The pharmaceutical industry is comprised of a myriad of active pharmaceutical ingredients, all applied to improving human health and quality of life. One of the requirements of reaping the health benefits of these drug products is maintaining them in a native or desired form. Such non-native forms can include, aggregated or fragmented structures. Unfortunately, the most widely used methods to detect non-native or denatured proteins require trained technicians, bulky instrumentation and large amounts of reagents. Deviation from the native structures can occur at all stages; from manufacturing and processing to storage. With these limitations in mind, a simplistic and highly sensitive in solution detection method was evaluated to visually detect denatured insulin proteins, utilizing gold nanoparticle aggregation via 3-Aminopropyltrethoxysilane. The insulin in this study was heat stressed using an 80°C water bath to create an accelerated heat stressed environment. The insulin, gold nanoparticle and aminosilane solution was then characterized utilizing, UV-Vis spectroscopy, dynamic light scattering and scanning electron microscopy. Captured images and resulting absorbance spectra of the trials demonstrated visual color changes detectable with the human eye as a function of the denaturation time. This work serves as an extended proof of concept for fast in solution detection methods for proteins that have experienced heat stress.

© The Author(s) 2016. Published by ECS. This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 License (CC BY, http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse of the work in any medium, provided the original work is properly cited. [DOI:10.1149/2.0041705jes] All rights reserved.
grade purity trisodium citrate reagent at a 99% purity used to reduce the gold chloride was obtained from Alfa Aesar. The aminosilane, 3-Aminopropyltriethoxysilane was procured from Sigma-Aldrich containing a 98% purity. All chemicals were dissolved in deionized water (DI H2O) without employment of any further purification methods.

Synthesis of the gold nanoparticles used within this study were prepared based on a one pot synthesis. Precursor solutions were prepared by dissolving gold chloride and trisodium citrate in deionized water. The reaction was carried out at approximately 100°C preparing the respective APTES, insulin solution and 0.8 mL of the gold nanoparticle (AuNp) solution. After thorough mixing, 0.1 mL of the respective APTES concentration is then added and subsequently characterized via ultraviolet visible spectroscopy. Protein denaturation was achieved by placing the diluted insulin in a glass vial submerged in a hot water bath containing a 98% purity used to reduce the gold chloride was obtained from Alfa Aesar. The aminosilane, 3-Aminopropyltriethoxysilane was procured from Sigma-Aldrich containing a 98% purity. All chemicals were dissolved in deionized water (DI H2O) without employment of any further purification methods.

Synthesis of the gold nanoparticles used within this study were prepared based on a one pot synthesis. Precursor solutions were prepared by dissolving gold chloride and trisodium citrate in deionized water. The reaction was carried out at approximately 100°C preparing the respective APTES, insulin solution and 0.8 mL of the gold nanoparticle (AuNp) solution. After thorough mixing, 0.1 mL of the respective APTES concentration is then added and subsequently characterized via ultraviolet visible spectroscopy. Protein denaturation was achieved by placing the diluted insulin in a glass vial submerged in a hot water bath set to 80°C. As the protein was denatured, 0.1 mL aliquots of the protein solution was removed, mixed according to the above mentioned procedure and characterized. To properly capture the instantaneous color of the protein, AuNps, insulin and APTES solutions, a picture was taken directly after the addition of APTES and then loaded onto the UV-Vis.

Scanning electron microscopy images were taken using the HITACHI FE-SEM SU8010 instrument at 8 kV accelerating voltage and 5 milliamps. Dynamic light scattering measurements were taken to determine the aggregate sizes produced as a function of the accelerated heat stress trials consisted of 0.1 mL of the 22.06 ug/mL insulin solution and 0.8 mL of the gold nanoparticle (AuNp) solution. After thorough mixing, 0.1 mL of the respective APTES concentration is then added and subsequently characterized via ultraviolet visible spectroscopy. Protein denaturation was achieved by placing the diluted insulin in a glass vial submerged in a hot water bath set to 80°C. As the protein was denatured, 0.1 mL aliquots of the protein solution was removed, mixed according to the above mentioned procedure and characterized. To properly capture the instantaneous color of the protein, AuNps, insulin and APTES solutions, a picture was taken directly after the addition of APTES and then loaded onto the UV-Vis.

