Controlled assembly of peptide-functionalized gold nanoparticles for label-free detection of blood coagulation Factor XIII activity

Rona Chandrawati and Molly M. Stevens
Department of Materials, Department of Bioengineering and Institute for Biomedical Engineering, Imperial College London, London SW7 2AZ, UK. E-mail: m.stevens@imperial.ac.uk; r.chandrawati@imperial.ac.uk

A highly sensitive label-free assay for the determination of blood coagulation Factor XIII activity is demonstrated through the controlled assembly of peptide-functionalized gold nanoparticles (AuNPs). Activated Factor XIII catalyzes the formation of covalent crosslinking between peptide chains through ε-(γ-glutamyl)-lysine bonds leading to the aggregation of the AuNPs and consequently a red-shift of the localized surface plasmon resonance. The selective engineering of nanoscale order over AuNP crosslinking via the formation of isopeptide bonds provides a new approach toward the design of nanoassemblies with precise control on the molecular level. The colorimetric assay reported here provides direct qualitative and quantitative analysis of Factor XIII activity with a limit of detection of 0.01 U mL^{-1}.

Factor XIII is a key enzyme in the blood coagulation pathway and has attracted significant attention due to its clinical importance in hemostasis, angiogenesis, wound healing, and tissue repair.[1–3] It belongs to the family of transglutaminases, and upon activation by thrombin and Ca^{2+} catalyzes the formation of highly-organized intermolecular crosslinks between glutamine and lysine residues on fibrin molecules, forming stable blood clots that prevent excessive bleeding from damaged blood vessels. Pathological Factor XIII deficiency is associated with severe life-threatening bleeding, impaired wound healing, intracranial hemorrhage, recurrent pregnancy losses, and cardiovascular and gastrointestinal disorders.[4–8] Over the past decades, various detection methods have been developed for the determination of Factor XIII activity, including gel electrophoresis,[9] enzyme-linked immunosorbent assay (ELISA),[10] and fluorescence resonance energy transfer (FRET)-based assays.[11] In commercially available Factor XIII assays, two main detection approaches are used: (i) detection of ammonia released during the transglutaminase reaction[12,13] or (ii) incorporation of labeled amine (fluorescent-, radio-, or biotin-labeled) into a glutamine residue.[14–16] The former approach is rapid, but it has relatively low sensitivity; the latter approach is time consuming, difficult to standardize, and often overestimates the Factor XIII levels.[17,18] Hence, despite a significant level of development, these techniques still fail to address the great need for specific and sensitive assays that allow for the detection of Factor XIII activity at clinically relevant concentrations, and Factor XIII deficiency remains the most underdiagnosed bleeding disorder.[18–20]

The development of state-of-the-art strategies to create bioresponsive nanomaterials has opened up new avenues for clinical diagnosis. In particular, gold nanoparticles (AuNPs) show great promise as outstanding building blocks for the detection of specific target biomolecules due to their unique optical properties that arise from the localized surface plasmon resonance (LSPR) phenomenon. LSPR gives rise to a characteristic absorbance peak in the visible region of the electromagnetic spectrum that can be tuned by manipulating the morphology and/or interparticle separation of the AuNPs.[21–25] The versatile surface chemistry available for functionalization of AuNPs facilitates the use of specific molecular recognitions to control the aggregation state of a suspension of particles, which results in a shift in the absorbance peak. This phenomenon allows colorimetric detection of a range of molecular analytes such as enzymes,[26–28] DNA,[29] proteins,[30] ions,[31] and small molecules.[32]

To advance the state-of-the-art of enzyme-responsive nanoparticles and to overcome the limitations of current Factor XIII detection techniques, here we utilize AuNPs as an excellent sensing platform and develop a label-free detection assay for blood coagulation Factor XIII activity based on the controlled aggregation of peptide-functionalized AuNPs (Fig. 1). Particles are functionalized with a Factor XIII reactive peptide domain containing glutamine or lysine residues, and the activity of the blood coagulation factor results in the formation of ε-(γ-glutamyl)-lysine isopeptide bonds, leading to particle aggregation, and therefore a significant decrease in interparticle separation distances. Qualitative and quantitative analysis can be achieved based on a color change of the AuNP solution and the red-shift of the LSPR, respectively, which is dependent on the concentration and activity of Factor XIII. In particular in this study, we: (i) surface functionalize AuNPs with custom-designed synthetic peptide substrates for Factor XIII; (ii) demonstrate the detection of Factor XIII activity based on the crosslinking and aggregation of peptide-functionalized AuNPs; and (iii) investigate the specificity and sensitivity of the assay and identify the limit of detection of Factor XIII.

