Biochemistry and Biophysics Reports 1 (2015) 1–7

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

# Study into the kinetic properties and surface attachment of a thermostable adenylate kinase

H.J. Hathaway [a], J.M. Sutton [b], A.T.A. Jenkins [a,*]

[a] Department of Chemistry, University of Bath, Claverton Down, Bath and North East Somerset, BA2 7AY, UK
[b] Technology Development Group, Public Health England, Porton Down, Salisbury, Wiltshire, UK

ARTICLE INFO

*Article history:*
Received 4 February 2015
Received in revised form
20 March 2015
Accepted 23 March 2015
Available online 31 March 2015

*Keywords:*
Thermostable adenylate kinase
Kinetic parameters
Plasma deposition
Cleaning efficacy
Enzyme inhibition

ABSTRACT

A thermostable adenylate kinase (tAK) has been used as model protein contaminant on surfaces, so used because residual protein after high temperature wash steps can be detected at extremely low concentrations. This gives the potential for accurate, quantitative measurement of the effectiveness of different wash processes in removing protein contamination. Current methods utilise non-covalent (physisorbtion) of tAK to surfaces, but this can be relatively easily removed. In this study, the covalent binding of tAK to surfaces was studied to provide an alternative model for surface contamination. Kinetic analysis showed that the efficiency of the enzyme expressed as the catalytic rate over the Michaelis constant ($k_{cat}/K_M$) increased from **8.45** ± 3.04 $mM^{-1} s^{-1}$ in solution to **32.23** ± 3.20 or **24.46** ± 4.41 $mM^{-1} s^{-1}$ when the enzyme was immobilised onto polypropylene or plasma activated polypropylene respectively. Maleic anhydride plasma activated polypropylene showed potential to provide a more robust challenge for washing processes as it retained significantly higher amounts of tAK enzyme than polypropylene in simple washing experiments. Inhibition of the coupled enzyme (luciferase/luciferin) system used for the detection of adenylate kinase activity, was observed for a secondary product of the reaction. This needs to be taken into consideration when using the assay to estimate cleaning efficacy.



## 1. Introduction

Efficient modelling of protein attachment to surfaces has generated wide interest within the scientific community over the years, largely owed to the significant number of implications this phenomenon is responsible for. Perhaps one of the most clinically relevant implications is the residual protein contamination of surgical equipment post cleaning and disinfection. Equipment such as endoscopes and other reusable tools are subject to vigorous disinfection cycles within the clinic, however there are certain biological species which are resistant to such cleaning processes. An example of which are prions, protease resistant, transmissible glycoproteins responsible for a range of transmissible spongiform encephalopathies including Creutzfeldt-Jacob disease (CJD) [1]. Therefore evaluation of proteinaceous surface contamination has become paramount in preventing the spread of disease within the healthcare setting. This has led to specific review and revision of processes for cleaning surgical instruments in the health service in England [2]. A current clinical model aimed at quantifying residual protein presence post disinfection utilises a thermostable adenylate kinase (tAK) enzyme, so used due to the resilient nature of the enzyme drawing potential comparison to equally resilient species such as prions.

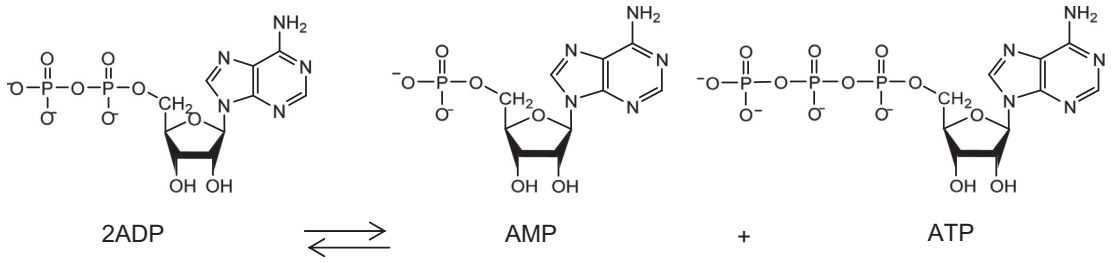
The enzyme adenylate kinase (AK) is a phosphotransferase enzyme which catalyses the reversible formation of adenosine triphosphate (ATP) and adenosine monophosphate (AMP) from adenosine diphosphate (ADP), as shown in Scheme 1.