The accelerated heat stress trials consisted of 0.1 mL of the 22.06 ug/mL insulin solution and 0.8 mL of the gold nanoparticle (AuNp) solution. After thorough mixing, 0.1 mL of the respective APTES concentration is then added and subsequently characterized via ultraviolet visible spectroscopy. Protein denaturation was achieved by placing the diluted insulin in a glass vial submerged in a hot water bath set to 80°C. As the protein was denatured, 0.1 mL aliquots of the protein solution was removed, mixed according to the above mentioned procedure and characterized. To properly capture the instantaneous color of the protein, AuNps, insulin and APTES solutions, a picture was taken directly after the addition of APTES and then loaded onto the UV-Vis.

The following experiment was executed to quantify the optical changes in the solutions containing denatured vs. non-denatured insulin. As previously described in the Experimental sections, 0.8 mL of the gold nanoparticle solution was mixed with 0.1 mL of the heat stressed insulin. To complete the trials, 0.1 mL of either 0.3% or 0.2% APTES was added to the solution and immediately characterized via ultraviolet visible spectroscopy. In Figure 1 we observe systematic blue shifts in the max peak wavelength and a decrease in the peak broadening as the heat stress time was increased for all trials. Specifically, within Figures 1a and 1c, the insulin was heat stressed at 80°C using 0.3% and 0.2% APTES respectively. In Figure 1c we observe a significant decrease in the peak broadening after only 1 minute of heat stress. The resulting absorbance spectra of the 1-minute trial in Figure 1c is almost indistinguishable from the control of gold nanoparticles and native insulin without the addition of APTES. In contrast, it takes 6 minutes for the trial containing 0.3% APTES to produce an appreciable decrease in the peak width. In Figure 1b the heat stress temperature was decreased to 70°C and we observe the temperature dependence of absorbance spectra as well. In Figure 1b it took 3 minutes of the accelerated heat stress to induce regression of the max peak wavelength back to approximately 500 nm. In Figure 1d a higher APTES concentration of 0.4% was utilized and the subsequent blue shifts were also monitored as a function of the accelerated heat stress.

The changes in the absorbance spectra of the samples correspond to visual in solution color detection of the denatured insulin utilizing the gold nanoparticles and APTES. Figure 2 displays the solution hues of the gold nanoparticles and APTES trials. In Figure 2a, the resulting solution hues of the 80°C heat stress and 0.3% APTES trials were captured. Within Figure 2a, as the insulin is heat stressed, the solution transitions from a purple hue back to a pink/red hue indicating visual detection at 6 minutes of heat stress. Figure 2b displays the 70°C heat stress and 0.2% APTES trial which produced a 3-minute detection with a similar trend of a purple to red color transition. Figure 1c exhibited the fastest visual detection of just 1 minute of accelerated heat stress at 80°C and 0.2% APTES, maintaining the purple to red hue. In contrast, at the higher APTES concentration of 0.4%, the 0-minute solution hue turned a deep purple and the visual indicator

Figure 1. Displays the absorbance spectra of the gold nanoparticles, insulin and APTES solutions. a) 80°C heat stress and 0.3% APTES, 6-minute detection. b) 70°C heat stress and 0.2% APTES, 3-minute detection. c) 80°C heat stress and 0.2% APTES, 1-minute detection. d) 80°C heat stress and 0.4% APTES, 12-minute detection.

