of colloid, 10 μl of salt and 10 μl of tested aqueous solution. The SERS spectra of these latter were immediately recorded. For enzymatic assays, the time was a critical factor; dilutions of the withdrawn reaction solution in silver colloid medium allowed having a snapshot of the reaction at a given time. Indeed, the reaction is highly slow down or stopped after dilution.

Enzyme kinetics assays: fluorescence versus SERS

For fluorescence measurements, substrates and compounds were previously dissolved in DMSO, with the final solvent concentration kept constant at 1–2% (v/v). The enzymatic reaction was performed at 37°C in a total volume of 100 μl with 100- μM VPR-AMC in KLK8 reaction buffer at pH 9 composed of Tris 50 mM, Tween20 0.02%. The hydrolysis of fluorogenic VPR-AMC substrate was monitored with $\lambda_{\text{exc}} = 355\text{ nm}$ and $\lambda_{\text{em}} = 460\text{ nm}$ at 37°C . For SERS measurements, the enzyme kinetics was investigated on the protease

KLK8 (10 nM) and VPR-AMC substrate (1 mM) in buffer activity in a total volume of 200 μl . Successive samples (10 μl) were collected at different time intervals from the reaction medium and subjected to measurement by SERS, as described above. The final contribution of DMSO was 1%. For inhibition assay with SERS detection, activities of untreated KLK8 (control) or KLK8 preincubated with a test compound (compound 4d) at a concentration corresponding to IC_{50} (40 μM) were registered during 15 min. Then reaction is launched using a 1 mM initial concentration of substrate VPR-AMC in an initial volume of 100 μl . Spectra of the inhibited reaction were recorded at various time.

Computational analysis

In the past two decades, a number of useful multivariate and other chemometric methods have been developed and applied to sets of Raman spectra. These include cluster analysis in order to identify distinct subsets of similar Raman spectra in an unsupervised manner,^[19] classification in order to group similar Raman spectra in a supervised manner,^[20] and curve resolution analysis, such as SIMPLISMA^[21] and MCR-ALS^[22] in order to obtain spectral estimates of the underlying pure components. Curve resolution is a particularly interesting form of analysis, because good spectral estimates of the underlying pure components often allow direct identification of the individual components. Another curve resolution method is BTEM algorithm.^[23,24] BTEM analyses large sets of multi-component spectra and provides a set of 'pure component' spectral estimates for the constituents present, without recourse to any a priori information. Furthermore, these spectral estimates often have outstanding signal-to-noise ratios, even for the minor or trace constituents present.^[25,26] BTEM has been successfully applied to Fourier transform infrared,^[23,24,27,28] Raman,^[23,29,30] nuclear magnetic resonance,^[31] mass spectrometry,^[32,33] UV-vis,^[34] Spatially Offset Raman Spectroscopy,^[35–37] Reflection Absorption Infra Red Spectroscopy (RAIRS)^[38] etc. Comparisons between BTEM analysis and other self-modelling curve resolution methods, in the context of vibrational spectroscopies, have appeared elsewhere.^[24,27]

The original SERS spectra were first de-spiked, to remove cosmic artefacts from the measured spectra, using an in-house algorithm written in MatLab. This provided a matrix of SERS spectra that was subsequently used as input for spectral reconstruction.

The BTEM algorithm was used for the blind spectral reconstruction, using no a priori data such as libraries. In BTEM analysis, spectral reconstructions are obtained one-by-one until all targeted features in the data set are exhausted. The basic idea behind BTEM is the search for the simplest underlying patterns in the data. In most cases, these simplest underlying spectra are normally the pure component spectra. Let k represent the number of spectra measured and let v represent the number of spectroscopic channels, then the intensity matrix $M_{k \times v}$ can be transformed to obtain each pure component spectral estimate. The procedure has been described in Widjaja & Garland.^[30,39,40] The set of data used corresponds to the nine spectra obtained as a function of time (Fig. 4a) during the kinetic assay, and two spectra of pure compounds in the same buffer, substrate VPR-AMC and protein.

