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1   **Spacer-enhanced chymotrypsin-activated peptide-functionalized gold**

2   **nanoparticles probes: a rapid assay for the diagnosis of pancreatitis**

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4   **Fang-Yuan Yeh[1], I-Hua Tseng[1], Shu-Hung Chuang[1,2], Chih-Sheng Lin[1, *]**

5   *1. Department of Biological Science and Technology, National Chiao Tung University,*

6   *Hsinchu, Taiwan*

7   *2. Department of Surgery, Mackay Memorial Hospital, Hsinchu Branch, Hsinchu,*

8   *Taiwan*

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10   **Running title:** Spacer-enhanced chymotrypsin-activated AuNPs-peptide probes
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12   *** Author for correspondence:**

13   Chih-Sheng Lin, Ph.D.

14   Department of Biological Science and Technology,

15   National Chiao Tung University,

16   No.75 Po-Ai Street, Hsinchu 300, Taiwan

17   Tel: 886-3-5131338

18   E-mail: lincs@mail.nctu.edu.tw

Abstract

Pancreatitis is the inflammation of the pancreas. Chymotrypsin, an indicator of pancreatic function, could serve as a biomarker for the diagnosis of pancreatitis. A gold nanoparticles (AuNPs)-based fluorescence assay was fabricated in this study to assay the activity of chymotrypsin. Peptides labeled with fluorophore were conjugated onto AuNPs as chymotrypsin-activated peptide-functionalized AuNPs probe (AuNPs-peptide probe). The detection sensitivity of the AuNPs-peptide probe toward proteolytic activity was significantly increased by using spacer-enhanced peptides with specifically designed lengths and charges. The limit of detection of the designed AuNPs-peptide probe decreased, enabling detection at the pM level within a 15 min detection time.

The AuNPs-peptide probe was used to evaluate chymotrypsin activity as an indicator of acute pancreatitis (AP) in a mouse model induced by cerulein challenge. The fecal chymotrypsin activity in cerulein-induced AP mice was significantly lower than that observed in controls. This is the first study to use an AuNPs-peptide probe for the diagnosis of pancreatitis using fecal specimens.

Keywords: chymotrypsin; gold nanoparticles; peptide, pancreatitis; molecular probe

18   **1. Introduction**

19   Pancreatitis is a disease in which the pancreas experiences under inflammation. It

20   occurs when digestive enzymes are activated before they are secreted into the

21   duodenum, allowing them to attack and damage the pancreas. In the United States, at

22   least 250,000 patients are admitted to the hospital each year for acute pancreatitis (AP),

23   making it the second most common gastrointestinal disease. The incidence of

24   pancreatitis continues to increase each year. The annual cost of caring for these patients

25   is approximately $4 – $6 billion [1]. Although the pathogenesis of AP is not fully

26   understood, most hypotheses are based on the concept of the premature activation of

27   digestive zymogens in the pancreas, leading to tissue necrosis by auto-digestion [2].

28   Measurements of plasma amylase and lipase levels are the most widely used methods

29   for AP diagnosis [3, 4]. However, false-positive amylase and lipase results could be caused

30   by abnormal conditions [5], and false-negative results could be obtained in hyperlipidemia

31   [6] or diabetic ketoacidosis [7, 8]. Furthermore, blood enzyme determinations are invasive

32   tube tests that are not routinely available for use in the diagnosis of AP. Therefore,

33   biochemical tests based on a single fecal sample serve can as a potential and valuable

34   diagnostic alternative [9]. Although trypsin preactivation is the principal cause of

35   pancreatitis, trypsin undergoes degradation in the distal small bowel, which make

36   trypsin a bad fecal marker [10]. Previous studies have shown that chymotrypsin can serve

37   as an indicator of pancreatic function and be related to pancreatic diseases [11, 12].

38   Chymotrypsin is a serine protease secreted from the pancreas into the duodenum, where

39   it becomes active. In the case of pancreas insufficiency secondary to pancreatitis, the

40   secretion of the enzyme is markedly reduced; therefore, reduced chymotrypsin activity

41   can be measured in pancreatitis. As with all fecal protease assays in conventional

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42   pancreatic function tests, fecal chymotrypsin can be utilized as a surrogate [13]. In the past

43   few decades, reports have described various proteases assays that are primarily based on

44   liquid chromatography, substrate zymography, enzyme-linked immunosorbent assays

45   (ELISA), radioisotopes, or chromogenic and fluorogenic substrates. However, these

46   techniques are often associated with cost-ineffective features such as being

47   time-consuming, expensive, discontinuous, or requiring specific instruments. Much

48   attention has been paid to resonance energy transfer (RET) for *in vitro* and *in vivo*

49   assays of proteases due to advances in fluorescence-based techniques [14-16]. Gold

50   nanoparticles (AuNPs) are a potential nanomaterial for RET-based assays; AuNPs of

51   less than 40 nm in diameter have the lowest scattering constant and therefore the highest

52   potential to quench fluorescence [17]. AuNPs have a superior quenching efficiency in a

53   broad range of wavelengths compared to other organic quenchers [17, 18].

54   We aimed to develop an efficient method to diagnose pancreatic disease by fecal

55   samples. Therefore, AuNPs-peptide probes for the detection of chymotrypsin activity

56   were fabricated. The FITC-labeled peptides were conjugated onto AuNPs as

57   chymotrypsin-activated peptide-functionalized AuNPs probe (*i.e.*, AuNPs-peptide

58   probe), and a specially designed peptide spacer that could significantly increase the

59   detection sensitivity was also introduced. The chymotrypsin activity in the feces of

60   cerulein-induced AP mice was detected, and the results confirmed that the fabricated

61   AuNPs-peptide probe could be effectively applied to the diagnosis of pancreatitis.

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66   ## 2. Results

67   ### 2-1. Fabrication of chymotrypsin-activated peptide-functionalized AuNPs probes

68   The fabrication of the protease-activated AuNPs-peptide probes is illustrated in **Fig.**

69   **1A**. In each probe, specific FITC labeled peptides were self-assembled onto AuNPs

70   through an Au-S bond by cysteine-gold interaction, of which Cys acted as an anchor.

71   The fluorescence of FITC is quenched by AuNPs, and the fluorescence can only be

72   recovered by protease activation, which hydrolyzes the peptide substrates allows FITC

73   to diffuse beyond the efficient quenching distance from the nanoparticles. Fluorescence

74   was measured using excitation/emission at 495/515 nm, and the change in fluorescence

75   intensity was used to estimate protease activity.

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77   ### 2-2. Characteristics of the AuNPs-peptide probes

78   The sizes of the synthesized citrate-capped AuNPs were estimated by TEM to be

79   14.6 ± 2.4 nm (**Fig. 1B**) and by dynamic light scattering (DLS) to be 22 nm (**Fig. 1C**).

80   The size of the AuNPs-P3 probe was estimated by TEM (**Fig. 1D**) and DLS (**Fig. 1E**) as

81   17.2 ± 1.1 nm and 29 nm in diameter, respectively. The citrate-capped AuNP and

82   AuNPs-peptide probe demonstrated absorption spectra with a localized surface plasmon

83   resonance (LSPR) band shift, with the absorption peak shifted from 520 to 525 nm (**Fig.**

84   **1F**). The chymotrypsin-activated AuNPs-peptide probe showed a similar absorption

85   spectrum to that of the with non-activated AuNPs-peptide probe.

86   Agarose gel electrophoresis was performed to identify the morphological changes

87   in the AuNPs after fabrication and protease activation. Changes in the bands could be

88   observed under visible light and UV-illumination. The gel shown in **Fig. 2A** (under

89   visible light) indicates differences in migration between the citrate-capped AuNPs, the

90   AuNPs-peptide probe, and the chymotrypsin-activated AuNPs-peptide probe. The

91   UV-illuminated fluorescence band of the AuNPs-peptide probe without chymotrypsin

92   activation was very weak; evidence for both free and bound peptide-FITC cleaved by

93   chymotrypsin are also given in **Fig. 2B** (under UV-illumination).