We designed two peptide substrates, Cys-Ala-Leu-Asn-Asn-Gly-Gln-Gly (CALNNGQG) and Cys-Ala-Leu-Asn-Asn-Gly-Lys-Gly (CALNNGKG). The rationale behind the design of these peptides is as follows: (i) conjugation of pentapeptide CALNN to the surface of AuNPs promotes stability of the particles at physiological salt concentration;[33] (ii) cysteine (C) residue facilitates the immobilization of the peptides to AuNP surfaces via thiol-gold chemistry; and (iii) glutamine (Q) and lysine (K) residues act as substrates for Factor XIII.

Surface functionalization of 15 nm-diameter spherical AuNPs with CALNNGQG or CALNNGKG peptides was assessed using UV-Vis spectrophotometry (Fig. 2a). The stability of AuNPs is very sensitive to the external environment of the particles. CALNNGQG rendered the particles stable at physiological salt concentration (PBS buffer – 10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4), evidenced by a characteristic LSPR peak at 522 nm (Fig. 2a, red), which is in agreement with previous results on CALNN-functionalized AuNPs.[33] Under the same physiological conditions, non-functionalized AuNPs aggregated instantly due to the charge screening effect and exhibited an absorbance peak at longer wavelength (Fig. 2a, black). This difference demonstrates the surface functionalization of the AuNPs with CALNNGQG peptide. CALNNGKG-capped AuNPs, on the other hand, caused a significant red-shift of ~40 nm in the SPR peak (Fig. 2a, orange). This is likely due to the strong interaction of amino groups of lysine residues with Au surfaces[34] that may alter the configuration of CALNN monolayer assembly, a key feature for maintaining the stability of AuNPs.[33] To obtain a stable suspension of lysine-modified AuNPs, we sought a feasible alternative peptide substrate. Poly(L-lysine) (PLL) is a synthetic peptide consisting of a long chain of lysine residues and has been used as a coating layer to obtain particles with excellent

colloidal stability.[35,36] As shown in the absorption spectrum, PLL-capped AuNPs remained dispersed as indicated by the characteristic LSPR peak at 522 nm (Fig. 2a, blue). The surface modification of AuNPs with the peptide substrates was further verified by zeta potential measurements (Fig. 2b). Upon adsorption of CALNNGQG or PLL, the zeta potential shifted significantly from −24 ± 4 mV for bare AuNPs (black) to 2 ± 8 mV and 19 ± 7 mV for CALNNGQG-capped (red) and PLL-capped (blue) AuNPs, respectively. The changes in the zeta potential of the AuNPs demonstrated the successful surface functionalization of the particles.

Having characterized the surface functionalization of AuNPs with suitable peptide substrates, we tested the enzyme-responsiveness of this system and demonstrated the detection of blood coagulation Factor XIII through covalent crosslinking between the peptide chains, leading to particle aggregation and changes in interparticle separation distances. A population of CALNNGQG- and PLL-capped AuNPs in PBS buffer pH 7.4 was incubated with Factor XIII in the presence of thrombin and $CaCl_2$, which are essential components for the activation of Factor XIII zymogen. To increase the sensitivity of the enzyme-responsive assay, the concentrations of the assay components were optimized and determined to be 20 U mL^{-1} thrombin and 1 mM $CaCl_2$ (Fig. S1, ESI†). This produced maximal and complete activation of Factor XIII to obtain the highest LSPR peak shift for the AuNP aggregation-based assay.

