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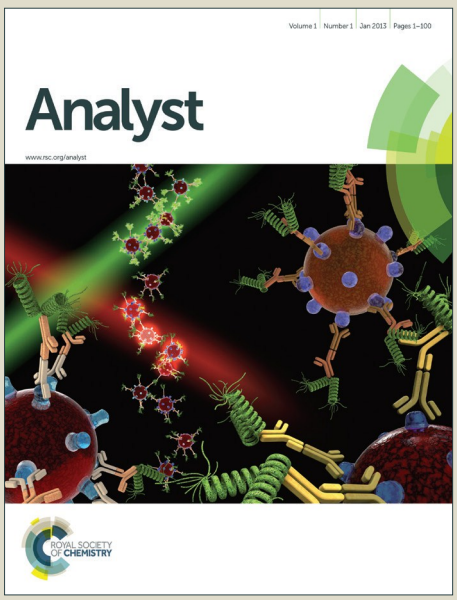

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Forensic determination of blood sample age using a bioaffinity-based assay

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A bioaffinity-driven cascade assay was developed to determine the time elapsed from the point a blood sample was left at a crime scene to the point of discovery. Two blood markers, creatine kinase (CK) and alanine transaminase (ALT), were utilized to determine the age of the blood spot based on their natural denaturation processes. The analysis with the proposed bioassay was performed in human serum samples, which underwent the aging process under environmental conditions that could be expected at crime scenes. The concentration of the markers in the sample was based on physiological levels present in healthy adults. These two markers were concerted in a biocatalytic cascade composed of two parallel subsystems, with each of them following the activity of one marker. Both markers have very distinct denaturation rates which would not allow them to be used in a single marker setup while still providing satisfactory results. However, by parallel tunable monitoring of both markers, it is possible to provide information of the blood sample age with low temporal error for a prolonged period of time. To mimic a possible real crime scene situation - the reliability of the proposed assay was then successfully tested on dried/aged serum samples (up to 5 days old) in environments with different temperatures.

Forensic investigation has revolutionized since biological samples have been included as part of the investigation process since they elucidate important facts about the crime scene and the crime itself. Biological traces found at crime scenes represent important leads in identification and the subsequent confirmation of possible suspects. Chemical and biochemical techniques utilized for the analysis of biological traces at crime scenes are the main scientific support of criminal investigations and subsequent prosecutions.[1] Forensic serology, a branch of forensic science, aims for the analysis of biological fluids, including but not limited to saliva and blood.[1] As bloodstains represent one of the major pieces of evidence utilized in forensic investigations,[2] they are routinely used to extract DNA (STR-DNA) by molecular biology techniques. This allows for the analysis of the DNA extracted from the blood through different techniques such as, 'DNA profiling' and 'DNA matching' which allow the identification of the donor.[3,4]

However, blood is not only a valuable source of information because of its genetic material, it is also valuable because of the unique composition of proteins and low molecular compounds present in the circulatory system. From the positions of blood spots, the crime can be re-constructed by analyzing blood splatter patterns[5,6] according to their geometry and distribution.[7] The age of a blood sample left at a crime scene can be a significantly important piece of information for the identification of the donor. For instance, multiple blood spots or splatters could be present at a crime scene and there would be no guarantee that every sample is relevant to the case. Having the ability to estimate the age of a blood spot would be significantly important in identifying blood that is relevant to a crime investigation and ruling out that which does not. Some spots could have been left days, weeks, or even months ago; to separately match every sample to an individual would be costly and time consuming. Currently, there is a technique available for distinguishing two individuals from overlapping bloodstains,[1,8] but with no technique to identify how old the samples are, they may be completely irrelevant. Being able to estimate the age of the blood samples left would allow investigators to exclude samples too old to be relevant and reduce the DNA work needed for the case. Nonetheless, the ability to estimate that age is still rather crude and underdeveloped.