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95   **2-3. Chymotrypsin activity assay by the AuNPs-peptide probes**

96   AuNPs fabricated with different peptide substrates (listed in **Table 1**) were

97   compared. Three fabricated AuNPs-peptide probes were used in the chymotrypsin

98   activity assay with a 1 h detection time at 37°C and pH 8.0. As shown in **Fig. 2C**, the

99   recovered fluorescence intensity upon chymotrypsin activation was in the following

100   decreasing order: AuNPs-P3 probe (6000 a.u.) > AuNPs-P2 probe (3000 a.u.) >

101   AuNPs-P1 probe (500 a.u.). Notably, only 5 ng mL^{-1} chymotrypsin was applied to the

102   AuNPs-P3 probe, whereas 500 ng mL^{-1} chymotrypsin was applied to the others. Further

103   investigation of the optimal detection range and time are documented in **Table 1**. The

104   required optimal detection time for each AuNPs-peptide probe decreased in the

105   following order: AuNPs-P1 probe (60 min) > AuNPs-P2 probe (30 min) > AuNPs-P3

106   probe (15 min). Because the AuNPs-P3 probe showed superior sensitivity, the following

107   experiments were conducted with this specific probe only.

108   To optimize the fluorescence assays using the AuNPs-peptide probe, the

109   AuNPs-peptide probe concentration was a prime concern because the quenching effect

110   of AuNPs is distance dependent. The concentration will affect the distance between

111   particles, and that the adjacent particles can participate in quenching fluorescence of

112   FITC. Different concentrations of the AuNPs-P3 probe (0.156 – 3.75 nM) were

113   incubated with a fixed concentration of chymotrypsin (7.5 ng mL^{-1}) for 15 min at 37°C.

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114   **Fig. 3A** shows that the optimal concentrations range of the AuNPs-peptide probe was

115   0.625 – 1.25 nM, whereas higher or lower concentrations resulted in poor performance,

116   as indicated by the minor change in fluorescence intensity.

117   The specificity of the AuNPs-peptide probe was also investigated by comparing the

118   use of serine proteases such as trypsin and chymotrypsin. Trypsin has a cleavage site at

119   arginine (Arg) in P3. The AuNPs-P3 probe (1.25 nM) was incubated with various

120   concentrations of trypsin and chymotrypsin (0.25 – 25 ng mL^{-1}), respectively, for 15

121   min at 37°C and pH 8.0. **Fig. 3B** shows that the AuNPs-peptide probe was highly

122   specific to chymotrypsin and displayed almost no change in fluorescence intensity in

123   the presence of trypsin.

124   The time-dependent emission spectrum of the AuNPs-P3 probe upon treatment

125   with 200 pM chymotrypsin is shown in **Fig. 4A**. The results indicated that the

126   fluorescence intensity reached a steady state after approximately 80 min at 37°C. The

127   reaction kinetics of the AuNPs-P3 probe and chymotrypsin is an important parameter to

128   in determining its biological applicability. To obtain the kinetic parameters, the

129   fluorescence signal at 515 nm was plotted as a function of time (**Fig. 4A**, left). The

130   pseudo-first-order rate, k_{obs}, was found to be 0.0424 s^{-1} by fitting the delta fluorescence

131   intensity data with a single exponential function. The best fit with a single exponential

132   function gave a $t_{1/2}$ of 16.3 min. Therefore, the detection time of the chymotrypsin assay

133   using the AuNPs-P3 probe was determined to be 15 min. The concentration correlation

134   was also displayed in **Fig. 4B**, where the AuNPs-P3 probe (1.25 nM) was incubated

135   with various concentrations of chymotrypsin (0.1 – 25 ng mL^{-1}) for 15 min at 37°C and

136   pH 8.0. The results showed higher fluorescence intensities with increasing

137   concentrations of chymotrypsin (**Fig. 4B**, right). The linear response to chymotrypsin,

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138   from 0.25 to 10 ng mL^{-1}, was well defined, with Δ FL intensity = 506.69 (chymotrypsin)

139   − 90.82 and R² = 0.99 (**Fig. 4B**, left).

140   The stability of the AuNPs-peptide probes was evaluated and the results showed

141   that the chymotrypsin assay using the AuNPs-P3 probe after 1 month of storage at

142   ambient temperature was still above 90% compared to fresh use (data not shown).

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144   **2-4. Cerulein-induced AP mice**

145   AP induction in the mouse model was conducted by a series of intraperitoneal

146   cerulein injections. The mice were sacrificed at 8, 10, or 24 h after the first

147   administration of saline (as control) or cerulein.

148   The collected plasma was analyzed by a Fujifilm clinical chemistry analyzer, in

149   which of the amylase and lipase levels were determined. The plasma amylase levels of

150   the cerulein-induced group were 27 and 49 (10^3 U L^{-1}) at the 8th and 10th h of sacrifice,

151   respectively, which were significantly higher than of the values from the saline group

152   (**Fig. 5A**). Moreover, the plasma lipase levels showed the same trend in the

153   cerulein-induced group, with levels of 2.3 and 3.4 (10^3 U L^{-1}) at the 8th and 10th h of

154   sacrifice, respectively, also significantly higher than of the values for the saline group

155   (**Fig. 5B**). The saline group in each sacrificed period showed the similar amounts of

156   plasma amylase (approximately 4,000 U L^{-1}) and lipase (approximately 500 U L^{-1}) in

157   each period. Both the plasma amylase and lipase levels showed at least a 3-fold increase

158   compared to the controls, indicating that AP induction was successful in this study.

159   The pancreatic tissues of the mice were collected to confirm trypsin preactivation

160   in AP occurrence. Extreme caution was exercised during the isolation of the pancreas

161   because these tissues can be easily confused with fatty tissue (**Supplemental Fig. S1A**),

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162   and beta cells can be identified by dithizone (DTZ) staining (**Supplemental Fig. S1B**

163   and **S1C)**. The AuNPs-P3 probe was used to analyze pre-activated chymotrypsin in the

164   pancreas. **Fig. 5C** shows that the pancreatic chymotrypsin level in the mice with

165   cerulein-induced AP was approximately $2.8 - 3.6$ ng chymotrypsin mg^{-1} total protein,

166   which was significantly higher than that observed in the control group.

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168   **2-5. Diagnosis of AP by duodenal and fecal chymotrypsin**

169   The collected eluent from the duodenum was analyzed using the AuNPs-P3 probe

170   to determine the level of chymotrypsin. The amount of duodenal chymotrypsin in the

171   mice with cerulein-induced AP showed a significant decrease in all three periods

172   compared with that of the control group (**Fig. 6A)**. The normal level of chymotrypsin

173   was approximately 45 μg in the duodenal eluent, and significantly decreased to

174   approximately 10 μg in the cerulein-induced group.

175   Fecal sample collection was conducted in 1 h-intervals for the entire 24 h time

176   course. The supernatant collected from the fecal protein extraction was applied to the

177   AuNPs-P3 probe (1.25 nM). Every 5 h period was classified into one group, thus

178   generating the following groups: $0 - 4^{th}$ h, $5 - 9^{th}$ h, $10 - 14^{th}$ h, $15 - 19^{th}$ h and $20 - 24^{th}$

179   h. The saline subjects showed changes in the cycle of chymotrypsin activity due to their

180   diet. The $0 - 4^{th}$ h, $5 - 9^{th}$ h and $20 - 24^{th}$ h groups from the mice with cerulein-induced

181   AP showed a significantly lower chymotrypsin level compared with those of the control

182   group (**Fig. 6B**).

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186   **3. Discussion**

187   **3-1. Fabrication of AuNPs-peptide probes**

188   The use of protease-activatable fluorophore-conjugated AuNP probes has recently

189   attracted considerable attention for the development of high-sensitivity detection

190   methods for physiological activities. AuNPs 15 nm in size offer a high potential to

191   quench fluorescence [17]; therefore, 15 nm AuNPs were used in this study, and

192   fluorophores such as FITC, were conjugated to the terminus of the peptide substrates.

193   The relationship between the AuNPs and the fluorophore is complex at very close

194   distances (< 10 Å), radiative rate enhancement dominates; at intermediate distances (20

195   – 300 Å), energy transfer is the dominant process; and at very large distances (> 500 Å),

196   fluorescence oscillations due to dipole-mirror effects take precedence [19]. According to

197   the size analysis results that the distance between AuNPs and FITC was approximately

198   13 – 35 Å, indicating quenching mechanism dominates this system. Based on the

199   nanometal surface energy transfer model, the quantum efficiency of the AuNPs-P3

200   probe was approximately 88.3 – 99.7%, theoretically [20]. The enzyme activity was

201   determined by the recovered fluorescence, which was defined as the difference between

202   the experiment group and the control group; hence the delta fluorescence intensity was

203   able to eliminate the background effect.