Results and discussion

Silver colloids for AMC detection

Several silver colloids were synthesized using the classical Creighton method.^[18] We have evaluated the impact of the

reaction medium components at the concentrations used in our assays (reaction buffer, DMSO, NaCl, protease) (Fig. S1, Supporting Information). Importantly, at the used concentrations, buffer, DMSO and enzyme do not alter the UV-vis spectra indicating that they do not perturb the colloidal suspension. As expected, NaCl acts as an aggregating agent. It plays an important role enabling the formation of 'hot spots' areas and monitoring electrostatic interactions with the analyte. Thus, we validated the use of silver colloid as SERS platform for application to enzymology. In addition, and to be consistent along all the set of experiments, the investigated molecules (AMC, VPR-AMC substrate) prior to analysis by SERS have been dissolved in the same buffer than that used in enzymatic assays.

Figure 2a shows a comparison of AMC signals (SERS vs Raman) and AMC embedded in the peptide substrate VPR-AMC. The analysed solutions of both compounds were dissolved in KLK8 reaction buffer at pH 9 prior to dilution in the colloidal suspension. Final pH in the colloidal suspension ranges from 7 to 8. Only free AMC radical results in an enhanced Raman signal with silver colloid leading to SERS responses. For aqueous solution of AMC in the absence of silver colloid, no Raman signal can be detected at same

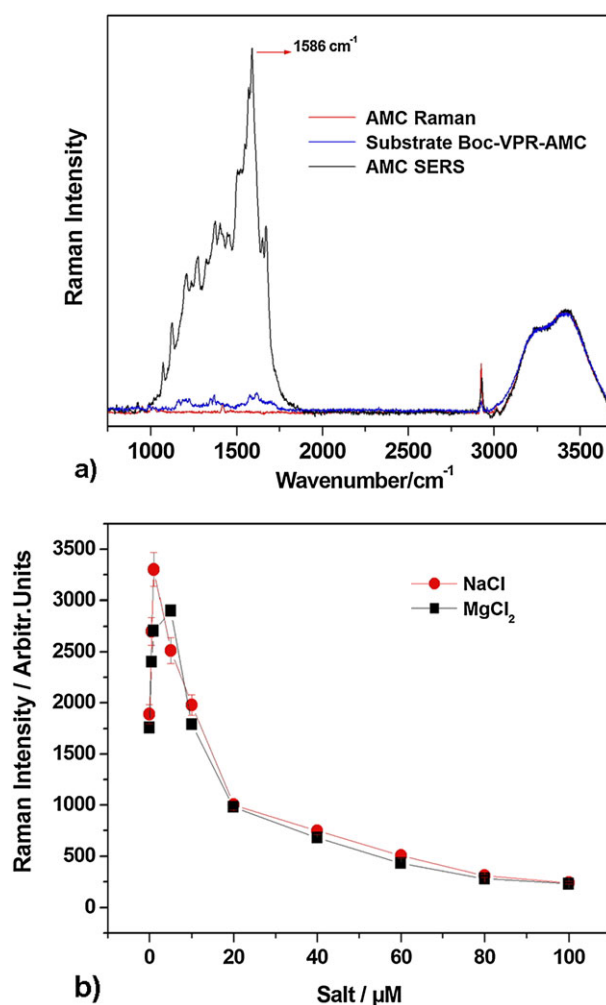


Figure 2. Raman and SERS spectra of free AMC and peptide substrate: a) Final concentrations were of 10 μM from stock solutions at 100 μM in KLK8 activity buffer, for classical Raman spectrum silver colloid was replaced by water; the most intense band at 1586 cm^{-1} is indicated in red; b) Evolution of 1586 cm^{-1} band intensity as the function of the concentration of NaCl and MgCl_2 salts. The final percentage of DMSO was 2% for all samples.

range of concentrations (10 μM) (Fig. 2a). Presence of C=O bonds and aromatic cycles promotes electronic interaction of AMC (Fig. 1b) with Ag° colloids.^[41–43] Consequently modes involving these chemical bonds were enhanced. Particularly in the SERS spectrum, the band at 1586 cm^{-1} was identified to be the most intense and was thus selected as a standard band for quantitation studies. As previously observed, salt may favour AMC/Ag colloid interaction and increase SERS signal. Both NaCl and MgCl_2 were tested (Fig. 2b) and enhance SERS effect because of charge screening by oppositely charged ions Na^+ and Mg^{2+} . AMC intensity at 1586 cm^{-1} was found optimum in the presence of 1 mM NaCl. In all the further SERS measurements, NaCl concentration was thus adjusted to a final concentration of 1 mM. Figure 2a shows that the VPR-AMC peptide substrate even at a concentration as high as $100\text{ }\mu\text{M}$ (compatible with this used in the enzymatic assays) gives rise to a very weak Raman signal. This could be explained by a lack of accessibility for AMC moiety; the peptide portion could prevent appropriate adsorption of AMC moiety onto silver particles.