The isolation and purification of a thermostable AK (tAK) from the archaebacterium *Sulfolobus acidocaldarius* in 1993 [3], paved the way for the significant development of proteinaceous surface contamination detection within the healthcare setting. The archaebacterium from which tAK is isolated is an extremophile found growing in volcanic springs in temperatures between 75 and 80 °C. The corresponding tAK isolated from this thermophile demonstrates a temperature optimum of around 90 °C and is stable to very low pH [4]. The novelty of tAK lies with its ability to withstand extreme conditions, tAK is currently in use in the clinical setting (WASHtAK [5]) as a model for highly surface adherent species commonly associated with residual surface contamination. The technology behind this device is detailed in a previous paper [6], which highlights that protein removal is modelled by tAK as an indicator of cleaning efficiency. Briefly,

*Abbreviations:* AK, Adenylate Kinase; tAK, Thermostable Adenylate Kinase; ATP, Adenosine Triphosphate; ADP, Adenosine Diphosphate; AMP, Adenosine Monophosphate; CJD, Creutzfeldt –Jacob Disease; RLU, Relative Light Units

* Corresponding author. Tel.: +44 1225 386118.
*E-mail address:* a.t.a.jenkins@bath.ac.uk (A.T.A. Jenkins).

http://dx.doi.org/10.1016/j.bbrep.2015.03.011




**Scheme 1.** tAK catalysed ATP formation.

in this system tAK is physisorbed to a polypropylene strip and is placed into a surgical equipment washer to undergo the standard disinfection cycle that reusable surgical equipment is subject to. Post wash cycle the polypropylene strip is removed and the residual tAK is exposed to its substrate (ADP) which is then converted to ATP. Subsequently the ATP concentration is measured via a well-known coupled enzymatic reaction involving a standard preparation of luciferase and its substrate luciferin. This assay has been extensively optimised over the years in order to accurately determine ATP concentration as a function of light output [7]. Ultimately the system relies on the excitation of luciferin to a higher energy level using ATP, the decay from this excited state to the ground state then leads to the generation of light in the form of bioluminescence. The full mechanism of bioluminescence emission from the luciferase/luciferin assay has been published elsewhere [8].

Previous studies on AK isolated from *Sulfolobus acidocaldarius* (in conjunction with the luciferase method of quantification) have been largely studied at high temperatures close to the optimum. One such study conducted at 70 °C [3] reported a $K_M$ value of 0.7 mM with ADP as the substrate in the presence of 5 mM MgCl. The study also reported a $k_{cat}/K_M$ (catalytic capacity) value of $2.8 \times 10^5\ M^{-1}\ s^{-1}$ based on a calculated theoretical $V_{max}$ value. The kinetic properties of this specific AK at 25 °C, which would be the case were it used for daily monitoring of cleaning efficacy, are absent in the literature both in solution and bound to surfaces. As such there is no direct comparison or evaluation of the effect on activity when the enzyme is restricted to a solid support, such as it is in its current clinical application.

There are a number of ways in which proteins can bind to surfaces, both the prevention and promotion of such a phenomenon has been vastly studied highlighting the effects of protein conformation/orientation, hydrophobicity/hydrophilicity, electrostatic nature of the support, binding temperature/pH and the structural stability of the protein in question [9,10]. The prevention of protein attachment to surfaces is of particular importance when considering the anti-fouling properties of a range of materials including, but not limited to biosensors, surgical equipment/medical devices, food containment and industrial equipment, the significance of which has been reviewed elsewhere [11].