204   The importance of peptide sequence design for the ligands coated on AuNPs has

205   been previously studied [21], because of the citrate-capped AuNPs showed poor stability

206   (as shown in **Supplemental Fig. S2B**). For a targetable probe used to detect protease

207   activity, the peptide sequence must be limited to specific hydrolysis substrates.

208   Therefore, it is essential to consider the overall effect of the peptide rather than only

209   effect of the particular amino acid. It has been previously shown that increasing the

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210   length of the peptide can increase the stability of peptide-capped AuNPs [22]. Moreover, a

211   previous study also proposed that peptide-modified AuNPs aggregation can be

212   controlled by electrostatic repulsion between the peptides [23]. The aim of peptide design

213   is to efficiently decrease the steric barrier caused by the densely packed layer of

214   peptides on the AuNP surface to increase enzyme access to the cleavage site. Hence,

215   this study examined two main concepts in peptide design, namely, length and charge.

216   Three different peptide substrates were compared, with each possessing the same

217   cleavage sites for chymotrypsin (**Table 1**). The length was tailored through the use of a

218   spacer of repeating Gly units, $(Gly)_5$ (the difference between P1 and P2), and the charge

219   was modified by substituting the non-charged $(Gly)_5$ spacer with a negatively-charged

220    $(Asp)_5$ spacer (the difference between P2 and P3). The pH was adjusted according to the

221   isoelectric point (pI) of the peptides to achieve a net negative charge on the peptide;

222   hence to stabilize the AuNPs-peptide probe (data not shown). Besides, the pH of the

223   AuNPs had no effect on aggregation (**Supplemental Fig. S2A**).

224   AuNPs fabricated with peptide ligands can improve the stability of particles [24], but

225   the AuNPs-P2 probe stability showed was worse than that of the citrate-capped AuNPs

226   (**Supplemental Fig. S2C**). Nevertheless, the use of a stabilizer such as BSA, efficiently

227   increased the stability of the AuNPs-P2 probe (data not shown). The negatively-charged

228   spacer, $(Asp)_5$, promoted electrostatic repulsion between the particles and increased the

229   stability of the AuNPs, which show good dispersion (**Supplemental Fig. S2D**).

230   DTT-based displacement was conducted to estimate the number of peptides conjugated

231   to each AuNP. Our results showed that the number of peptide substrates (9 – 13

232   residues) conjugated to the 15 nm AuNPs was approximately 1,000 – 1,800 peptides per

233   AuNP or 2.5 – 1.5 peptides nm^{-2}. Compared with previous reports involving a short

234   peptide (2 – 6 residues) found approximately 200 – 900 peptides per AuNP 12 – 12.5

235   nm in size, our results showed a higher conjugation ratio [22, 25, 26]. In other words, the

236   synthesized AuNPs-peptide probes were compactly packed with peptides. The

237   advantages of the densely packed monolayers are as follows: (1) more loading peptides

238   provide more chances for proteases to hydrolyze and activate the AuNPs-peptide probes,

239   and (2) compact coverage increase the stability of AuNPs-peptide probes compared to

240   that of citrate-capped AuNPs.

241   The zeta potential indicates the degree of repulsion between adjacent, similarly

242   charged particles in dispersion. Colloids with a high zeta potential (negative or positive)

243   are electrically stabilized, whereas those with low zeta potentials tend to coagulate or

244   flocculate. A value of 25 mV (positive or negative) can be taken as the arbitrary value

245   separating lowly-charged surfaces from highly-charged surfaces [27]. Park et al. showed

246   that the zeta-potential value of AuNP to AuNPs-peptide-fluorescent protein changed

247   from -26.44 ± 2.58 mV to 5.46 ± 2.44 mV [28] .Compared with our results, this value is

248   less stable. The zeta potentials of the citrate-capped AuNPs and AuNPs-P3 probe in our

249   study increased from -34.7 ± 13.8 mV to -39.2 ± 15.9 mV; this improvement can be

250   attributed to the $(Asp)_5$ spacer.

251

252   **3-2. Establishment of chymotrypsin activity assay using the AuNPs-peptide probe**

253   A review of AuNPs-based biosensors for enzyme activity documented that the

254   number of published AuNPs-based protease assays is relatively low (< 100 publications)

255   and only a few proteases (kinases, MMPs, and caspases) have appeared frequently to

256   date [29]. This study presents a new target for AuNPs-based protease assays and a novel

257   investigation of the effect of spacer length and charge on sensitivity. The specially

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258   designed peptide chain consisted of four elements: an anchor, a spacer, a sequence

259   hydrolyzed by proteases, and a fluorophore. According to the results presented in **Fig.**

260   **2C**, the three fabricated probes showed different sensitivities in detecting chymotrypsin.

261   The AuNPs-P2 probe showed a 5-fold increase in the delta fluorescence intensity

262   compared to that observed in the AuNPs-P1 probe. It has been demonstrated that longer

263   spacers on gold glyconanoparticles, which are AuNPs modified with a variety of

264   saccharide molecules, enhance binding affinity to the target (lectins) [30]. The results also

265   corresponded to our observation that a longer spacer could result in a larger distance

266   between the ligands at the same ligand density, hence reducing steric hindrance when

267   the target approaches the ligands, making the ligands more accessible for interaction

268   with the target.

269   The detection sensitivity of the AuNPs-P3 probe was superior (>100-fold)

270   compared to that of the AuNPs-P2 probe. In our study, we proposed that the $(Asp)_5$

271   residues played a novel role as an extending anchor that decreased the steric hindrance

272   to the enzyme and lowered the pI of the peptide, increasing particle stability. Asp also

273   excluded the absorbed citrate on the AuNP, due to its repulsive interactions with the

274   citrate carboxylates [22]. Hence, the repulsions between the chains and the citrate ions on

275   the surfaces of the AuNPs maintained the dispersion of the peptides. The mechanism

276   behind the increase in sensitivity using of a charged peptide spacer in functionalized

277   AuNP-peptide probes remains to be explored. A few studies have shown that an

278   enhancement of enzyme activity is possible using nanoparticles modified with

279   polyvalent-conjugates. $(Arg)_6$ was used in the generation of cationic peptide formed

280   nanoparticles that possessed significantly enhanced antimicrobial properties [31].

281   Functionalized dextranated magnetic nanoparticles with different valency of small

282   molecules have shown an over 10^4-fold increase in avidity to the molecule-binding

283   protein [32]. Algar et al. have previously demonstrated that an increase in the charge of

284   polyvalent QD-peptide conjugates can significantly enhance the sensitivity to trypsin

285   cleavage of the peptide conjugates [33]. To the best of our knowledge, this study is the

286   first report of the use of polyvalent peptide spacers to enhance enzyme activity in a

287   AuNPs-based system for proteinase activity determination.

288   The enhancement of enzyme sensitivity through the use of spacers might also be

289   due to the electrostatic complementarity between $(Asp)_5$ and chymotrypsin. A previous

290   study showed that anionic functionalized nanoparticles were highly effective

291   surface-based inhibitors of chymotrypsin; this activity was attributed to the electrostatic

292   complementarity between the carboxylate end groups and the halo of cationic residues

293   located around the periphery of the active site in chymotrypsin [34].

294   Morphological changes in AuNPs with different treatments can be observed by

295   agarose gel electrophoresis [35]. The mobility of the citrate-capped AuNPs increased upon

296   modification with negatively-charged peptides, namely, the AuNPs-peptide probe. The

297   mobility was further increased in the chymotrypsin activated AuNPs-peptide probe due

298   to the loss of residues (**Fig. 2A**). The quenching phenomenon between AuNPs and FITC

299   is confirmed in **Fig. 2B**; chymotrypsin activation is shown to turn on the fluorescence.