As expected, the presence of the released peptide was not detected on the Raman spectra. Indeed, the peptide is devoid of aromatic amino acids and only bears an arginine. Furthermore it is worth noting that in a recent study McIntosh *et al.* have reevaluated the value of guanidinium pKa. The measured pKa was 13.8 which is substantially higher than the known of 12.^[44] We can thus confirm that Arg guanidium is protonated in our conditions.

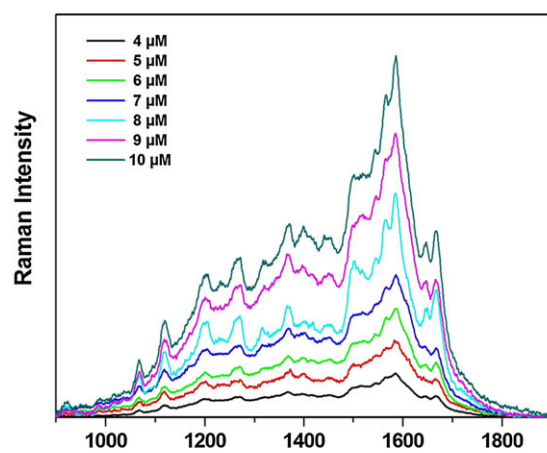
This first observation is in accordance with the fact that SERS can distinguish AMC radical and VPR-AMC. Proteolytic activity could thus be potentially followed using this molecular probe.

Quantitative analysis

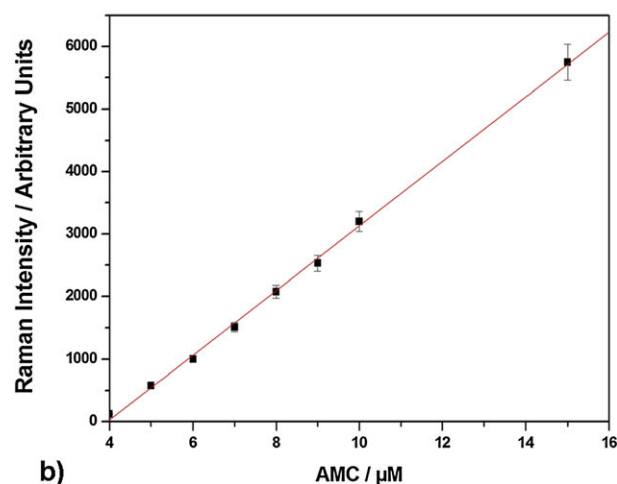
Comparative dose–responses of AMC were performed in the concentration range of $0.5\text{--}10\text{ }\mu\text{M}$ in the reaction buffer using both SERS and fluorescence spectroscopy in order to quantify released AMC after reaction with KLK8 (Fig. 3). In the presence of colloids, Raman signal can be observed for a concentration as low as $4\text{ }\mu\text{M}$ (Fig. 3a). This range of concentrations corresponds to these potentially released after proteolytic action of KLK8. No negative impacts or overlapping signals of the reaction media were noticed on SERS measurements as also previously observed by UV–vis spectroscopy.

Figure 3a presents the SERS spectra of AMC as a function of concentration, in the presence of 1 mM NaCl in silver colloid and 1–2% of final DMSO corresponding to standard percentage in enzyme reaction solutions. The SERS spectra are comparable to those recorded by Vogel *et al.*,^[45] with variable relative intensities. Within the concentration range used, coumarin adsorption on the silver surface amplifies the band at $1586\text{--}1568\text{ cm}^{-1}$ attributed to a complex stretching vibration involving the C=O (Table 1). The bands in the range of $600\text{--}750\text{ cm}^{-1}$ corresponding to ring stretching are not strongly amplified suggesting that coumarin is not adsorbed on colloid *via* the aromatic rings.^[45–49] DMSO also presents Raman response in the presence of silver colloid (Table 1, Fig. 2a). These DMSO characteristic bands and the absorption bands of water can be used for intensity normalization as the percentage of DMSO in the solution is constant (1 or 2%).^[48]