Plasma activated materials have generated considerable interest in terms of functionalising solid supports to promote protein attachment to surfaces, and this may be useful if one wishes to accurately model the removal of tightly adhered proteins, including prions, The highly energised state of plasma has the potential to modify the outermost layer of a polymer surface by deposition of various functional groups. The successfully deposited functional groups (which can vary from amines to carboxyl groups depending on the monomer used) can then go on to react with a variety of species which would otherwise remain unreactive towards the inert polymer support. Not only does plasma deposition provide a predominantly solvent free alternative to wet chemistry, which in turn reduces the amount of chemical waste, it is also a largely 'controllable' process, meaning surface modification can be achieved homogeneously with the final composition tailored towards the intended application [12]. Numerous biomolecules have been successfully anchored onto polymer supports following plasma activation of the surface, many of which have been reviewed elsewhere [13]. Plasma activation of surfaces to improve coating has been used on a large scale in the automotive industry, and this suggests that such processes should be scaleable if required for high volume applications.

One of the main concerns when considering the possibility of utilising surface bound proteins is the apparent loss of activity when restricting biologically active species to a solid support. Random attachment (disregarding the orientation of the protein) can often result in the modification of critical resides within the protein structure which can directly affect the overall stability of the complex. This in turn can have repercussions in terms of accessibility of the protein in question, often as a result of steric hindrance affecting access to particular biologically active sites within the protein structure. One must also consider the impact of utilising potentially charged functional groups (such as those commonly found in lysine and aspartic acid residues), the masking or complete removal of such a charge can have detrimental effects in terms of protein stability [14]. An advantage of using a thermostable adenylate kinase which is largely resistant to extreme pH is the resilient nature of the protein itself, tAK is essentially a stable protein reducing its capacity to denature when anchored to a solid support, thus increasing the ways in which immobilisation can be carried out.

To date there have been no studies carried out evaluating the kinetic behaviour of tAK and the subsequent coupled luciferase system when considering surface binding. This report aims to detail the catalytic activity and surface binding properties of tAK when immobilised onto a solid support with the intention of potentially providing a more robust challenge for use in cleaning process validation and modelling.

## 2. Materials and methods

### 2.1. Materials

tAK was obtained from the Technology Development Group, Public Heath England (PHE – Salisbury UK). Its isolation, expression in *Escherichia coli* and purification has been detailed previously [6,15]. Mucin from porcine stomach was purchased from Sigma-Aldrich (Poole, Dorset, UK). ATP Reagent (luciferase/luciferin/divalent metal ions and stabilisers), Diluent C (reconstitution solution for ATP Reagent), tris-EDTA buffer, ADP and ATP standard solutions were all purchased from BioThema (Sweden).

AMP, ethanol, maleic anhydride and preformed phosphate buffered saline (PBS) tablets (pH 7.4) were purchased from Sigma-Aldrich (Poole, Dorset, UK).

### 2.2. Methods

#### 2.2.1. Generation of an ATP standard curve

ATP standard solutions were prepared via serial dilution from stock (0.01 mM) to give a range of concentrations from $1 \times 10^{-3}$ mM to $1 \times 10^{-8}$ mM, diluted in tris-EDTA buffer. 100 μl of ATP standard was added to each well of a white 96 well polystyrene microtitre plate. Luminescence output was recorded for 3 s using the bioluminescence module on a BMG Labtech Spectrastar plate reader. ATP Reagent (standard preparation of luciferase, luciferin, magnesium ions and stabilisers) was prepared via reconstitution with Diluent C at room temperature, 30 μl was injected after 3 s using the inbuilt injector system. Luminescence output was recorded for a further 27 s at 25 °C. 5 replicates were performed for each ATP concentration.

#### 2.2.2. tAK in solution

ADP standard solutions were prepared via serial dilution from stock (0.2 mM) to give a range of concentrations from 0.1 mM to 0.0016 mM, diluted in tris-EDTA buffer. A 0.8 ng/μl tAK standard solution was serial diluted from stock (2.32 μg/μl) using a solvent system consisting of 80% water, 20% ethanol and 0.1% hog mucin to give a range of tAK concentrations – 0.4, 0.2, 0.1 ng/μl [15,16]. 100 μl of ADP standard was added to each well of a white 96 well polystyrene microtitre plate. ATP Reagent was prepared as before and a 2 component injection system was used; 10 μl tAK and 30 μl ATP Reagent were mixed and luminescence output was monitored for 5 s prior to injection and continued for a further 55 s at 25 °C post injection. This was performed in triplicate for each enzyme concentration.