Fig. 3a shows the UV-Vis absorption spectra of the AuNP mixtures in the absence or presence of Factor XIII. Upon introduction of 0.5 U mL^{-1} Factor XIII, the enzyme catalyzed the formation of ε-(γ-glutamyl)-lysine isopeptide bonds between glutamine residues of CALNNGQG peptide and lysine residues of PLL, with particle crosslinking giving a significant red-shift of the LSPR peak, from 522 to 559 nm. This shift could be monitored in real time and the kinetics of the aggregation process showed that a maximum LSPR peak shift was obtained over an incubation time of 120 min (Fig. 3b). When the AuNP mixtures were exposed to only one of the assay components (i.e., Factor XIII, thrombin, or $CaCl_2$ alone), no shift of the LSPR peak was observed (Fig. 3b). This observation confirmed that the aggregation of the particles is dependent on the activity of Factor XIII rather than simply the presence of the assay components. Corresponding transmission electron microscopy (TEM) images and photographs of the peptide-functionalized AuNP solutions before and after incubation with Factor XIII are shown in panels (i) and (ii) of Fig. 3c and d, respectively. The TEM images confirmed the state of aggregation of the AuNPs, which can be detected by the naked eye through a red-to-blue color change. Thus, the correlation between the aggregation state of the AuNPs and their optical properties facilitates qualitative detection of Factor XIII activity.

To the best of our knowledge, this is the first report describing the controlled crosslinking of AuNPs through the formation of ε-(γ-glutamyl)-lysine isopeptide bonds, allowing precise spatial control of materials at the nanoscale. This selective engineering of nanoscale order advances the state-of-the-art of enzyme-responsive molecular recognition for self-assembly of AuNPs, which not only plays a crucial role toward the development of new tools for diagnostics, but also serves as a promising route for the design of molecular-scale devices.

To further confirm that the crosslinking of the peptide chains on AuNPs and the particle aggregation is specific to Factor XIII activity, control experiments were performed with potential interfering enzymes such as lysozyme and phospholipase. As shown in Fig. 3e, the

signal intensities of peptide–AuNP mixtures incubated with lysozyme or phospholipase are negligible, and only in the presence of Factor XIII is a significant LSPR induced. Similarly, when glutamine or lysine residues were replaced (i.e., Factor XIII was added to a population of CALNNGQG-capped AuNPs alone, PLL-capped AuNPs alone, or a mixture of CALNN- and PLL-capped AuNPs), crosslinking of the particles cannot be formed (Fig. 3e).

Fig. 3f summarizes the shift of the LSPR peak upon introduction of various concentrations of Factor XIII (0.5 U mL^{-1} down to 0.01 U mL^{-1}) into a population of CALNNGQG- and PLL-capped AuNPs in the presence of 20 U mL^{-1} thrombin, 1 mM $CaCl_2$, and 1 mg mL^{-1} human serum albumin (HSA). The addition of HSA, which is the most abundant protein in serum, to the AuNP mixture did not induce particle aggregation. The limit of detection of Factor XIII in this AuNP assay is derived from the calibration curve (Fig. 3g), and is defined as the lowest concentration of Factor XIII that yields a signal three times higher than the standard deviation of the blank.[37] The limit of detection of Factor XIII activity based on the aggregation of the peptide-functionalized AuNPs is determined to be 0.01 U mL^{-1}. This represents a clinically relevant level of Factor XIII (~0.05 U mL^{-1}) that is not readily detectable by the state-of-the art diagnosis[18] and the present assay shows great promise for the development of diagnostic tools for the detection of Factor XIII deficiency. The high sensitivity of this assay is attributed to the specific recognition and subsequent crosslinking of peptide substrates by activated Factor XIII, with the unique plasmonic properties of the AuNP leading to highly specific and sensitive detection of target biomolecules.

We have demonstrated a highly sensitive label-free detection approach for Factor XIII activity based on controlled aggregation of peptide-functionalized AuNPs, which provides qualitative and quantitative analysis with a limit of detection of 0.01 U mL^{-1}. The current assay not only presents new and exciting opportunities en route to the development of a diagnostic test for Factor XIII deficiency, but also demonstrates precise nanoscale control over AuNP crosslinking via the formation of ε-(γ-glutamyl)-lysine isopeptide bonds. This further advances the state-of-the-art of enzyme-responsive molecular-mediated bridging for AuNP self-assembly and serves as a promising route for the design of nanoassemblies with precise control on the molecular level. We anticipate the simplicity of the colorimetric assay coupled with high specificity and sensitivity will be valuable in prompt identification and early intervention for controlling potential life threatening complications.