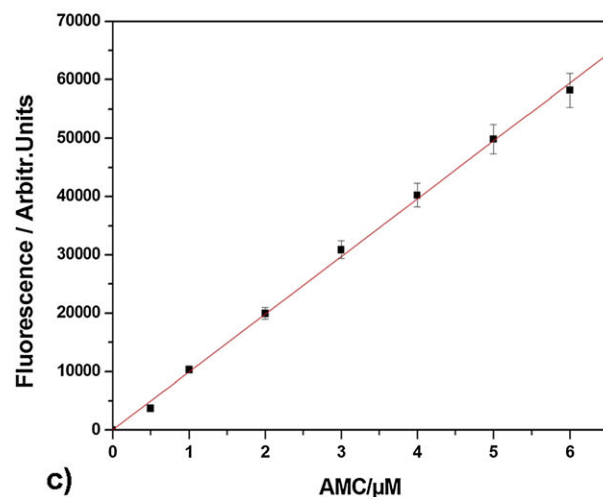
Figure 3b shows that the Raman intensity increases linearly as a function of AMC concentration, from $4\text{ to }15\text{ }\mu\text{M}$. In this range, the intensity of the band at 1586 cm^{-1} can be used as a marker to quantify coumarin/silver interaction, and consequently to monitor AMC release after reaction with KLK8. The same linear curve is observed with fluorescence measurements in the range of $0.5\text{--}6\text{ }\mu\text{M}$,



a)



b)



c)

Figure 3. AMC SERS signal: a) Variation of SERS intensity of AMC band at 1586 cm^{-1} as a function of AMC concentration in the presence of 1% DMSO, 1 mM NaCl and 80% of silver colloid; b) standard curves of AMC as a function of the concentration expressed as 1586 cm^{-1} SERS band intensity c) AMC fluorescence units at 460 nm.

concentrations that correspond to the maximum of expected released amounts and level of detection using standard spectrometer parameters (Fig. 3c).

Table 1. Main SERS observed bands and assignments of amino-methyl coumarin

Wavenumber (cm ⁻¹)	Assignments (after ^[45–49])
3014, m	DMSO
2928, vs	DMSO
1665, 1643, s	C=O, C=C, ring stretching; NH ₂ deformation
1587, 1568, vs	C=O, C=C, CC, ring stretching
1501, s	CH bending; C=C stretching/CH wagging
1000–1370, m	CH bending; CO, CC, C—NH ₂ stretching; NH ₂ rocking; ring breathing

Abbreviations: very strong (vs); strong (s); medium (m); weak (w).

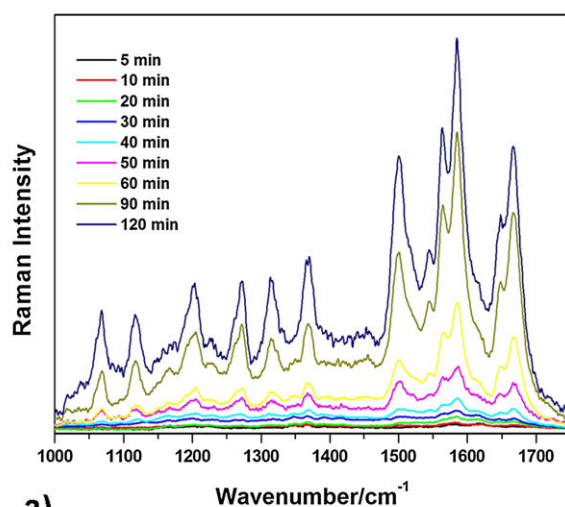
Kinetics monitoring: SERS versus fluorescence spectroscopy

The enzyme kinetics as followed by SERS were investigated with KLK8 (10 nM) and VPR-AMC substrate (1 mM) in the initial reaction mixture before dilution in the colloidal solution. This substrate concentration was adjusted in order to have an optimum SERS detection during the investigated time scale. The time course of the reaction was followed using the sampling method: successive samples were withdrawn over time from the reaction medium and subjected to successive measurement by SERS. Thus, registered SERS spectra illustrated the composition of reaction media at a given stage of the reaction.