#### 2.2.3. Adsorption of tAK to polypropylene surface

A 0.8 ng/μl tAK standard solution was serial diluted from stock as before to give the same range of tAK concentrations. 50 μl of tAK was added to each well of a white 96 well polypropylene microtitre plate. This was allowed to dry down at 37 °C for 2 h with agitation. 30 μl ATP Reagent was then added to each well. ADP standard solutions were prepared via serial dilution from stock to give the same range of concentrations diluted in tris-EDTA buffer. Luminescence output was recorded for 5 s prior to the injection of 100 μl ADP and continued for a further 55 s post injection at 25 °C. This was repeated in triplicate for each concentration of enzyme with each concentration of ADP.

#### 2.2.4. Plasma activation of polypropylene surface

Plasma deposition was carried out using a plasma reactor unique to the research group as previously detailed [17]. White 96 well polypropylene microtitre plates were placed inside the plasma reactor chamber; 0.1 g maleic anhydride was added to the Young's flask, before being reduced to $10^{-3}$ mbar pressure. The flask then underwent 3 freeze thaw cycles using liquid nitrogen in order to remove any water. The plasma was ignited and pulsed at a duty cycle of 1 s (on)/40 ms (off) with a peak power of 40 W input for 10 min. tAK was added to the plasma deposited maleic anhydride microtitre plates immediately after deposition (at 37 °C with agitation for 2 h). Successful deposition was confirmed with Fourier-Transform Infrared Spectroscopy (FT-IR) – Supplementary information. A peak at 1788 $cm^{-1}$ represents the carbonyl stretching frequency of the newly deposited anhydride moieties. It is likely that the primary amines found in a number of amino acid residues within tAK can go on to react with the anhydride ring retained within the deposited maleic anhydride present on the modified polymer surface [17]. A potential mechanism for the reaction of tAK with plasma deposited maleic anhydride is shown in Fig. 1.

#### 2.2.5. Luciferase inhibition

Inhibition experiments were undertaken using AMP and luciferase. A concentration of $1 \times 10^{-5}$ mM ATP was chosen based on previous experiments as this concentration of ATP produces light output in the mid-range of the experimental setup. The AMP concentration was varied from 1 mM to $1 \times 10^{-4}$ mM. 100 μl of each ATP/AMP solution was added to each well of a white 96 well polypropylene microtitre plate, luminescence output was monitored for 5 s prior to the injection of 30 μl of ATP Reagent. The reaction was further monitored for 55 s at 25 °C, and again performed in triplicate.

#### 2.2.6. Washing experiments

Washing experiments were performed after immobilisation of tAK by pipetting 100 μl of either water or PBS buffer into each well followed by manual swirling of the plate before removal via pipette. This was repeated 3 times for each well.

## 3. Results and discussion

### 3.1. Standard curve

A standard curve was produced in order to quantify the initial rate of reaction of subsequent tAK experiments to ATP concentration for use in kinetic parameter calculations. The standard curve is shown in Fig. 2 demonstrating a linear correlation between light output and ATP concentration. From this graph it was possible to relate ATP concentration to the rate of light production when considering the enzyme catalysed formation of ATP.

### 3.2. The kinetic properties of the tAK enzyme as evaluated following physical adsorption onto polypropylene.

Different concentrations of tAK were immobilised under standard conditions (80% water, 20% ethanol, 0.1% mucin, reacted for 2 h at 37 °C with agitation) Data is shown as relative light units (Fig. 3). The initial rate of reaction was calculated between 5 and 15 s by means of tangents and plotted as a function of ADP concentration, as shown in Fig. 4.