Due to the degree of denaturation and other changes, blood samples have the potential to provide the time that has elapsed since the blood left the originator's body. Numerous techniques have been suggested for this purpose over the past decades, but due to the lack of practicality they

have never been applied in real settings.[9] In 1930, the pioneer in this area, Schwarzacher, attempted to find the relationship between the solubility of blood in water and its age.[10] Spectrophotometry analysis was first applied in 1960 by Patterson[11], where he determined that the changing color of a bloodstain is dependent on environmental conditions by recording the bloodstain's reflectance. Later, in 1983, Tsutsumi studied changes in individual proteins present in bloodstains.[12] More recent methods include electron paramagnetic resonance (EPR)[13], high performance liquid chromatography (HPLC)[14], oxygen electrodes[15], RNA degradation[16], near infrared (NIR) spectroscopy[17], and atomic force microscopy (AFM).[18]Altogether, the vast majority of these techniques require sample preparation and need to be performed in a laboratory setting, preventing the possibility to perform direct analysis at the crime scene. In addition to these requirements, these techniques have yet to report any environmental influences that will play a key factor in the decomposition of the sample, such as humidity, temperature or exposure to light.[18]

This investigation developed a novel tunable parallel assay of biomarkers for forensic determination of the age of blood samples left at crime scenes. Two protein markers, the enzymes creatine kinase (CK; E. C. 2.7.3.2) and alanine transaminase (ALT; E. C. 2.6.1.2), were used in a concerted manner to determine the age of bloodstain samples.[19-22] The mean concentrations of markers were based on their physiological level present in a healthy adult, CK (100 mU/mL) and ALT (20 mU/mL).[19-22] Previously, an elevation of some of these markers was used as an indication of various illnesses and injuries.[23-27] In addition, the combination of these two markers has been used to distinguish the ethnic origins and gender of their donors.[28,29] However, those markers were not used for the determination of the age of a sample, where their concerted parallel decomposition in time is followed.

We designed and optimized a biocatalytic cascade where parallel determination of the activity of both markers was followed in two parallel branches/subsystems, each following the enzymatic activity decay of one marker. Both subsystems represent the tunable elements; by modulation of their respective performances, the time for which the assay can follow the sample age with low temporal error and increased reproducibility can be flexibly prolonged or shortened.

Scheme 1 represents the entire biocatalytic cascade (CK/ALT), which follows the enzymatic activities of both blood markers within the aging process. In the proposed cascade, performed in 50 mM glycyl-glycine buffer solution at pH 7.95 (see experimental details in the ESI), the biocatalytic reaction of the CK marker was coupled with the consequent reaction

catalyzed by pyruvate kinase (PK; E.C. 2.7.1.40) to produce adenosine triphosphate (ATP) and pyruvate. In a parallel manner, the ALT marker was also producing pyruvate via the deamination of alanine (Ala) with α-ketoglutaric acid (KTG) acting as a co-substrate. The last biocatalytic step is the reduction of pyruvate into lactate by lactate dehydrogenase (LDH, E.C. 1.1.1.27), with the simultaneous consumption of NADH, optically readable at 340 nm (Scheme 1).

As indicated in Scheme 1, segments A and B, respectively, indicate each subsystem. In this case, segment A represents the CK pathway, while segment B is composed of the ALT pathway. In the first sets of experiments each pathway was analyzed and optimized independently, where the cascade was following only one marker, CK or ALT, respectively. This allowed for the individual evaluation of the performance of each independent marker. To accomplish this, human serum samples spiked with the appropriate concentrations of markers were placed on a glass surface and underwent an aging process for variable periods of time (from 0 up to 120 hours) while incubated at 40°C. The human serum samples were naturally dried by the environment and re-suspended with water just before measurements were taken. The biocatalytic cascade was activated by mixing the sample with the necessary enzymatic cascade substrates, co-substrates and auxiliary enzymes that were previously dissolved in 50 mM glycyl-glycine buffer (pH 7.95). Afterwards, the samples were immediately subjected to a continuous optical measuring at λ = 340 nm, to monitor the consumption of NADH. The conditions for the subsystems and the entire cascade were identical. Also, for each measurement taken a set of three (n=3) human serum samples, containing the markers was analyzed.

As previously indicated, the aging process was followed at a rather high temperature (40^{o}C), which is not expected at most crime scenes, but allows us to pronounce the aging effect on the particular markers. Fig 1, part A shows the real time response (oxidation of NADH, as shown in Scheme 1) of the biocatalytic cascade subsystem following the CK marker. The bar diagram inset (Figure 1B) illustrates the decay in time response where the output signal is plotted as a function of blood sample age. Fig 1 shows the rather fast decay of the marker activity where after 6 hours of the aging period the CK activity is reduced to approximately 20 % of its original level. This shows that the CK branch of the sensing cascade provides a low error of blood age sample determination, but its overall performance decreases drastically for samples older than 6 hours due to the lack of marker activity. To address this problem and simultaneously improve the tenability of the presented bioanalytical paradigm, ALT was also evaluated as a marker.