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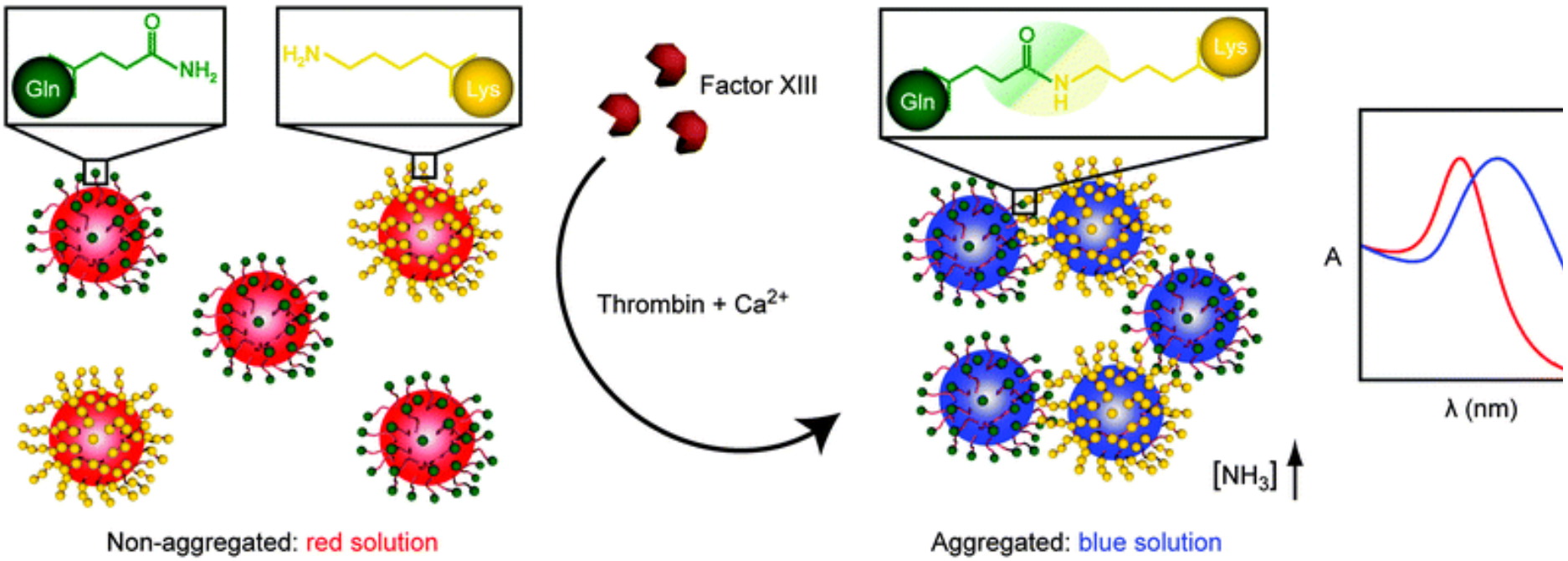

Fig. 1 Schematic illustration of label-free detection of blood coagulation Factor XIII activity based on controlled assembly of peptide-functionalized gold nanoparticles (AuNPs). Particles are functionalized with peptides containing glutamine (Gln) or lysine (Lys) residues and the activity of Factor XIII results in the formation of covalent crosslinking between the peptide chains through ε-(γ-glutamyl)-lysine bonds, leading to particle aggregation. This highly sensitive assay allows qualitative (color change of AuNP solution) and quantitative (red-shift of the localized surface plasmon resonance) analysis that is dependent on the concentration and activity of Factor XIII.