A curious observation is the apparent drop in initial rate at a concentration of 0.05 mM ADP when using the highest concentration of tAK (40 ng). A possible explanation for this could be product inhibition, which is outcompeted at high substrate concentrations. This trend was not seen when lowering the concentration of tAK. The curves were fitted to the Hill Eq. (1), using a non-linear curve fit function generated within Origin graphing software, the fitting values for Fig. 4B are shown in Table 1. The Hill equation is a slight modification of the traditional Michaelis–Menten equation, allowing for a certain degree of cooperativity (represented by the $n$ value) in a multi-substrate reaction. tAK binds two molecules of ADP in a random *bi bi* sequential fashion [18], meaning there is an increased likelihood of cooperativity. This phenomenon is also predicted due to the sigmoidal nature of curve.

$$v = \frac{V_{max}[S]^n}{K + [S]^n} \quad (1)$$

The Michaelis–Menten Eq. (2) was employed in order to quantify enzyme activity, which normally accounts for simple one substrate binding kinetics, however random sequential kinetics will simplify to the Michealis–Menten model provided that $k_2 \ll k_{-1}$, which has been confirmed as being the case for AK [19], whereby the dissociation of bound nucleotides has been shown to be the rate limiting step. $V_{max}$ values were obtained via extrapolation and again produced using Origin graphing software,

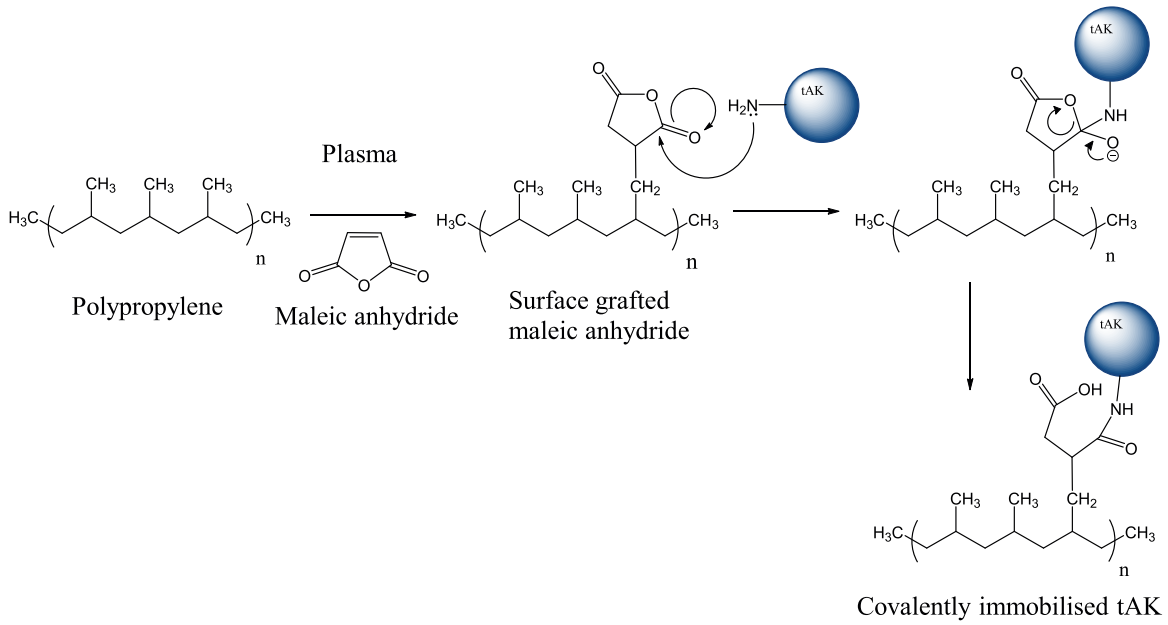



**Fig. 1.** Putative reaction scheme showing the reaction between plasma deposited maleic anhydride on polypropylene with tAK.