300   The UV-illuminated gel shows that the fluorescent band with poorer mobility was

301   attributed to the loss of the $(Asp)_5$ residues (Lane 4 and Land 5). The quenching effect

302   of AuNPs is very important; therefore, the concentration of the AuNPs-peptide probe

303   was considered due to its influence on the distance between individual particles. **Fig. 3A**

304   shows the optimal concentration range; higher or lower concentrations led to smaller

305   changes in fluorescence intensity, and higher concentrations especially resulted in

306   considerable decreases in intensity. The higher concentration likely had a negative

307   impact due to the quenching effect whereas the lower concentration likely provided

308   insufficient loading of the peptide substrates to detect protease activity. Additionally, the

309   specificity of the AuNPs-peptide probe was investigated using the serine protease

310   trypsin. Trypsin was chosen because it is structurally similar to chymotrypsin and

311   follows the same secretion route in the digestive system. Trypsin has one cleavage site

312   in the peptide substrate (P3); however, **Fig. 3B** shows that the AuNPs-peptide probe was

313   unable to detect trypsin but exhibited high specificity to chymotrypsin.

314

315   **3-3. Application of AuNPs-peptide probe to the diagnosis of AP**

316   The AP mouse model was successfully established in this study (**Supplemental**

317   **S3**). Mice with cerulein-induced AP showed increased levels of plasma amylase and

318   lipase. We aimed to confirm the presence of premature trypsin in AP based on increased

319   pancreatic chymotrypsin activity [36]. Pancreatic acinar cells produce the inactive

320   precursor of chymotrypsin, chymotrypsinogen, which is carried in the pancreatic juice

321   through the pancreatic duct into the duodenum and then activated by trypsin. The

322   chymotrypsin activity in the pancreas of cerulein-induced AP mice was analyzed by the

323   AuNPs-P3 probe because of their high sensitivity. **Fig. 5C** supports the hypothesis that

324   pancreatic chymotrypsin activity is significantly increased in mice with

325   cerulein-induced AP, with chymotrypsin activity found to be increased by 3-fold

326   compared to that of in the controls. Few works have discussed the elevated activity of

327   chymotrypsin in the pancreas with AP. In a cerulein-induced rat model, a 3-fold

328   increase in chymotrypsin activity has been observed relative to the normal reference [36],

329   which is consistent with our results. The reported maximal pancreatic injury at the 12th h

330   [37] was also similar to our findings that the 10th h showed the highest level of pancreatic

331   chymotrypsin.

332   However, there were inconsistencies in the pancreatic chymotrypsin, plasma

333   amylase and lipase activity levels at the 24th period, at which time levels of pancreatic

334   chymotrypsin remained abnormal, whereas the other enzymes had reached normal

335   levels. We were thus prompted to examine the two sources of chymotrypsin secretion.

336   Chymotrypsin activity as an indicator to estimate the occurrence of pancreatitis usually

337   refers to fecal chymotrypsin [12]; however, the chymotrypsin in duodenal fluid was also

338   investigated in this study. The duodenal contents of cerulein-induced AP mice were

339   collected after proper fasting to exclude the effect of food. The amount of chymotrypsin

340   in mice with cerulein-induced AP significantly decreased in the duodenal fluid in all

341   periods compared with that of the saline subjects (**Fig. 6A**). The duodenal chymotrypsin

342   activity in mice with cerulein-induced AP showed a 25% decrease relative to that of

343   controls. Acinar cell injury leads to pancreas malfunction, resulting in a decrease in

344   chymotrypsin activity in the duodenum. This study is the first report to investigate the

345   potential of duodenal chymotrypsin as a reliable index for AP in a mouse model. The

346   duodenal chymotrypsin activity provides a direct estimate of pancreatic injury; however,

347   it offers one disadvantage in that it is invasive to obtain duodenal fluid. But for broader

348   applications, the AuNPs-peptide probe has the potential in surgical applications,

349   allowing real-time visualization of pancreatic juice leakage during surgery [38].

350   Normally, for clinical diagnosis, 24 to 72 h stool collection is needed for protease

351   determination [12, 39]. The use of fecal chymotrypsin as an indicator of pancreatitis

352   diagnosis usually refers to chronic pancreatitis; hence, we tried to find the applicability

353   in acute pancreatitis. For stool productive species like mouse, that one collection of

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354 stool is not reasonable for experimental design; therefore, we conducted a 24 h

355 collection of stool with 1-h intervals. Intra peritoneal cerulein injection can cause

356 stomachache and loss of appetite; hence, fasting beforehand might encourage the mice

357 to eat and therefore to produce stool. A continuous 24 h stool collection not only

358 provided a time course for mouse digestion but also showed the change of fecal

359 chymotrypsin activity in the mouse model of AP. Because of individual and digestion

360 rate differences, the 24 h stool collection was categorized into groups to average

361 performances. The saline subjects showed a cycle change in chymotrypsin activity due

362 to their diet. Base on overall average, the cerulein-induced group had lower fecal

363 chymotrypsin activity than that of controls, which the dividing fecal chymotrypsin as

364 abnormal status was approximately 30 ng mg^{-1} of stool. Especially the $0 - 4^{th}$ h, $5 - 9^{th}$

365 h and $20 - 24^{th}$ h groups of cerulein-induced AP mice showed significantly lower fecal

366 chymotrypsin activity (30%) relative to that of controls (**Fig. 6B**). It has been proposed

367 that a fecal chymotrypsin below 3 U g^{-1} of stool (approximately 75 ng mg^{-1} of stool)

368 suggests advanced chronic pancreatitis in human [40]. The advantage of fecal

369 chymotrypsin over duodenal chymotrypsin is its ease of specimen collection. Moreover,

370 based on the high sensitivity of the AuNPs-P3 probe, only $10 - 20$ mg of feces is

371 required for the assay, making monitoring feasible.

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373

374 **4. Experimental section**

375 **4-1. Chemicals**

376 All chemicals were analytical reagent grade and used without further purification.

377 Sodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), calcium chloride ($CaCl_2$), Triton X-100, hydrogen

378   tetrachloroaurate (III) (HAuCl$_4$ • 3H$_2$O), Brij™ 35 solution 30% (w/v), polyethylene

379   glycol (#P2139, Mw 8000), DL-dithiothreitol (#D5545), dithizone (#D5130),

380   collagenase type I (#C9891), chymotrypsin (#C4129) were obtained from

381   Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile (ACN), were obtained

382   from Merck (Darmstadt, Germany). Tris-HCl, dulbecco's phosphate buffered saline

383   (DPBS), were purchased from Invitrogen (San Diego, LA, USA). Sodium chloride was

384   purchased from USB (Cleveland, OH, USA). Nanopure water (18 MΩ• cm; Millipore,

385   Bedford, MA, USA) was used in all experiments and to prepare all buffers.

386

387   **4-2. Apparatus**

388   Absorption spectra of AuNPs and fabricated-AuNPs were analyzed by UV-Vis

389   spectrophotometer (SpectraMax 190; Molecular Devices Corporation, Sunnydale, CA,

390   USA). Absorbance values of protein assay were recorded at 595 nm using a UV-Vis

391   spectrophotometer (Molecular Devices Corporation). The fluorescence signals of

392   AuNPs-peptide probe were analyzed by fluorescence spectrophotometer (F-2700;

393   Hitachi, Tokyo, Japan). Enzyme activities in plasma/serum were analyzed by Fujifilm

394   clinical chemistry analyzer (FUJI DRI-CHEM 3500; Fujifilm Corporation; Tokyo,

395   Japan). The gel electrophoresis analyses were performed with horizontal electrophoresis

396   system (Mini-Sub Cell GT; Biorad, Corston, UK). Microscope equipped with a

397   high-resolution video camera (BX51; Olympus, Tokyo, Japan). Size examination of

398   AuNPs and fabricated-AuNPs were examined by transmission electron microscopy,

399   TEM (JEM-1230; JEOL, Tokyo, Japan) operated at 100 kV and equipped with CCD

400   camera, and dynamic light scattering, DLS (Brookhaven Instruments Corporation,

401   Holtsville, NY, USA). Particles analyzed by Zetasizer Nano (Malvern Instruments,

402   Worchestershire, UK) and use disposable solvent resistant micro cuvette (ZEN0040) at

403   room temperature.

404

405   **4-3. Preparation of AuNPs**

406   AuNPs were prepared by citrate reduction method according to the reported

407   procedure [35, 41]. Colloidal AuNPs (15 nm in diameter) were prepared as follows: 50 mL

408   of 1 mM chloroauric acid solution was heated with oil bath till boiling, and followed by

409   the addition of 5 mL of 38.8 mM sodium citrate solution. The color of the solution

410   would turn from yellow to color less to black and to wine red. After the solution was

411   cooled to room temperature, the product of 15 nm AuNPs solution was preserved at 4°C.