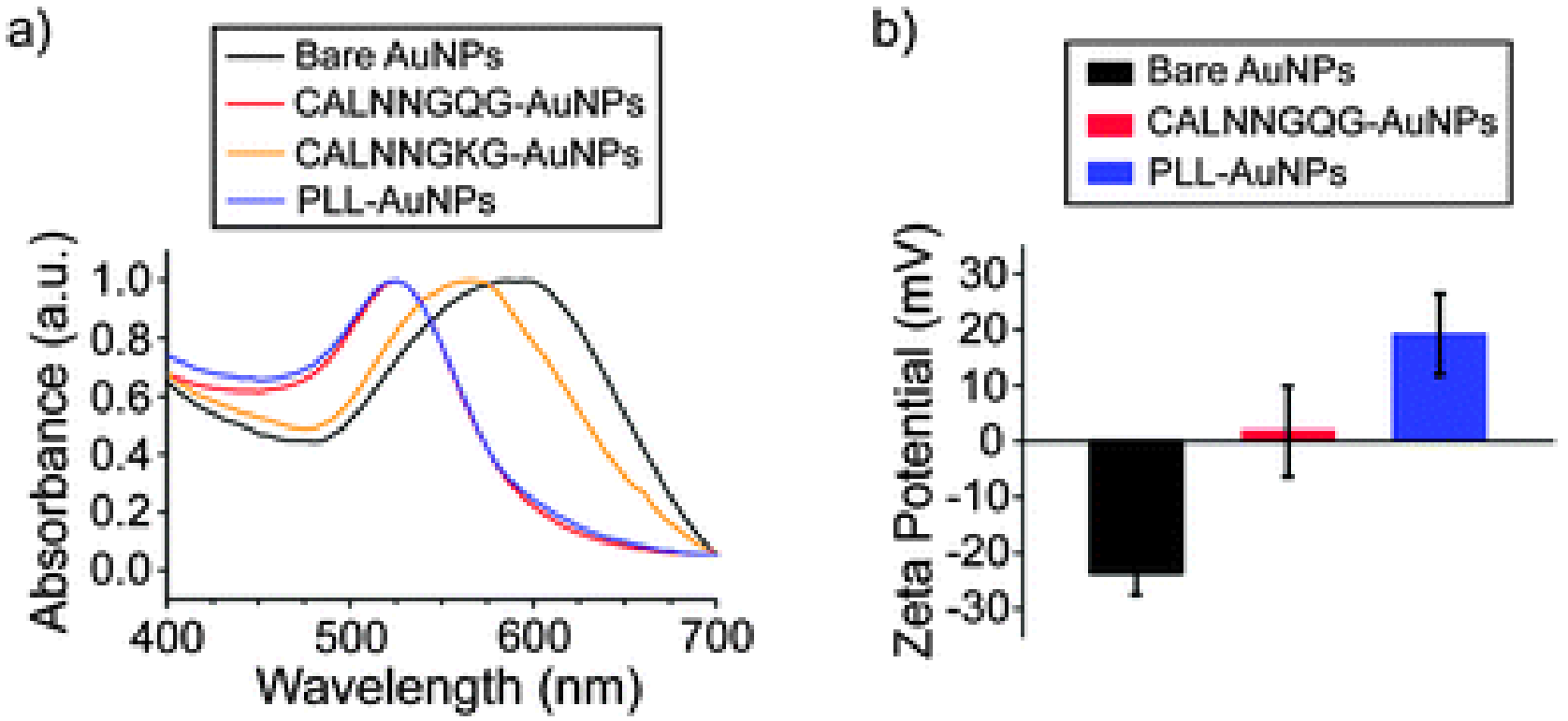

Fig. 2 Peptide-functionalized AuNPs at physiological conditions (PBS buffer pH 7.4). (a) UV-Vis absorption spectra of bare AuNPs (black), CALNNGQG-capped AuNPs (red), CALNNGKG-capped AuNPs (orange), and PLL-capped AuNPs (blue). The surface modification of AuNPs with CALNNGQG peptide and PLL yielded particles with excellent colloidal stability at physiological salt concentrations. (b) Zeta potential measurements of bare AuNPs (black), CALNNGQG-capped AuNPs (red), and PLL-capped AuNPs (blue) verified the surface functionalization of the particles.