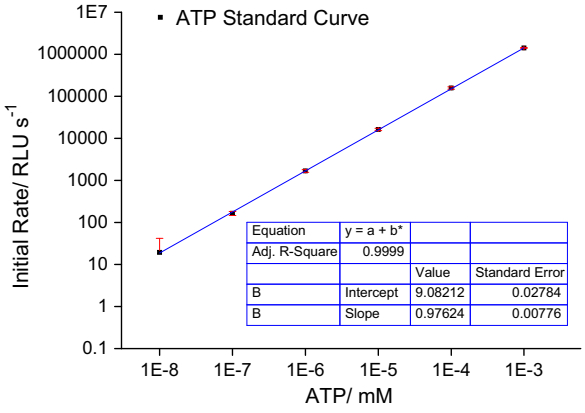



**Fig. 2.** ATP standard curve used to correlate light output to ATP concentration in tAK experiments.

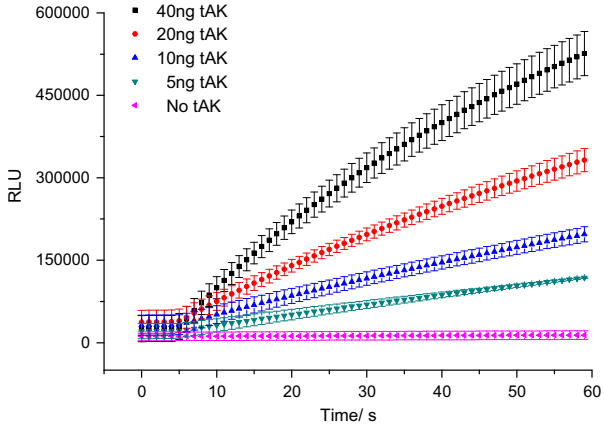



**Fig. 3.** Luminescence output (RLU) plotted as a function of tAK concentration over time. This figure shows the reaction of tAK adsorbed to polypropylene using a substrate concentration of 0.1 mM ADP. Data is shown as Relative Light Units (RLU) with error bars representing standard deviation. The data is an average of at least 3 independent repeats.

they were then compared with the standard curve in order to quantify maximum rate of reaction in terms of moles per litre per second of ATP produced. $k_{cat}$ values were then calculated based on the molecular weight of tAK (69 kDa)

$$v = \frac{V_{max}[S]}{K_M + [S]} \quad (2)$$

### 3.3. tAK bound to plasma deposited maleic anhydride on polypropylene

In an attempt to increase the binding and retention of tAK to polypropylene, maleic anhydride was coated using a plasma deposition method. Deposition was carried out under conditions previously optimised for maximum surface retention of functional groups [20]. Initial rate data for 40 ng and 5 ng tAK is shown in Fig. 5, and again the reduction in rate of reaction is present at mid-range ADP concentrations. This was mirrored for all tAK concentrations.

The decrease in rate of reaction in the mid-range of ADP concentrations presented when tAK was covalently bound to the surface of the polymer was not seen when considering tAK in solution and was only observed at high tAK concentration when adsorbed to the polymer surface. This may suggest some form of inhibition caused by the restriction of the anchored enzyme, possibly as a result of a decrease in the rate of diffusion due to obstructed access to the enzyme's active site, or as a result of steric hindrance. However the difficulty in assessing this hypothesis lies with the use of a coupled system. Whilst the apparent decrease in rate of reaction may be as a result of the anchoring of the enzyme to a surface, it may equally be due to interference from the luciferase system. It has long been known that luciferase can be inhibited by AMP [7] if the concentration of AMP reaches a concentration whereby it outcompetes ATP, a decrease in luminescence will be observed due to inhibition. Whilst this is not seen in solution when using the same concentration of reactant, it could be possible that due to a change in the rate of diffusion of nucleotides away from tAK in its anchored state (AMP being smaller and thus potentially diffusing away faster than the larger ATP molecules), the inhibition effect is enhanced.