412   The size of AuNPs was confirmed by TEM and DLS, and the concentration was

413   estimated by Beer's law with absorbent peak at 520 nm with an extinction coefficient of

414    $3.94 \times 10^8 \ M^{-1} \ cm^{-1}$.

415

416   **4-4. Preparation of AuNPs-peptide probes**

417   Three peptide substrates were used in the protease activated AuNPs-peptide probes

418   and the sequences were documented in **Table 1**. The difference between peptide#1 (P1,

419   GPLGLAG(Hyp)C) and peptide#2 (P2, GPLGLARGGGGGC) is length which the

420   anchor increase with a spacer of repeating glycine, $(Gly)_5$. The difference between P2

421   and peptide#3 (P3, GPLGLARDDDDDC) is the spacer of non-charged $(Gly)_5$ to

422   negatively-charged aspartic acid, $(Asp)_5$. However, three of them have the same

423   cleavage sites to chymotrypsin. The peptide-FITC was synthesized commercially by

424   Genesis Biotech (Taipei, Taiwan). The peptide included a cysteine (Cys) in the

425   C-terminal containing a thiol group (-SH) which could conjugate on to AuNPs through

18

426   Au-S bond. The probes were synthesized as following: the prepared AuNPs were

427   adjusted to proper pH by 1 N HCl or 1 N NaOH and then the concentration was

428   adjusted with ddH$_2$O to OD = 1, which was 980 μL of 2.5 nM AuNPs. AuNPs were

429   mixed with 10 μL of 1 mg mL^{-1} peptide-FITC and 10 μL of 0.01 M phosphate buffer

430   containing 0.1% SDS and 0.3 M NaCl, and then the mixture were shaken for 12 h, 40 ×

431   *g* at room temperature. AuNPs-peptide probes were purified by two rounds of

432   centrifugation. After first centrifugation (10,000 × *g* for 20 min), the supernatant was

433   carefully removed and added 500 μL 2% (wt/wt) PEG. After the second centrifugation

434   (11,000 × *g*, for 20 min), the supernatant was removed and resuspended with ddH$_2$O of

435   AuNPs-P3 probe or 0.1% BSA of AuNPs-P1 and AuNPs-P2 probes.

436

437   **4-5. Gel electrophoresis of AuNPs-peptide probes**

438   Gel electrophoresis analysis modified from Hanauer' protocol was used to confirm

439   the change of AuNPs after fabrication and protease (chymotrypsin) activation [40]. The

440   morphological change could be observed from visible red bands of AuNPs and

441   UV-illuminated fluorescent bands. Agarose gels (1.5%) were used and prepared with

442   0.5X TBE buffer. All samples loaded with 35% glycerol for increasing density as the

443   ratio of 7:1 (in volume). The citrate-capped AuNPs mixed with very small amount of

444   10% SDS (1 μL) for migration. The gels were run in a horizontal electrophoresis system

445   for 30 min at 110 V in 0.5X TBE buffer. Gel images were taken by a digital camera

446   under visible light and UV-light; besides, the images might be processed with small

447   linear contrast adjustments in order to obtain the true representation.

448

449

450   **4-6. Dithiothreitol-based displacement**

451   Dithiothreitol (DTT)-based displacement was utilized for separating peptides from

452   their AuNP. DTT will reactively displace the ligands from surface sites because it is

453   much more reactive toward AuNP compared with most ligands of interest [42]. In this

454   study, the conjugate ratio of peptide-FITC to AuNPs could be quantified by

455   fluorescence intensity. Fluorescence was measured using excitation/emission of

456   495/515 nm. DDT-based displacement was conducted as following: prepared 50 mg

457   mL^{-1} DTT (dissolved in pH 6.5 PB buffer, 0.1 M) and then added 500 μL DTT to

458   suspend AuNP probe sediment (0.625 nM) for 12 h incubation at room temperature.

459   The total fluorescence of loading peptide substrates was defined after proper dilution

460   and acquired a linear correlation between peptide-FITC concentration and fluorescence

461   intensity, and the supernatant of DTT displacement also processed with same procedure

462   to acquire the linear correlation. Dilution buffer was TTC buffer (50 mM Tris, 10 mM

463      $CaCl_2$, 150 mM NaCl and 0.05% Brij 35, pH 8.0).

464

465   **4-7. General procedure for chymotrypsin activity measurements by**

466   **AuNPs-peptide probe**

467   For the standard chymotrypsin activity assay, chymotrypsin (25 kDa) prepared in

468   1mM HCl with 2 mM $CaCl_2$ and stored at −20°C. Different concentrations of

469   chymotrypsin were diluted with pH 8.0 TTC buffer and added into AuNPs-peptide

470   probe (125 μL) to comprise 250 μL of mixture, and then incubated at 37°C. All of the

471   solutions were analyzed with fluorescence spectrophotometer (Hitachi F-2700), using

472   excitation/emission at 495/515 nm. Delta fluorescence intensity is the difference of

473   recovered fluorescence between protease-activated AuNPs-peptide probe and

474   non-activated control group.

475

476   **4-8. Animals**

477   Female C57BL/6 mice, purchased from the National Laboratory Animal Center

478   (NLAC, Taipei, Taiwan), were housed at the Laboratory Animal Center, National Chiao

479   Tung University, under standard conditions. Female mice of 6 to 8 weeks of age were

480   used in this study. All experimental procedures were carried out in accordance with the

481   guidelines of the Institutional Animal Care and Use Committee of National Chiao Tung

482   University. Every effort was made to minimize the suffering of the animals and the

483   number of animals used.

484

485   **4-9. Cerulein-induced AP mouse model**

486   The cerulein-induced AP mouse model was developed according to a previous

487   protocol [43], with minor modifications. The mice were induced to develop AP by 4 doses

488   of intraperitoneal injections of cerulein (200 $\mu g\ kg^{-1}$) at 2-h intervals. Each group

489   consisted of four mice. The control subjects were equally treated with 0.9% NaCl (10

490    $\mu L\ mg^{-1} 2\ h^{-1}$, 4 IP injections). The mice were sacrificed at 8, 10, or 24 h after the first

491   administration of saline (as a control) or cerulein. Mice were fasted for 6 h prior to

492   sacrifice, after which the blood, duodenum, and pancreas were collected.

493   For the time courses of fecal chymotrypsin in AP model experiments, the mice

494   were divided into two groups. All mice were subjected to 12 h fasting prior to injections.

495   AP (n = 4) was induced by 4 doses of cerulein (200 $\mu g\ kg^{-1} 2\ h^{-1}$), and the control

496   subjects (n = 3) were equally treated with 0.9% NaCl (10 $\mu L\ mg^{-1} 2\ h^{-1}$). All feces were

497   subjected to lysis as previously described.

498   **4-10. Sample collection**

499   For the fecal chymotrypsin assay, the feces were weighed and preserved at 4°C

500   before extraction. The fecal protein extraction process was as follows: one feces (10 –

501   25 mg per feces) was dissolved in 0.5 mL fecal protein extraction buffer (250 mM NaCl,

502   50 mM $CaCl_2$, and 0.25% Triton X-100) for 5 min with continuously mixing by vortex

503   mixer followed by centrifugation (10,000 × g, 1 min), and then the supernatant was

504   collected.

505   For the chymotrypsin assays in the duodenal eluent and pancreas, the mice were

506   sacrificed with CO_2 inhalation. Blood was collected by direct cardiac puncture and

507   mixed with heparin to acquire plasma after centrifugation (3,000 × g, 10 min at 4°C).

508   The first part of the small intestine followed the demarcation set by Shang [44] to

509   determine the duodenum. The duodenal contents were eluted with 1 mL DPBS and

510   collected the eluent. The duodenal eluent was treated by centrifugation (13,000 × g, 10

511   min at 4°C), and the supernatants were collected. The pancreas tissues were

512   homogenized with 1 mL PRO-PREP protein extraction solution (iNtRON

513   Biotechnology, Seongnam, South Korea). The homogenates were centrifuged at 13,000

514   × g for 10 min at 4°C. All the supernatants were collected and stored at −80°C until

515   further analysis. The protein concentrations of the supernatant were measured using a

516   Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

517

518   **4-11. Statistical analysis**

519   All data were reported as the mean ± standard deviation (SD) for the specified

520   number of replicates indicated in the caption. Statistical significance was determined by

521   a two-tailed Student's t-test with 95% confidence for unpaired observations. A p value

522   less than 0.05 was considered to be significant.