In figure 2, part A shows the real time response of the samples analyzed with branch B – following only ALT level – incubated at 40°C for up to 120 hours. Part B of figure 2, shows that the enzymatic activity of this particular marker undergoes a constant decay within the aging process more than 50% of the enzymatic activity remains after 120 hours. This would allow the determination of the blood sample age even beyond this time interval. On the other hand, low decay of the signal in combination with a low signal change overall will be responsible for a high temporal error of the blood sample age determination, which can be a drawback, especially in shorter aging times.

Fig. 3A shows an example of real time responses in which both paths of the cascade are active. Fig. 3B (bar diagram) illustrates the overall signal decay in time for the samples which underwent the aging process, combining paths A and B of the cascade. The resulting response forms a compromise within the system; for shorter times, it offers a significantly better response than a single (ALT) marker output, while the cascade is able to provide a more significant output, than that of CK alone, at prolonged aging periods. The combination of both branches in the fully assembled cascade, the CK marker provides the majority of the output when the sample is rather young (as we know this marker undergoes a fast deactivation), while the ALT marker is responsible for the signal in longer aging periods. This proposed assay offers a parallel analysis of both markers' activity within the aging process and balances both branches to compromise the performance between low error and a prolonged age determination. The performance of each of the subsystems and the combined paths was compared, as can be seen in Fig.4. This figure illustrates the difference in absorbance in the different time intervals where the two pathways and the entire system where evaluated. The combination of both subsystems allowed for the "tuning" of the sensing cascade, where both reaction branches where optimized for longer/shorter ageing intervals.

For the proposed CK/ALT tunable multi-marker cascade, the effect of temperature on the aging process was also examined. Human serum samples spiked with markers underwent an aging process to create an initial database of optical outputs of blood samples submitted to different degradation times and temperatures. The samples were incubated at 18 °C, 25 °C (room temperature) and 40 °C to mimic real different scenarios that can be encountered in a forensic investigation. The samples were also analyzed for up to 120 hours (5 days). Time zero corresponds

to the analysis of the freshly prepared sample. The remaining times represent the time that the sample had been incubated at that particular temperature. The samples that were incubated at 18 °C, the lowest temperature, expectably shows the lowest decomposition decay, while an elevated temperature, such as 40 °C, caused the faster denaturation (Fig. 5). The difference in optical responses among samples incubated at the same temperature, and among samples that vary in temperature incubation, makes this biocatalytic assay a perfect fit for the forensic investigation setting. These results show the potential of the proposed approach pre-calibrated cascade for a wide range of temperatures. Consequently, when an unknown blood sample is found at a crime scene where the environmental conditions are known, the output signal can be attributed to a particular time that translates to when the blood sample left the circulatory system.

Conclusions

This investigation showed that the combination of multiple enzyme markers, such as CK and ALT, can be used as a tool to determine blood spot ages at a crime scene. This study has also shows the advantage of having a parallel marker sensing cascade, over a single marker cascade where the combination of two markers provides improved information about the sample properties compared to a single marker. Due to the differences in the denaturation rates of various markers present in body fluid, a single marker assay may not allow for a reliable determination of blood sample age. For instance, markers with high stabilities would continue to provide a signal for long periods of time, but would also cause a high percentage of error in the analysis. On the other hand, other markers that have a rapid denaturation, such as CK, cannot be used for a prolonged period of time, but would provide a lower chance of error. In our parallel assay, we use a combination of both types of markers, long life and short life respectively, to provide a "compromised" response. By using the high sensitivity of markers with short half-lives together with the stability of markers with longer lives, the response was more reliable. This, apart from a prolonged time horizon, also allowed for "tuning" of the sensing cascade by optimizing of both reaction branches for longer/shorter ageing intervals.

These experiments confirm the applicability of the multi-marker CK/ALT biocatalytic assay for the analysis of the age of blood samples. In a real crime scene, these biomarkers will be found in bloodstains; therefore, these experiments were performed in human serum solutions dried on common glass surfaces, to closely mimic the real crime scene samples. The environmental

conditions, such as temperature, that the samples were exposed to, affect the enzymatic activity of the markers. Enzymatic activity varies drastically with time and temperature; therefore, the samples in this investigation were incubated at different temperatures. These factors of enzyme denaturation will allow for the construction of a trustful database, in which the age of the blood sample can be revealed on-site in forensic investigations.

This approach showed that the multi-marker bioanalytic assay paradigm can be successfully used for reliable determination of biological sample age in forensic analysis. Because of its simplicity and robustness, this methodology aims to be adapted as a component of a forensic field kit, moreover it can potentially be used by nonscientific trained personnel at crime scenes.

