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PEGylation modulates the interfacial kinetics of proteases on peptide-capped gold nanoparticles†

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Fluorescence unquenching measurements of protease-dependent release of fluorescent biomolecules from peptide-capped gold nanoparticles reveal the effect of the monolayer composition on enzyme kinetics.

Understanding and controlling the interactions of biomolecules with nanomaterials is of critical importance for their application in biosensors, *in vivo* imaging, nanomedicine and for the design of more complex self-assembled nanodevices.[1] Enzymes, *e.g.* kinases, proteases, nucleases, *etc.*, can be used to modify nanoparticle bioconjugates providing tools to detect enzyme activity,[2–4] release drugs or control the formation of nanoparticle assemblies.[5,6] Pioneering work by Rotello's group has shown how the activity and substrate specificity of a nanoparticle-bound enzyme can be modulated by tuning the nanoparticle physicochemical properties.[7,8]

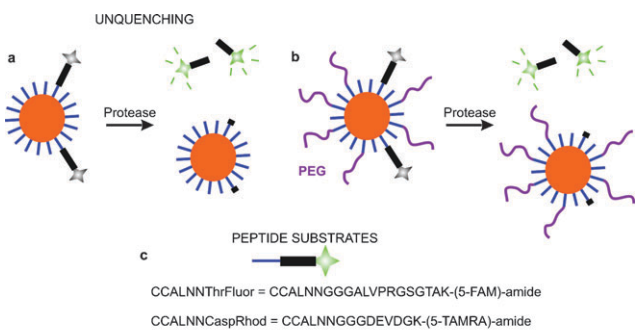
Here we show that peptide-capped gold nanoparticles incorporating a protease substrate can be tailored to measure the activity of a variety of proteases. Importantly, the proteolytic activity is quantitatively described by an interfacial kinetic model and can be modulated by tuning the nanoparticle–protease interaction using polyethylene glycol (PEG) modified peptides.

The measurement principle is shown in Scheme 1. Gold nanoparticles are functionalized with a mixed self-assembled monolayer (SAM). The mixed SAM includes a matrix peptide which provides colloidal stability as well as an extended functional peptide which can be cleaved by one of the proteases and is terminated by a fluorophore. When the functional peptide is intact most of the fluorescence is quenched. The activity of the protease results in "unquenching", *i.e.* an increase of the fluorescence signal as the dye is released from the nanoparticle surface. A PEG modified peptide (CCALNNPEG) can be incorporated in the mixed layer which should result in molecular crowding and reduction in non-specific binding. Gold nanoparticles with an average diameter of 8.8 nm were synthesized by citrate reduction (size distribution: Fig. S1, ESI†).

To obtain quantitative kinetic measurements of enzyme activity, it is necessary to determine the number of fluorescent peptides per nanoparticle. Ideally, this should be done independently of the enzyme assay itself. We have therefore used KCN to dissolve the gold nanoparticle core resulting in the disappearance of the plasmon band[9] and the increase, on the same timescale, of the fluorescence signal (Fig. S2 and S3, ESI†). The dissolution kinetics of the gold core are observed to be strongly dependent on the nature of the monolayer probably reflecting the diffusion of the cyanide ions within the monolayer (Fig. S3, ESI†). In most cases, the fluorescence signal reaches a plateau within one hour and this value can be used to deduce the number of fluorophores per nanoparticles using the appropriate calibration curve (Fig. S4, ESI†).

To evaluate the influence of the monolayer on enzymatic activity, we compared the activity of thrombin on peptide-capped monolayer with or without a proportion of PEGylated peptides incorporated. An example showing the increase in fluorescence as a function of time upon addition of the enzyme is shown in Fig. 1a. The specificity is demonstrated using a D-peptide resulting in complete suppression of the peptide degradation. It is worth noting that to transform the fluorescent levels into concentration of released fluorophores, a correction must be applied to take into account the fact that the measurements take place in an absorbing medium. The absorbance of the nanoparticles is non-negligible and its impact can be quantitatively described by a simple model (Fig. S5, ESI†).

To obtain reliable kinetic measurements, we followed the same approach as Nayak *et al.* for the interfacial activity of enzymes acting on substrates immobilized on flat self-assembled monolayers.[10] For both the PEGylated and non-PEGylated nanoparticles, we performed the fluorescence measurements for grafting densities of the enzyme substrate ranging from 0 to 20 pM cm^{-2}. The grafting densities reported in Fig. 1b and c are deduced from the nanoparticle size and a



Scheme 1 Unquenching of nanoparticle-bound fluorescent peptides by enzymatic cleavage is influenced by the monolayer composition.