523

524

5. Conclusions

526   Chymotrypsin can be used as an indicator of pancreatic function. The established

527   AuNPs-peptide probe was used in chymotrypsin assays showing high sensitivity at the

528   pg mL^{-1} level and requiring only 15 min to detect enzyme activity. The sensitivity of the

529   AuNPs-peptide probe was greatly increased through the use of a spacer-enhanced

530   polyvalent peptide, $(Asp)_5$. This process is a one-step biological recognition element

531   assembly procedure that circumvents the use of additional stabilizers and enhances

532   stability.

533   A cerulein-induced AP mouse model was established in this study, and the

534   AuNPs-peptide probe was successfully used to analyze chymotrypsin activity to assess

535   pancreatic function. The plasma amylase and lipase levels were compared to the

536   chymotrypsin activities in the duodenal eluent, the pancreas and the feces. The increase

537   in pancreatic chymotrypsin activity indicated the pre-activation of trypsin in AP.

538   Meanwhile, the chymotrypsin activity in the duodenal eluent provided a direct index for

539   the estimation of pancreatic injury, although it required an invasive procedure. The fecal

540   chymotrypsin assay serves as a non-invasive method for the diagnosis of AP, and the

541   fecal chymotrypsin below 30 ng mg^{-1} of stool suggests AP. The AuNPs-peptide probe

542   was successfully applied in the rapid detection of pancreatic disease-related

543   chymotrypsin activity. Moreover, the AuNPs-peptide probe could potentially be used to

544   monitor the leakage of pancreatic juice during surgery to avoid postoperative pancreatic

545   fistulas. This study also provides a platform that could be applied in the daily

546   monitoring of fecal chymotrypsin activity and is non-invasive. This is the first study to

547   use AuNPs-peptide probes in practical disease diagnosis.

548

549   **6. Acknowledgments**

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1 **Figure 1.** (A) Schematic illustration of the protease activated fluorescent

2 self-assembled AuNPs (AuNPs-peptide probe); (B) TEM image of citrate-capped

3 AuNPs; (C) DLS result of citrate-capped AuNPs; (D) TEM image of AuNPs-P3 probe;

4 (E) DLS measurement of AuNPs-P3 probe; and (F) absorption spectra of

5 citrate-capped AuNP, AuNPs-P3 probe and chymotrypsin (500 ng mL^{-1} for 15 min at

6 37°C) activated AuNPs-P3 probe.

7

8 **Figure 2.** (A) Agarose gel showing the migration of the bands of AuNPs and

9 fabricated- AuNPs, which appear red in color; (B) the UV-illuminated gel shows the

10 recovered fluorescence after chymotrypsin activation. For both (A) and (B), Lane 1 =

11 citrate-capped AuNPs; Lane 2 = peptide substrate, P3; Lane 3 = AuNPs-P3 probe;

12 Lane 4 = P3 digested by chymotrypsin; and Lane 5 = AuNPs-P3 probe activated by

13 chymotrypsin. (C) Fluorescence emission spectra of three different AuNPs-peptide

14 probes activated by chymotrypsin after 1 h incubation.

15

16 **Figure 3.** Detection characteristics of the AuNPs-P3 probe. (A) Concentration

17 optimization; and (B) detection specificity to serine proteases, including trypsin (open

18 diamond) and chymotrypsin (close diamond). Error bars (SD) represent the data from

19 three independent measurements.

20

21 **Figure 4.** Chymotrypsin activity assay using the AuNPs-P3 probe. (A) Time course of

22 chymotrypsin (5 ng mL^{-1}, 200 pM) treatment. The solid line represents the best fit to

23 a single exponential function, where y = 5699.0523·(1-e$^{-0.0424\,x}$) and R² = 0.995; and

24 (B) the relationship between chymotrypsin concentration and fluorescence intensity in

25 a 15 min detection time was determined. The linear correlation from 0.25 to 10 ng

26   mL^{-1} of chymotrypsin was confident, for which Δ FL = 506.69 (chymotrypsin) −

27   90.82 and R^2 = 0.989. Error bars (SD) represent the data from three independent

28   measurements.

29

30   **Figure 5.** Chymotrypsin activity in biological samples from mice sacrificed at 8 h

31   (C8), 10 h (C10), or 24 h (C24) after the first administration of saline (control) or

32   cerulein. (A) Plasma amylase, (B) plasma lipase, and (C) pancreatic chymotrypsin.

33   Error bars (SD) represent data from three independent detections; * and ** indicate

34   statistical significance at *p*-value < 0.05 and < 0.01, respectively.

35

36   **Figure 6.** (A) Duodenal chymotrypsin in mice with cerulein-induced AP; (B) 24 h

37   time course of fecal chymotrypsin in a cerulein-induced AP mouse model. Every 5 h

38   period was classified into one group; the grey bar indicates the saline group (control)

39   and the white bar indicates the cerulein-induced group. Error bars (SD) represent the

40   data from three independent detections; * and ** indicate statistical significance at