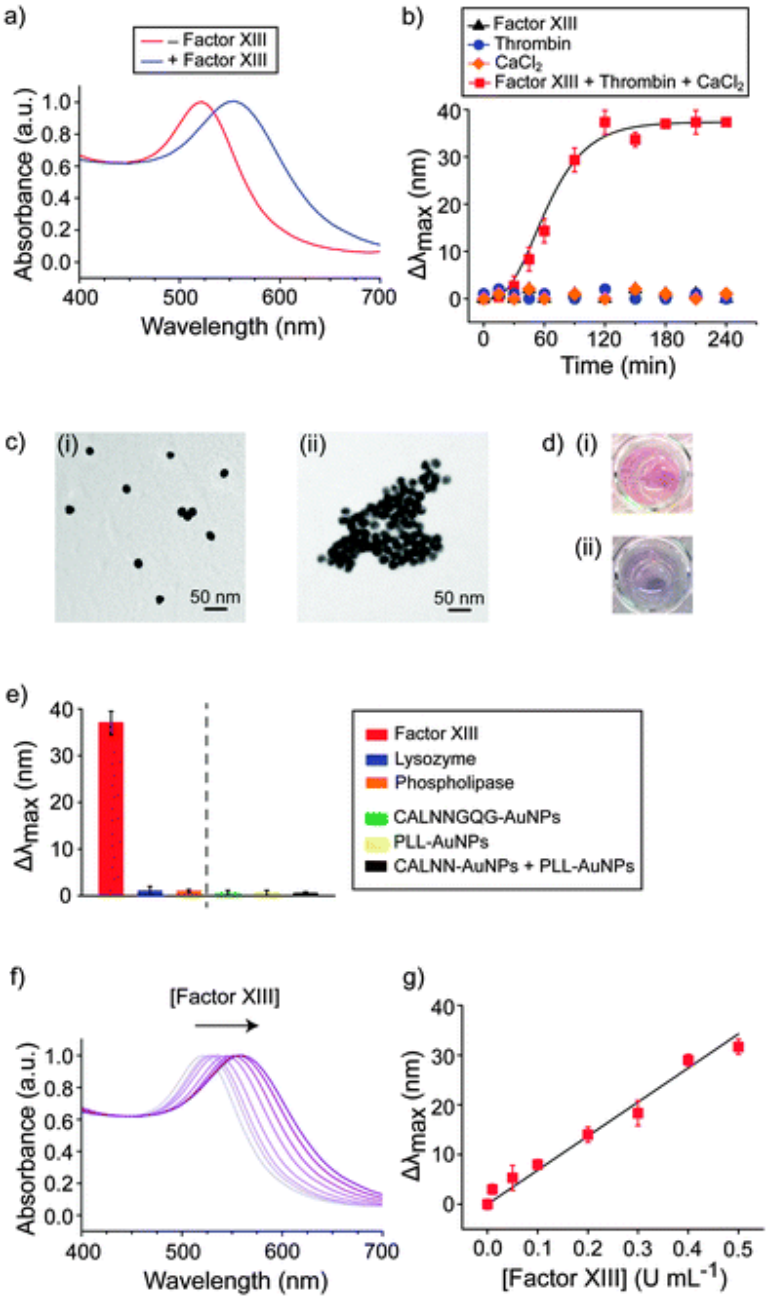

Fig. 3 Detection of blood coagulation Factor XIII activity based on controlled assembly of peptide-functionalized AuNPs. (a) UV-Vis absorption spectra of peptide-functionalized AuNPs before (red) and after (blue) exposure to Factor XIII in the presence of 20 U mL^{-1} thrombin and 1 mM $CaCl_2$. (b) Kinetics of the AuNP LSPR peak shifts induced by Factor XIII-mediated catalysis. (c) Transmission electron microscopy images and (d) photographs of the AuNP solutions before (i) and after (ii) exposure to Factor XIII. (e) LSPR peak shift of the AuNP solution in the presence of other enzymes or in the absence of peptide substrates, demonstrating the specificity of the AuNP-based assay solely mediated by Factor XIII activity. (f) UV-Vis absorption spectra of peptide-functionalized AuNPs after exposure to various concentrations of Factor XIII (0.01 U mL^{-1} to 0.5 U mL^{-1}) in the presence of 20 U mL^{-1} thrombin, 1 mM $CaCl_2$, and 1 mg mL^{-1} human serum albumin. (g) Red-shift of the LSPR peak as a function of concentration of Factor XIII (R^2 = 0.988), demonstrating the sensitivity of the AuNP-based assay for the detection of Factor XIII activity.