Results and Discussion

The following experiment was executed to quantify the optical changes in the solutions containing denatured vs. non-denatured insulin. As previously described in the Experimental sections, 0.8 mL of the gold nanoparticle solution was mixed with 0.1 mL of the heat stressed insulin. To complete the trials, 0.1 mL of either 0.3% or 0.2% APTES was added to the solution and immediately characterized via ultraviolet visible spectroscopy. In Figure 1 we observe systematic blue shifts in the max peak wavelength and a decrease in the peak broadening as the heat stress time was increased for all trials. Specifically, within Figures 1a and 1c, the insulin was heat stressed at 80°C using 0.3% and 0.2% APTES respectively. In Figure 1c we observe a significant decrease in the peak broadening after only 1 minute of heat stress. The resulting absorbance spectra of the 1-minute trial in Figure 1c is almost indistinguishable from the control of gold nanoparticles and native insulin without the addition of APTES. In contrast, it takes 6 minutes for the trial containing 0.3% APTES to produce an appreciable decrease in the peak width. In Figure 1b the heat stress temperature was decreased to 70°C and we observe the temperature dependence of absorbance spectra as well. In Figure 1b it took 3 minutes of the accelerated heat stress to induce regression of the max peak wavelength back to approximately 500 nm. In Figure 1d a higher APTES concentration of 0.4% was utilized and the subsequent blue shifts were also monitored as a function of the accelerated heat stress.

The changes in the absorbance spectra of the samples correspond to visual in solution color detection of the denatured insulin utilizing the gold nanoparticles and APTES. Figure 2 displays the solution hues of the gold nanoparticles and APTES trials. In Figure 2a, the resulting solution hues of the 80°C heat stress and 0.3% APTES trials were captured. Within Figure 2a, as the insulin is heat stressed, the solution transitions from a purple hue back to a pink/red hue indicating visual detection at 6 minutes of heat stress. Figure 2b displays the 70°C heat stress and 0.2% APTES trial which produced a 3-minute detection with a similar trend of a purple to red color transition. Figure 1c exhibited the fastest visual detection of just 1 minute of accelerated heat stress at 80°C and 0.2% APTES, maintaining the purple to red hue. In contrast, at the higher APTES concentration of 0.4%, the 0-minute solution hue turned a deep purple and the visual indicator
Figure 2. Displays the solution hues of insulin, gold nanoparticles and APTES. a) 80°C heat stress and 0.3% APTES, 6-minute detection. From left to right: AuNps, AuNps/Insulin, 0 min, 1 min, 3 min, 6 min, 12 min and Negative control consisting of AuNps/APTES. b) 70°C heat stress and 0.2% APTES, 3-minute detection. From left to right: AuNps, AuNps/Insulin, 0 min, 1 min, 3 min, 6 min, 9 min, and Negative control consisting of AuNps/APTES. c) 80°C heat stress and 0.2% APTES, 1-minute detection. From left to right: AuNps, AuNps/Insulin, 0 min, 1 min, 3 min, Negative control of AuNps/APTES. d) 80°C heat stress and 0.4% APTES, 12-minute detection. From left to right: AuNps, AuNps/Insulin, 0 min, 3 min, 9 min, 12 min, 15 min, 18 min.

Figure 3. Displays the gold nanoparticles, insulin and 0.3% APTES solution trials on a p-type silicon at 1.0 um. a) Non-denatured insulin. b) Denatured insulin. occurred after 12 minutes to a lavender purple. If the heat stress of the insulin was continued, it is projected that there would be a defined level of insulin denaturation that would produce a red hue and give a second detection marker for the 0.4% APTES concentration trial.

In order to further characterize the in solution detection of denatured insulin, the trials were imaged using scanning electron microscopy and the size and size distributions of the samples were quantified via dynamic light scattering. Figure 3 displays the scanning electron microscopy images of the gold nanoparticles, insulin and 0.3% APTES solutions. In Figure 3a we observe the large scale aggregation of the non-denatured insulin, gold nanoparticles and 0.3% APTES solution which was drop casted and air dried on a p-type silicon. This aggregation corresponds to the visual change in the solution color of the insulin and gold nanoparticle solution from red to purple upon the addition of APTES. In contrast to Figure 3a, we see in the denatured insulin trial (Figure 3b) a less densely packed morphology with minimal aggregation visible.