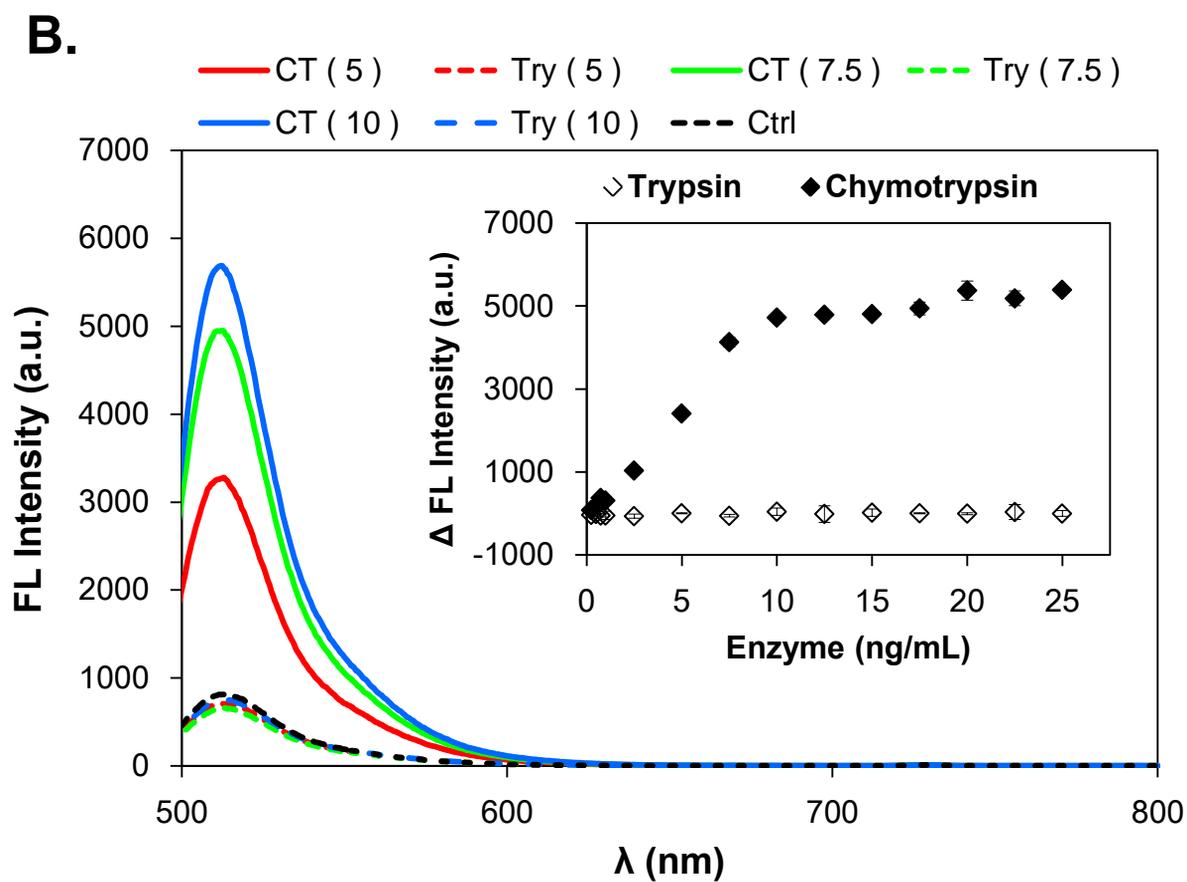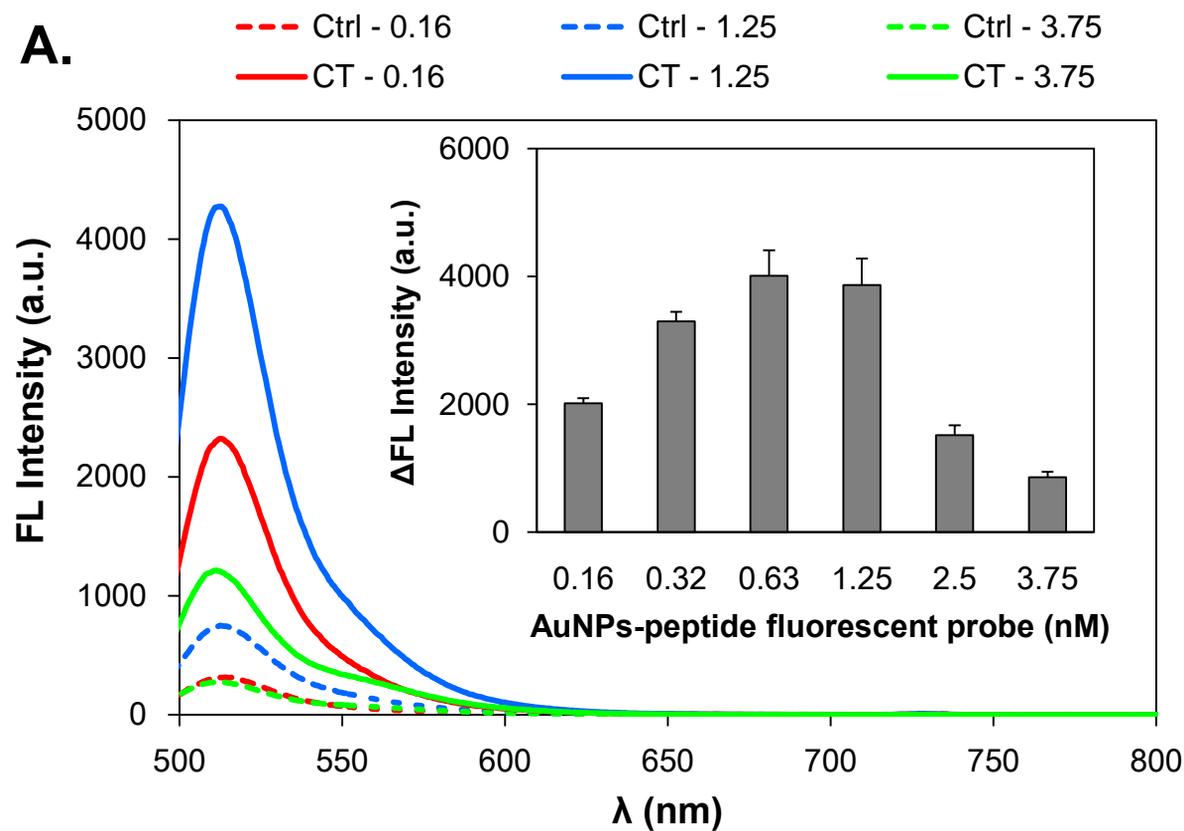41   *p*-value < 0.05 and < 0.01, respectively.