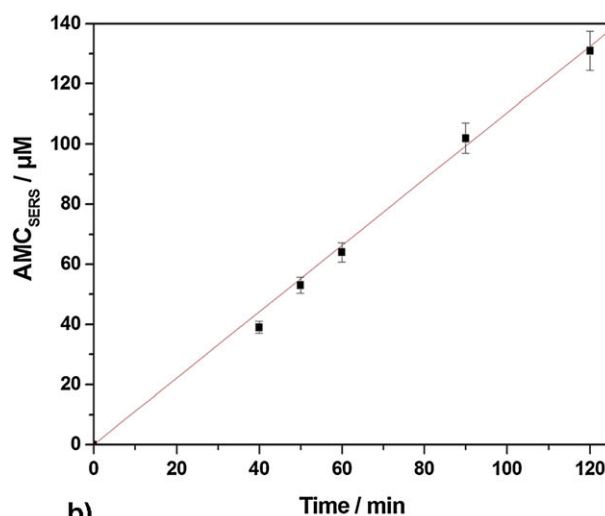
Figure 4a presents the time-course release of AMC from VPR-AMC substrate hydrolysis by KLK8 studied by SERS. It is clearly shown that the intensity of typical bands of AMC (see above) increased over the time. As expected, the obtained spectrum for released AMC is highly comparable to commercial AMC however with slight differences that could be explained in part by potential differences in protonation state of amine group (NH₃ vs NH₂) and different orientations of AMC on the colloid surface. Moreover, the response obtained on Fig. 4a during AMC release is not sensitive to the presence of peptide (that could be adsorbed on the colloid surface) as the same global results were obtained with fluorescence and SERS. No interaction between the free peptide and colloid could be pointed out in the conditions used. Overall the same Raman bands are observed with a better resolution for the spectra of released AMC. In both spectra, the most intense band remains at 1586 cm⁻¹. To determine the velocity of the reaction, amount of released AMC were deduced from the intensity of the 1586 cm⁻¹ band using standard curve (Fig. 3b) in comparison to fluorescence assay (Fig. 3c). The initial velocities that illustrate the amounts of released AMC per time unit were estimated to 1115.2 ± 10.7 nM AMC.min⁻¹ and 985.7 ± 14.4 nM AMC.min⁻¹ for respectively SERS experiments and fluorescence assay (Figs. 4b and 4c). These results seem very convergent and allow validating SERS as a potential tool for the monitoring of the enzymatic activity with AMC functionalized substrates.

BTEM analysis

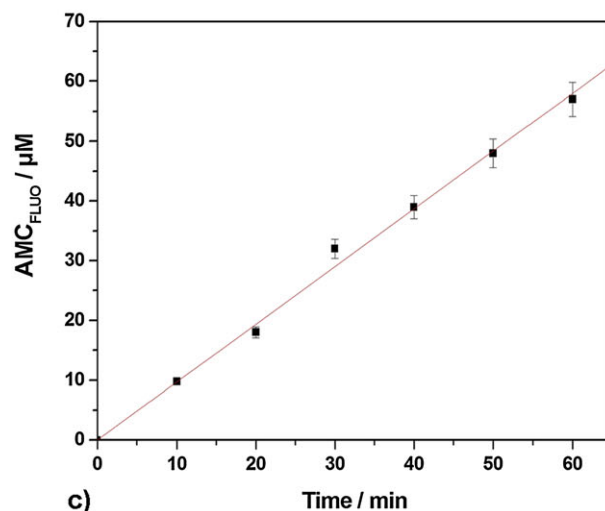
To consolidate our data, a BTEM analysis was performed and provided 4 'pure component' spectral estimates (Fig. 5), and comparisons to libraries showed that these estimates correspond to AMC (Spectrum A), buffer and VPR-AMC (Spectrum B), Glass tube contribution (Spectrum C) and water (Spectrum D). The computational analysis allowed to conclude that there is no intermolecular interactions and that spectra corresponded to pure species namely in the



a)



b)



c)

Figure 4. KLK8 enzymatic activity as followed by SERS: a) SERS spectra of VPR-AMC after reaction with KLK8 as a function of time; standard curves for kinetics of cleavage reaction measured in b) and followed by fluorescence spectroscopy in c).

case of released AMC. Additionally, this analysis validates the use of the calibration curve obtained with pure compounds.