To quantify the extent of aggregation within the denatured and non-denatured insulin trials, the size and size distribution of the samples were measured via dynamic light scattering. In Figure 4 we observe the size and size distribution for the trials containing, gold nanoparticles and native insulin. Here we observe no appreciable difference between the two curves corresponding to both solutions maintaining a red hue as observed in the captured solution images of Figure 2a. The majority of the gold nanoparticles in the solution ranged from 11–20 nm. In Figure 4b we observe the size and size distributions for the denatured insulin and non-denatured insulin containing 0.3% APTES. The non-denatured trial within Figure 4b exhibited an increase in the size and size distribution of the gold nanoparticle aggregates in contrast with the denatured curve which was very similar with respect to size and size distribution of the controls. In Figure 4b we observe a size range from 18–122 nanometers for the non-denatured insulin trial. Within Figure 3, we observe micron scale aggregation, however within the dynamic light scattering technique only submicron aggregation is observed. We believe this difference is due to differences in sample measurement times. Immediately after mixing the gold nanoparticle, insulin and APTES solution, the trials were characterized using the

Figure 4. Displays the size and size distribution of the trials solutions a) Gold nanoparticles, gold nanoparticles/insulin. b) Gold nanoparticles/non-denatured insulin/0.3% APTES and gold nanoparticles/Denatured insulin/0.3% APTES.
Dynamic light scattering technique. However, in order to image the samples via scanning electron microscopy, the samples were air dried overnight. During that time, the samples can continue to aggregate until they are immobilized on the surface of the silicon as the water evaporates.

The gold nanoparticles utilized in this study were synthesized via a one pot hot injection method using trisodium citrate as the reducing agent. This method produced gold nanoparticles that are capped with negatively charged citrate molecules, creating a stabilized particle through repulsive forces. Upon the addition of APTES to the gold nanoparticle solution, dipoles are formed and initiate the observed aggregation of the AuNps. The dipoles are induced due to the positively charged amine group on APTES molecule; which is electrostatically attracted to the negatively charged citrate groups on the surface of the AuNps. The newly formed dipoles are higher in energy and so to align with the laws of entropy, the AuNps aggregate to minimize effects of the dipole causing aggregates which then stimulates a visual color change.

In Figure 5, the size and size distribution of the non-denatured and denatured insulin as a function of the accelerated heat stress exposure time was quantified. The size majority of the non-denatured insulin samples exhibited a diameter of about 4.5 nm, which is indicative of hexamer formation. Within the denatured trials ranging from 1–20 minutes of heat stress, a systematic increase in the measured diameter size was observed. It is important to note that the insulin is aggregating and this is the cause of the larger size and size distribution as opposed to individual particle growth. In the 1-minute heat stress trial we observe a size majority of 102 nm for the aggregated insulin. As the heat stress is further applied, we observe 172 nm and 223 nm size diameter aggregates for the 5-minute and 10-minute accelerated heat stress trials (Figure 5). Within the 20-minute heat stress trial we observed two size diameter maxima at 204 nm and 530 nm.

Without the addition of APTES, the gold nanoparticle solution is relatively mono disperse and maintains an optical absorbance of about 500 nm. These values were comparable to previously reported sizes and corresponding wavelengths. The amine group present on APTES is electrostatically attracted to the negatively charged citrate surface of the gold nanoparticles. This coulombic attraction causes a dipole in the gold nanoparticles, increasing the overall energy state of the particle. To decrease this energy state, the gold nanoparticles aggregate which induces a visual color change in the solution. Therefore, aggregation is induced as a result of dipoles that form on the gold nanoparticles due to the presence of APTES. Maintaining the concentration of APTES, the extent to which these dipoles are formed are also dependent on the concentration of insulin present in solution and the state of the insulin (denatured or non-denatured).

Based on the mode of aggregation due to the presence of APTES, we propose the following mechanism for the visual detection method based on the observed insulin aggregation (Figure 4). Without insulin present in solution, the addition of 100 uL of 0.2% and 0.3% APTES will produce a blue hue within the gold nanoparticle solution. With the addition of 100 uL of the insulin solution in the presence of gold nanoparticles and APTES, a purple color is observed instead. However, as the protein begins to denature, the insulin agglomerates forming barriers that prevent the interaction of the APTES with the gold nanoparticles as a function of the extent of insulin denaturation (Figure 6). We propose that as the insulin is heat stressed, it more effectively blocks the creation of the dipoles thus preventing the gold nanoparticles from closing the inter-particle space and limiting the degree of aggregation. This causes a blueshift in the absorbance spectra of the solutions as the heat stress is applied to the protein, thus producing visual means of detection.