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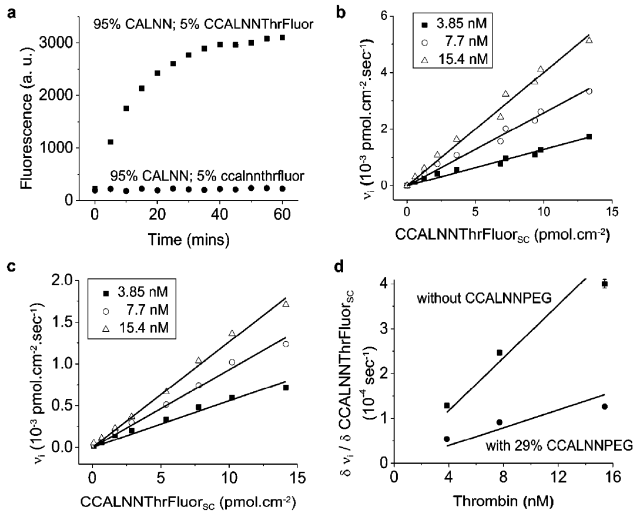

Fig. 1 Interfacial kinetics of thrombin activity on peptide-capped nanoparticles. (a) L-Peptide but not D-peptide fluorophore is cleaved by thrombin; initial rates as a function of substrate surface concentration for three different enzyme concentrations for (b) CALNN-capped nanoparticles and (c) CALNN–CCALNNPEG 7/3-capped nanoparticles; (d) slopes of the linear fits in (b) and (c) as a function of thrombin concentration. In b, c and d, the linear fits are single parameters, *i.e.* the lines are constrained to pass through the origin. Each experiment was repeated in duplicate using the same batch of nanoparticles. The slopes were measured from the 1 to 5 minute time interval after thrombin addition.

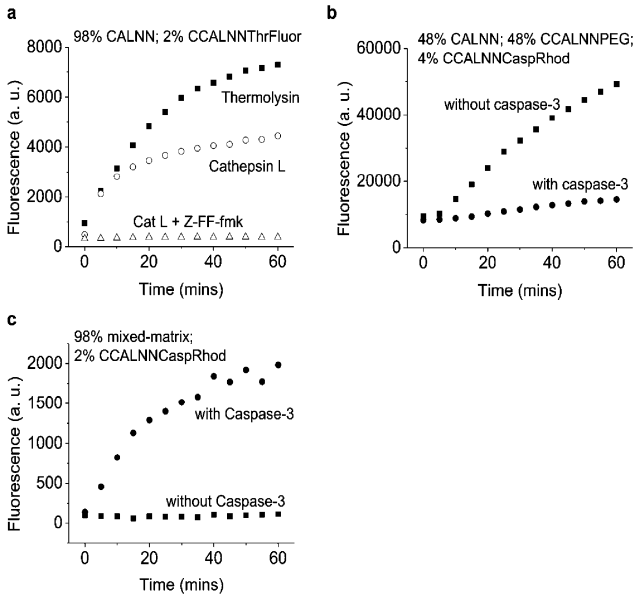

Fig. 2 Optimizing the monolayer allows the assay to operate for a variety of enzymes. (a) Thermolysin and cathepsin L also cleave the CCALNNThrFluor peptide, and cathepsin L function is diminished in the presence of the irreversible cathepsin L inhibitor Z-FF-fmk; (b) caspase-3 assay requires DTT as a reducing agent, and is not compatible with the use of CCALNNPEG NPs; (c) mixed-matrix NPs can be used with DTT and assay for caspase-3.

precise measurement of the number of fluorescent peptides per nanoparticle through dissolution of the nanoparticle core as explained above.