Further development in this area will be oriented toward the incorporation of this and similar approaches into portable lateral flow strip-like devices. The research into these approaches is currently under development in our laboratory.

Scheme and figure captions

Scheme 1. The biocatalytic multi-marker cascade in which (A) represents CK pathway, (B) represents ALT pathway, and (A) and (B) together represent the entire biocatalytic cascade, CK/ALT. The abbreviations used in the scheme are: CK (creatine kinase), PK (pyruvate kinase), LDH (lactate dehydrogenase), ALT (alanine transaminase), Crt (creatine), Crt-P (creatine phosphate), ATP (adenosine 5'-triphosphate), ADP (adenosine 5'-diphosphate), NAD^+ (β-nicotinamide adenine dinucleotide), NADH (β-nicotinamide adenine dinucleotide reduced), PEP (phospho(enol)pyruvic acid), Pyr (pyruvate), Lac (lactate), Ala (alanine), KTG (α-ketoglutaric acid), and Glu (glutamic acid). The exact information of chemicals used is giving in the experimental section (ESI).

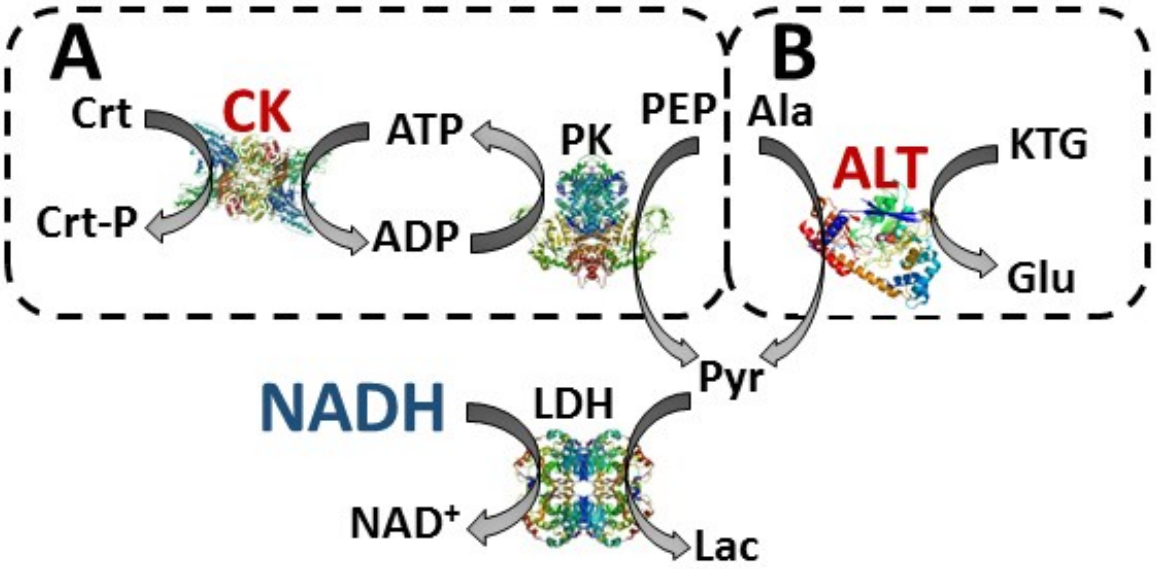
Figure 1. (A) Change in absorbance (λ = 340 nm) corresponding to the consumption of NADH upon operation of the CK- subsystem. These traces correspond to samples (n = 3) that mimic bloodstains, incubated at 40 °C from 0 hours to 120 hours, as indicated in the legend. (B) Inset bar diagram represents the change in absorbance at λ = 340 nm, after 30 minutes of assay completion. ESI includes the exact composition of reactant solutions used.

Figure 2. (A) Change in absorbance (λ = 340 nm) corresponding to the consumption of NADH upon operation of the ALT- subsystem. These traces correspond to samples (n = 3) that mimic bloodstains, incubated at 40 °C from 0 hours to 120 hours, as indicated in the legend. (B) Inset bar diagram represents the change in absorbance at λ = 340 nm, after 30 minutes of assay completion. ESI includes the exact composition of reactant solutions used.

Figure 3. (A) Change in absorbance (λ = 340 nm) corresponding to the consumption of NADH upon operation of the CK/ALT representing the whole parallel bioaffinity cascade. These traces correspond to samples (n = 3) that mimic bloodstains, incubated at 40 °C from 0 hours to 120 hours, as indicated in the legend. (B) Inset bar diagram represents the change in absorbance at λ = 340 nm, after 30 minutes of assay completion. ESI includes the exact composition of reactant solutions used.