**Conclusions**

Through this work we demonstrate the ability to visually detect denatured insulin utilizing concentration dependent induced aggregation of gold nanoparticles. The need to visually detect denatured or non-native proteins are crucial as the adverse effects of non-native structures can cause sever patient discomfort and a decrease in drug potency. This phenomenon poses a continuous problem within the pharmaceutical industries and can have detrimental and long lasting effects on patient quality of life. Therefore, this work provides a fast, facile and visual detection method for the presence of denatured proteins. Continued proof of concepts with other biological analytes such as antibodies and antibody conjugates using this system may prove...
to be a highly effective visual detection method during all stages of a drug product lifetime including; procession, formulation, filling, packing, shipping and storage.

Acknowledgments
This work is supported by the NSF-CREST (CNBMD) grant number HRD 1036494 and NSF-IGERT.

References
1. U. S. Congress, Office of Technology Assessment, Pharmaceutiical R&D: Costs, Risks and Rewards, (1993).
2. N. Lumelsky1, O. Blondel1, P. Laeng, I. Velasco, R. Ravin, and R. McKay, Science, 292, 1389 (2001).
3. The National Institute of Diabetes and Digestive and Kidney Diseases, Prediabetes and Insulin Resistance (2016).
4. J. Brange and L. Langkjoer, Pharm Biotechnology, 5, 315 (1993).
5. B. Prugovečki, I. Pulić, M. Toth, and D. Matković-Čalogović, Croatica Chemica Acta, 85, 435 (2012).
6. A. Papaioannou, S. Kuyucak, and Z. Kuncic, PLoS ONE, 11, 1 (2016).
7. R. Fu and M. Joseph, Agilent Technologies, 2 (2011).
8. P. R. Shorten, C. D. McMahon, and T. K. Soboleva, Biophysical Journal, 93, 3001 (2007).
9. S. Shahani and L. Shahani, Hong Kong Medical Journal, 21, 553 (2015).
10. T. Pham, J. B. Jackson, N. J. Halas, and T. R. Lee, Langmuir, 18, 4915 (2002).
11. E. FarrokhTakin, G. Ciofani, G. Puleo, V. Giuseppe, C. Filippeschi, B. Mazzolai, V. Piazza, and V. Mattoli, International Journal of Nanomedicine, 8, 2319 (2013).
12. S. Eustisa and M. El-Sayed, Chem. Soc. Rev., 35, 209 (2006).
13. T. Pham, J. B. Jackson, N. J. Halas, and T. R. Lee, Langmuir, 18, 4915 (2012).
14. E. FarrokhTakin, G. Ciofani, G. Paleo, V. Giuseppe, C. Filippeschi, B. Mazzolai, V. Piazza, and V. Mattoli, International Journal of Nanomedicine, 8, 2319 (2013).
15. J. Liao, Y. Zhang, W. Yu, L. Xu, C. Ge, K. Liu, and N. Gu, Colloids and Surfaces A: Physicochem., 223, 177 (2013).
16. T. Pham, J. B. Jackson, N. J. Halas, and T. R. Lee, Langmuir, 18, 4915 (2002).
17. E. FarrokhTakin, G. Ciofani, G. Paleo, V. Giuseppe, C. Filippeschi, B. Mazzolai, V. Piazza, and V. Mattoli, Journal of Nanomedicine, 8, 2319 (2013).
18. B. Chithrani, A. Ghazani, and W. Chan, ACS Nano, 4, 3689 (2010).
19. S. Eustisa and M. El-Sayed, Chem. Soc. Rev., 35, 209 (2006).
20. T. Pham, J. B. Jackson, N. J. Halas, and T. R. Lee, Langmuir, 18, 4915 (2012).
21. E. FarrokhTakin, G. Ciofani, G. Paleo, V. Giuseppe, C. Filippeschi, B. Mazzolai, V. Piazza, and V. Mattoli, International Journal of Nanomedicine, 8, 2319 (2013).
22. J. Liao, Y. Zhang, W. Yu, L. Xu, C. Ge, K. Liu, and N. Gu, Colloids and Surfaces A: Physicochem., 223, 177 (2013).