We found that the initial rate, ν_i, is proportional to the density of substrate on the monolayer CCALNNThrFluor$_{SC}$ (Fig. 1b and c). The initial rates also depend on the enzyme concentration: the initial rates alone cannot be used to compare enzymatic reactions. We can therefore measure the slopes $\delta\nu_i/\delta$CCALNNThrFluor$_{SC}$ for three different enzyme concentrations (Fig. 1c). Nayak *et al.* and Gutiérrez *et al.* have demonstrated that the value of k_{cat}/K_M for the interfacial reaction can be deduced from the slopes of $\delta\nu_i/\delta$CCALNNThrFluor$_{SC}$ as a function of the enzyme concentration.[10,11] As shown in Table 1, the value of k_{cat}/K_M when the substrate is embedded in the PEG containing monolayer is reduced by two thirds (Fig. 1d). These results show that the value of k_{cat}/K_M can be used to compare the effect of the surrounding monolayer on the enzyme process. The results are in qualitative agreements with Nayak *et al.*, who reported a similar decrease in k_{cat}/K_M for the enzyme cutinase acting on valerate-presenting alkanethiolate self-assembled monolayer when the substrate is "hidden" in increasingly long hydroxy-terminated alkanethiolates.

Table 1 k_{cat}/K_M of CALNN or PEGylated nanoparticles

Nanoparticle capping peptide	k_{cat}/K_M/M^{-1} s^{-1}
CALNN	$0.27 \pm 0.02 \times 10^9$
CALNN–CCALNNPEG 7/3 ratio	$0.09 \pm 0.01 \times 10^9$

Similarly, here the effect of the PEG is to significantly reduce access to the enzyme substrate.

To demonstrate that "unquenching" from fluorescently labelled peptide-capped nanoparticles constitutes a general approach to the evaluation of protease activity, we then prepared nanoparticles as artificial substrates for thermolysin, cathepsin L and caspase-3. Thermolysin and cathepsin L are two broad specificity proteases and they are able to cleave the peptide CCALNNThrFluor (Fig. 2a). The specificity of the cathepsin L cleavage can be proved by adding an inhibitor resulting in a complete stable background level fluorescence signal.

One potential limitation of the application of gold nanoparticles is ligand exchange. The activity of intracellular enzymes usually requires strongly reducing conditions which are attained with typically millimolar concentration of DTT (dithiothreitol) in the activity buffer. For the protease caspase-3, a key protein involved in apoptosis, the recommended 10 mM of DTT result in the rapid release of the monolayer by ligand exchange when a mixed CALNN–CCALNNPEG layer is used (Fig. 2b). It is however possible to circumvent this limitation by using "mix matrix"[12] NPs (Fig. 2c): no significant ligand exchange is observed when the same peptide is inserted in the mix matrix and those particles can be applied to measure caspase-3 kinetics. Using the KCN dissolution method, mix matrix NPs do not significantly dissolve in 30 mM KCN under boiling conditions indicating a remarkable resistance to the penetration of small ions (or in this context, DTT) in the layer (Fig. S6, ESI†).

The quenching efficiency of peptide-capped NPs can also be precisely determined by plotting the very low fluorescence signal from the nanoparticle as a function of

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the fluorescence after dissolution of the core (Fig. S2, ESI†). For 8.8 nm gold nanoparticles functionalized with CCALNNGGGALVPRGSGTAK-(5-FAM)-amide, the fluorescein is 96.0 ± 0.5% quenched when incorporated into a CALNN monolayer, and 96.7 ± 0.5% quenched when incorporated into a CALNN–CCALNNPEG monolayer.

Previous work on nanoparticle-based detection of enzyme activity includes aggregation-induced colour change, SERRS and fluorescence unquenching.[13,14] The latter has been applied *in vivo* using an IR fluorophore for the detection of extracellular matrix metalloprotease.[15] McKenzie *et al.* determined the number of biomolecular ligands per nanoparticles using either a protease or DNase, and fluorescence unquenching.[16]

For future advanced biomedical applications of nanoparticles, it will be necessary to combine on a single multifunctional nanoparticle properties which can be antagonistic such as the surface presence and accessibility of an active functional motif (which may contain charged or hydrophobic residues) while retaining a good resistance to non-specific binding and full colloidal stability. PEG has been shown to be very efficient at preventing non-specific binding to the extent that PEG entirely prevents nanoparticles from internalization in live cells[17,18] and, as we show here, also strongly reduce accessibility of substrates at the nanoparticle surface.

In summary we have measured quantitatively how PEGylation decreases enzymatic kinetics on a nanoparticle-bound substrate. The quantitative measurements presented in this work open the road to the fine tuning of the properties of multifunctional nanoparticles for *in vivo* applications.

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