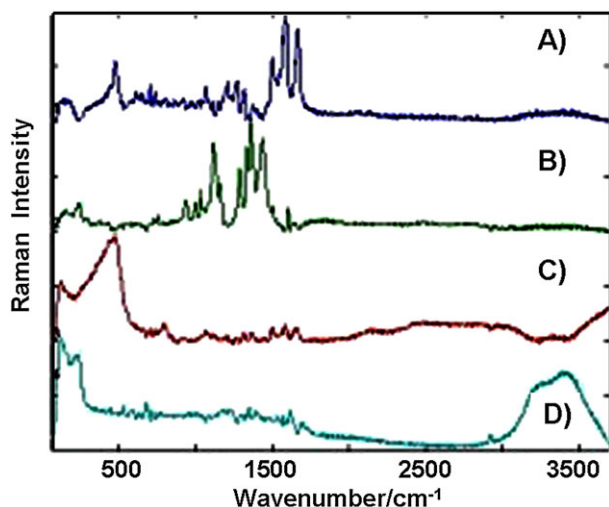


Figure 5. BETM analysis. Calculated spectra of the components in the SERS enzymatic mixture (A) released AMC, (B) Substrate, buffer, (C) Glass and (D) Water.

Protease inhibition as detected by SERS

In a final part of our investigations, we examined whether SERS could be useful for the evaluation of protease inhibitors (Fig. 6). In this goal, coumarin-based inhibitors developed in our lab were evaluated.^[17,50,51] These latter are known to perform irreversible inhibition of kallikreins through a suicide mechanism.^[17] Here, we selected the compound 4d (Fig. 1c) for its great inhibition potency. It is worth noting that the SERS signal of the inhibitor is very poor despite its coumarinic nature and did not interfere with AMC signal. Furthermore, no impact of compound 4d on silver colloid integrity was observed at concentration range used in this study (data not shown). The enzyme (10 nM) was preincubated 15 min in the presence of 40 μ M of 4d inhibitor ($IC_{50} = 4 \mu$ M) to completely inactivated KLK8 in 100- μ l initial volume of reaction buffer. It is shown that even after 120-min time contact with substrate (Fig. 6),

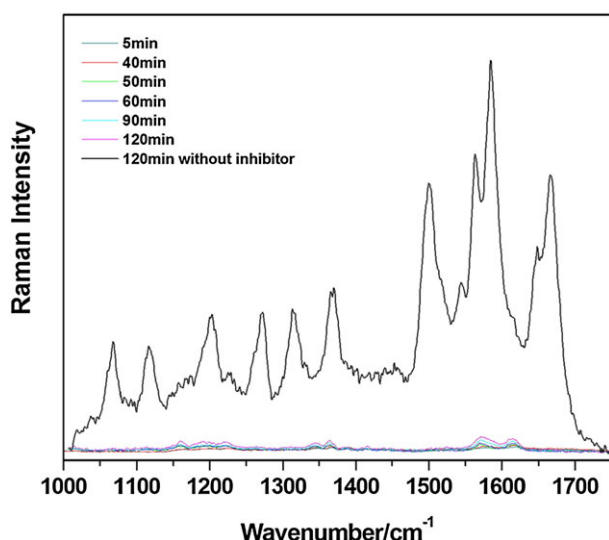


Figure 6. Inhibition of KLK8 by selected compound 4d. KLK8 was preincubated at 37 °C with 40 μ M of 4d inhibitor during 15 min. The control spectrum corresponding to the released AMC after 120 min in the absence of inhibitor is also shown.

the free AMC signal was not recovered. This means that KLK8 was efficiently inhibited. The protease activity could not be restored even after dilution in silver colloid, while the control reaction retains all the typical spectral properties as observed in Fig. 4a. This finding is in accordance with an irreversible inhibition as previously established using fluorescence assay.^[17]

Conclusions

In the present study, we have established for the first time that AMC group could be a useful SERS reporter in the presence of Creighton silver colloids to both detect serine protease activity and investigate their inhibition. The KLK8 was used as model of protease. We evidenced that only AMC released upon proteolytic processing gave rise to a SERS signal but not the AMC incorporated within the synthetic substrate. It has been established that DMSO, a widely used solvent in drug discovery, at percentage ranges used in inhibition evaluations did not interfere with AMC signal. The intensity of the band at 1586 cm^{-1} was validated as a marker to monitor AMC release after enzyme reaction. Furthermore, a computational analysis of the whole set of spectra was performed and showed that each component of the reaction medium has an individual and pure contribution excluding thus non-specific intermolecular interactions.

Acknowledgements

This work was supported by the CONVERGENCE Research Program of Université Pierre et Marie Curie (UPMC), the Institut National pour la Recherche Médicale (INSERM) and the Centre National de la Recherche Scientifique (CNRS). The authors are grateful to the French Ministry of Research and Education for PhD fellowship of F.S.

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