Table 1. Peptide sequences corresponding to the chymotrypsin cleavage sites, the pH used during assembly onto the AuNPs, and the optimal detection range and time of the fabricated AuNPs-peptide probes for the assay of chymotrypsin activity.
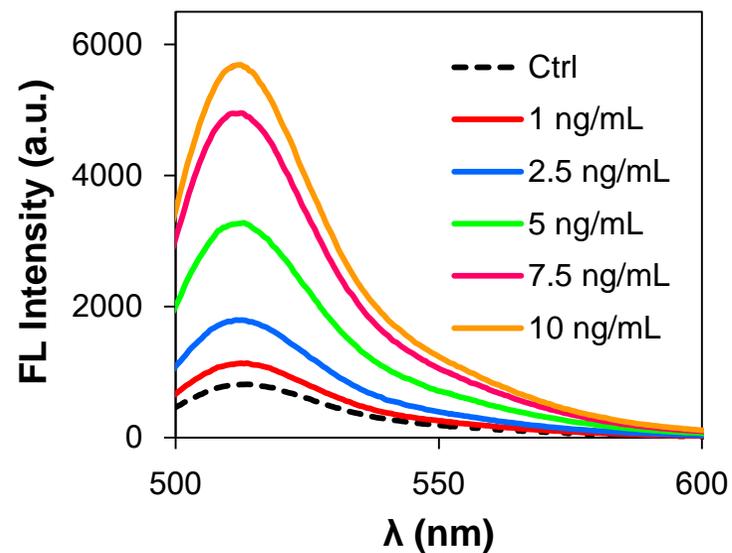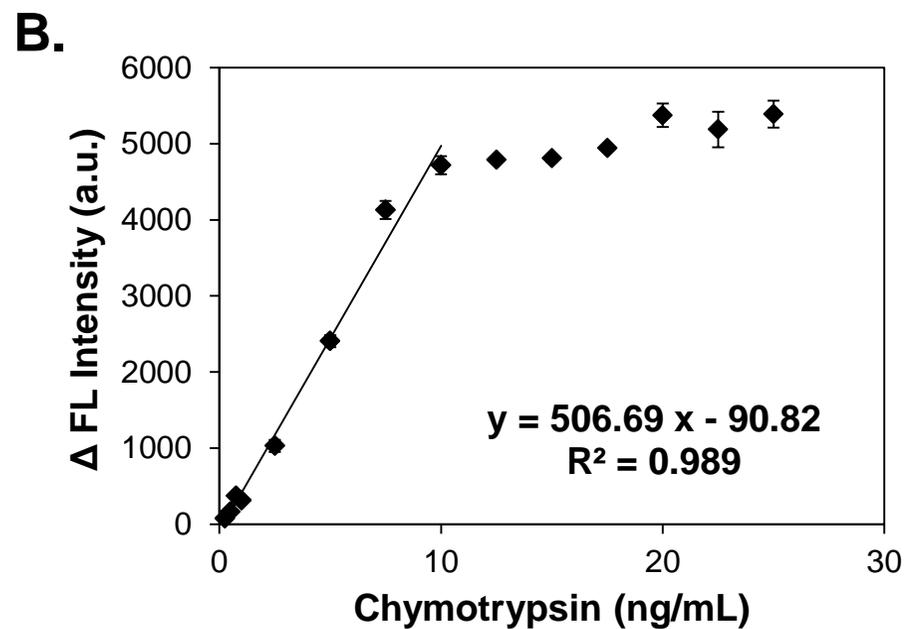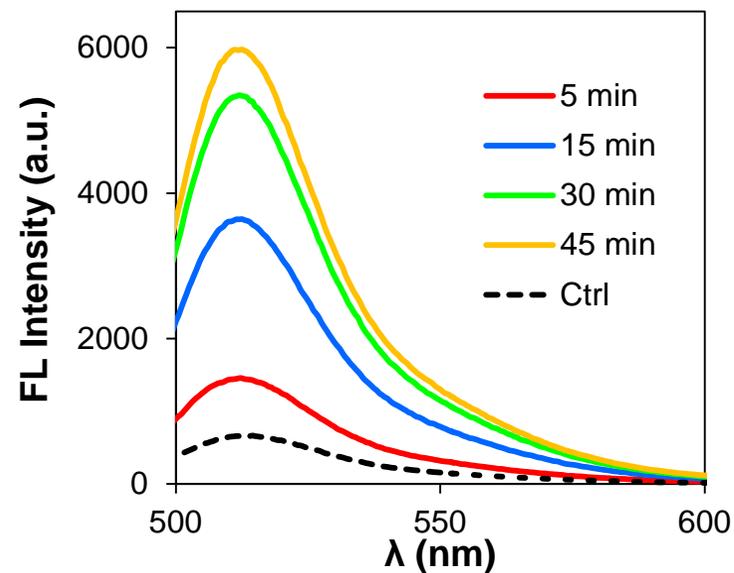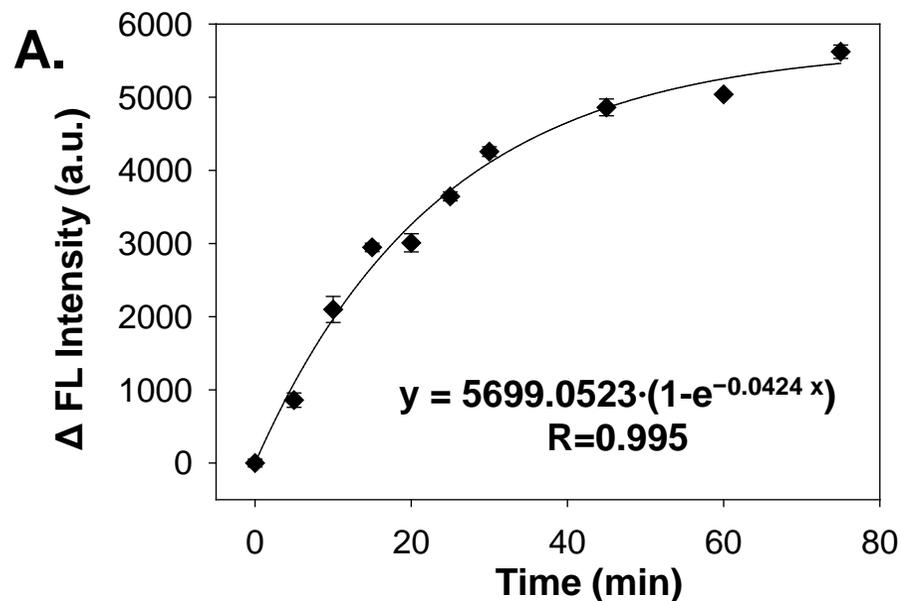
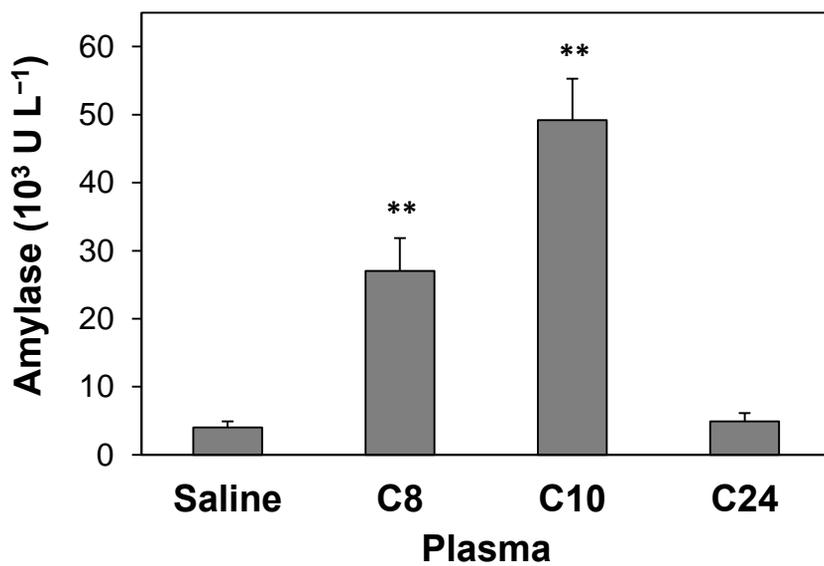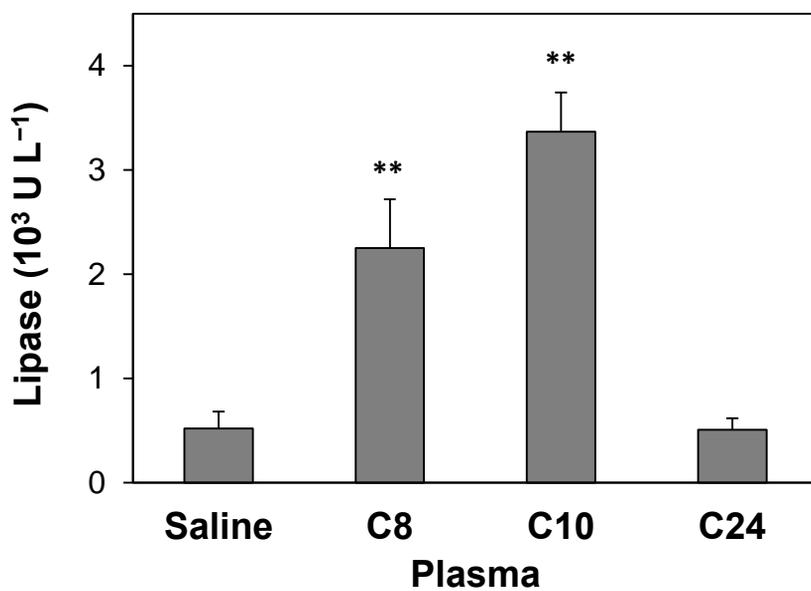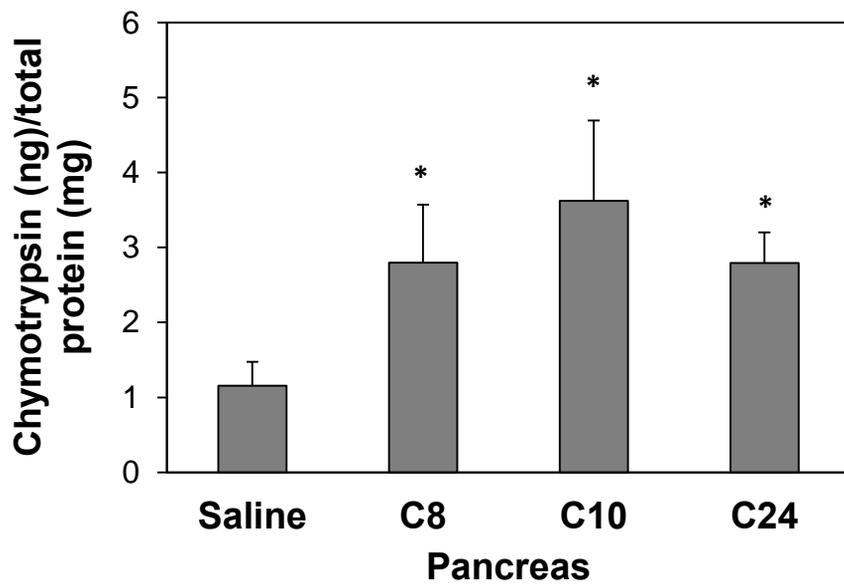| No.	FITC-Acp-peptide	pI	AuNPs fabrication pH	Optimal detection range (ng mL^{-1})	Optimal detection time (min)
P1	GPL ↑ GL ↑ AG(Hyp)C	5.3	7.4	100 – 500	60
P2	GPL ↑ GL ↑ ARGGGGGC	7.8	10.0	25 – 300	30
P3	GPL ↑ GL ↑ ARDDDDDC	3.6	5.6	0.25 – 10	15

The symbol (↑) indicates the potential cleavage sites cleaved by chymotrypsin. The pI of the peptide substrate was predicted using the online tools — Peptide Property Calculator, Genscript. The optimal detection range of the AuNPs-P1 probe showed a linear correlation of Δ FL = 0.78 (chymotrypsin) + 7.29, R^2 = 0.98. The optimal detection range of the AuNPs-P2 probe showed a linear correlation of Δ FL = 4.95 (chymotrypsin) + 82.35, R^2 = 0.99. The optimal detection range of the AuNPs-P3 probe showed a linear correlation of Δ FL = 506.69 (chymotrypsin) + 90.82, R^2 = 0.99.

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Figure 1

A.

FITC (excited)

Peptide substrate

+

AuNPs (15 nm)

FITC (quenched)

Protease
(chymotrypsin)

B.

C.

Intensity (%)

Diameter (nm)

D.

E.

Intensity (%)

Diameter (nm)

F.

Absorbance (OD)

AuNPs
AuNPs-peptide probes
AuNPs-peptide probes + chymotrypsin

Wavelength (nm)

Figure 2

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Figure 3

Figure 4

A. $y = 5699.0523 \cdot (1-e^{-0.0424 \, x})$
R=0.995

B. $y = 506.69 \, x - 90.82$
$R^2 = 0.989$

Figure 5

A.

B.

C.

Figure 6

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A.

B.