Figure 4. Absorbance change at λ = 340 nm, corresponding to the consumption of NADH after the analysis of the two different paths in the proposed biocatalytic assay, as well as the entire biocatalytic assay. Time zero corresponds to the analysis of the freshly prepared sample (n = 3). The rest of the samples were re-suspended in water after they underwent aging at 40 °C. The black square represents the CK path. The red circle represents the ALT path and the blue triangle represents the entire biocatalytic cascade, CK/ALT. The zero time intervals correspond to the freshly prepared samples without drying. The exact information of chemicals used is giving in the experimental section (ESI).

Figure 5. Absorbance change at λ = 340 nm, corresponding to the consumption of NADH after the analysis of the samples by the proposed CK/ALT biocatalytic assay. Samples (n = 3) were re-suspended after undergoing the aging process under the different temperatures: 40 °C (black square), 25 °C (red circle) and 18 °C (blue triangle), up to 120 hours. The zero time intervals correspond to the freshly prepared samples without drying. The exact information of chemicals used is giving in the experimental section (ESI).



Scheme 1

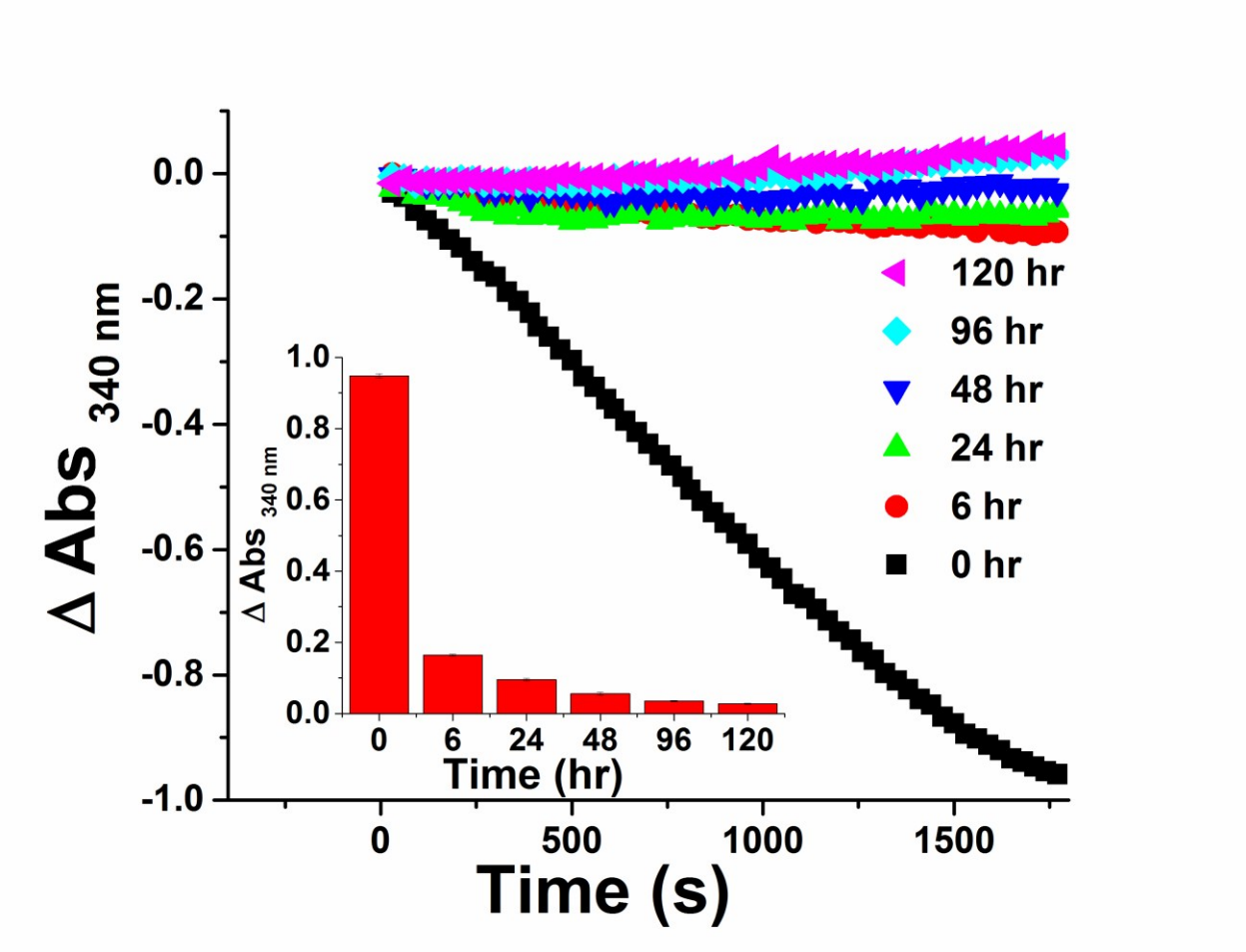


Figure 1

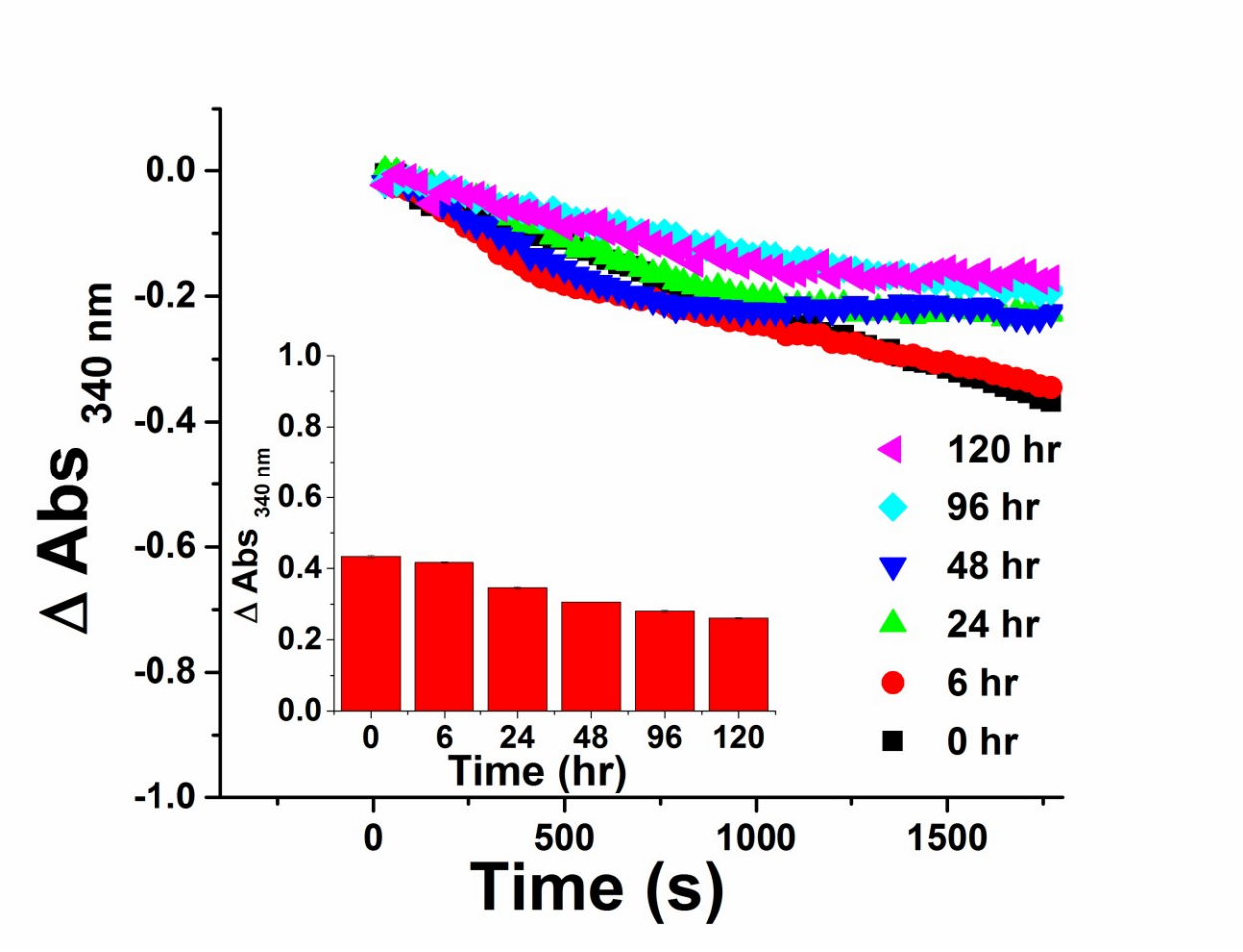


Figure 2

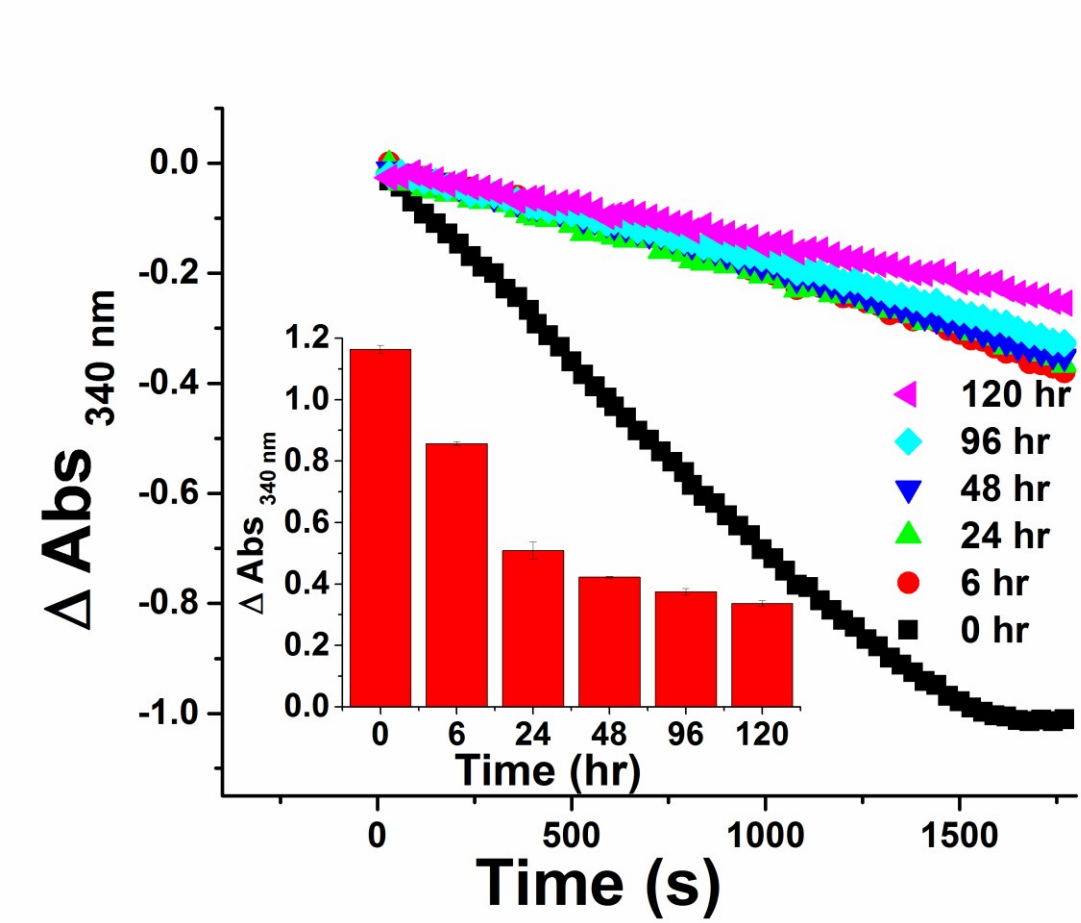


Figure 3

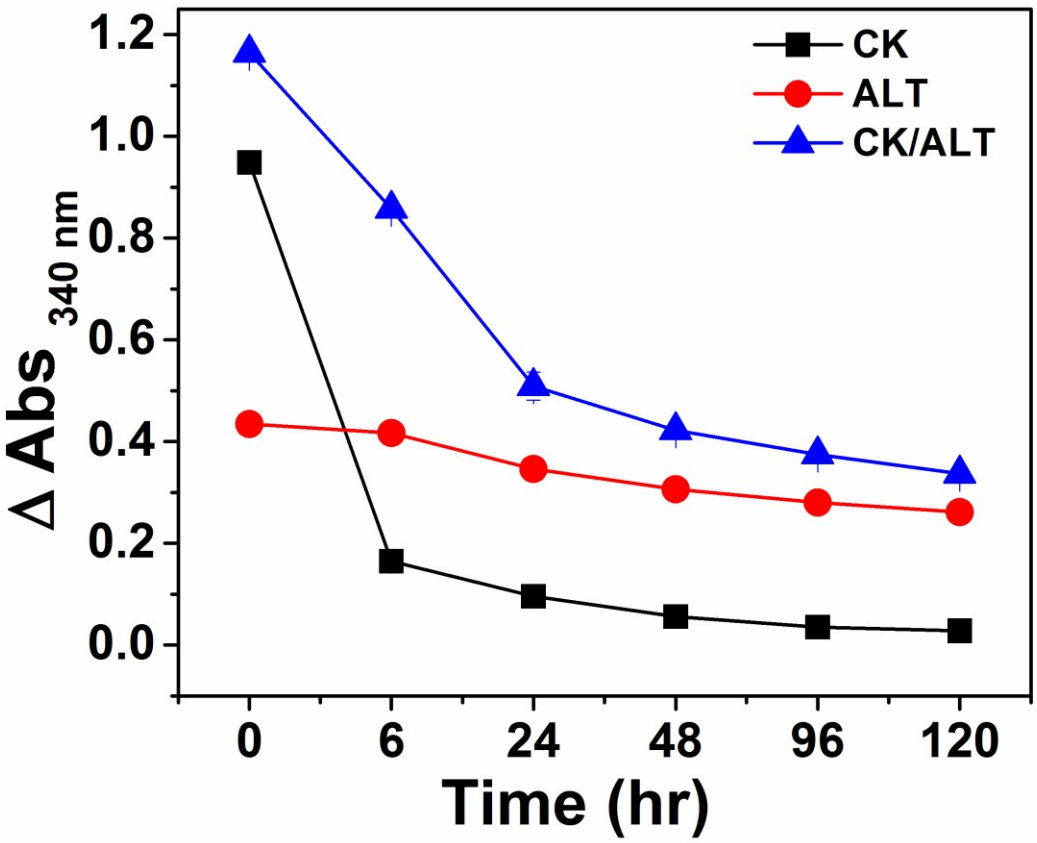


Figure 4

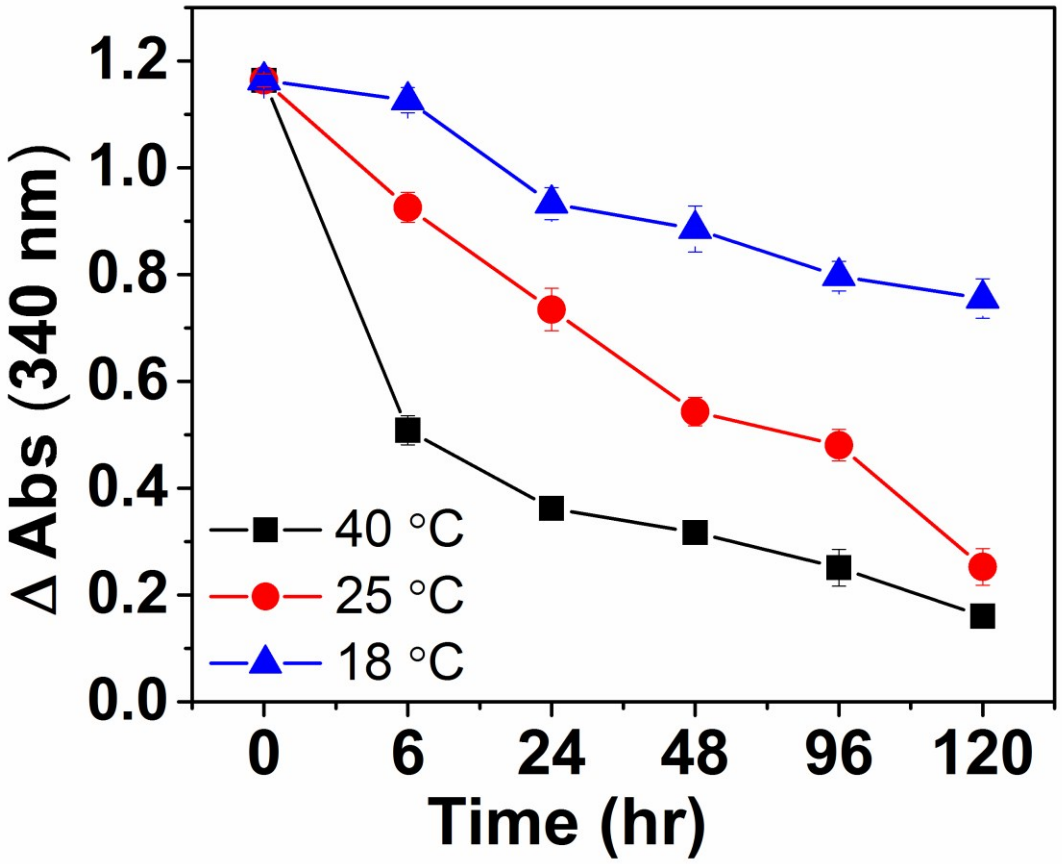


Figure 5

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