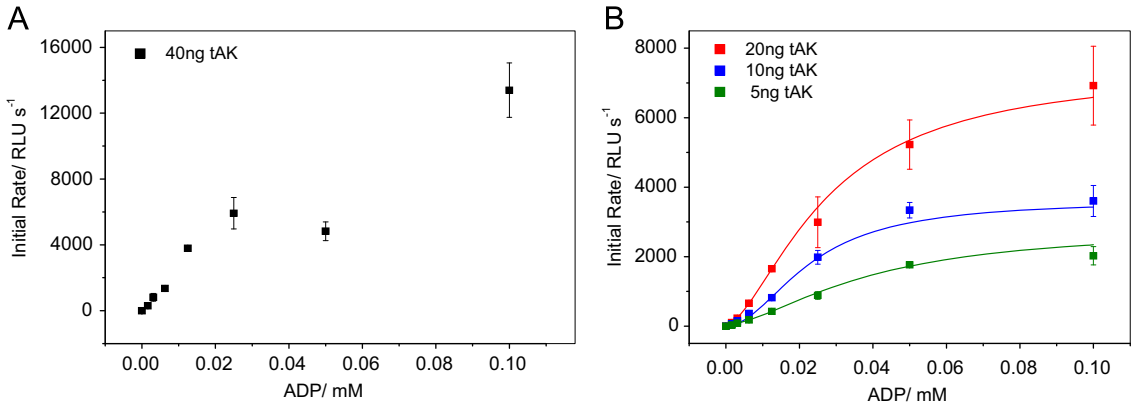



**Fig. 4.** Comparison of initial rate of reaction (RLU $s^{-1}$) when using a high tAK concentration (A) and for lower tAK concentrations (B) adsorbed to polypropylene. Tangents were fitted according to a linear fit function within Origin graphing software between 5 and 15 s.

**Table 1**
Summary of the fitting values and the standard error for 20, 10 and 5 ng of tAK from Fig. 4B according to the Hill Equation.

| tAK (ng) | Adjusted *R*-squared value | $V_{max}$ (RLU $s^{-1}$) | $K_M$ (mM) | *n* Value |
|---|---|---|---|---|
| 20 | **0.942** | **7420.79** ± 4355.85 | **0.028** ± 0.020 | **1.60** ± 0.63 |
| 10 | **0.939** | **3619.22** ± 1371.42 | **0.023** ± 0.011 | **1.97** ± 0.94 |
| 5 | **0.998** | **2024.45** ± 147.18 | **0.027** ± 0.003 | **1.73** ± 0.21 |

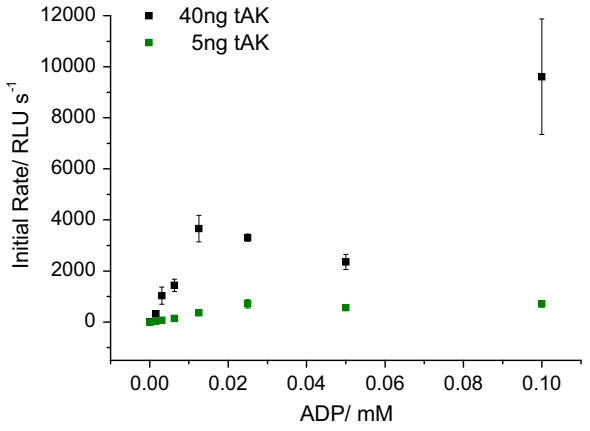



**Fig. 5.** Initial rate of reaction (RLU $s^{-1}$) plotted as a function of ADP concentration for two tAK concentrations covalently bound to maleic anhydride deposited onto polypropylene.

### 3.4. Inhibition studies

The inhibition of luciferase by various AMP concentrations was investigated in order to assess whether the coupled system can indeed be inhibited by a secondary product of the primary reaction in question. Fig. 6 shows the initial rate of light production when utilising luciferase to turn over ATP whilst increasing the concentration of AMP. From the standard curve a concentration of $1 \times 10^{-5}$ mM ATP should give an initial rate of reaction of around 16,000 RLU $s^{-1}$. Therefore it has been shown that even a very small amount of AMP can inhibit the coupled system involved in monitoring tAK activity.

To avoid this effect impacting on the measurement of tAK activity during assessment of wash processes it is important that a large excess of ADP is used to ensure that it outcompetes any AMP for binding to the enzyme. This approach has been used in previous studies looking at the use of tAK to monitor wash efficacy in different settings [5,15,16]. In addition, the reaction that

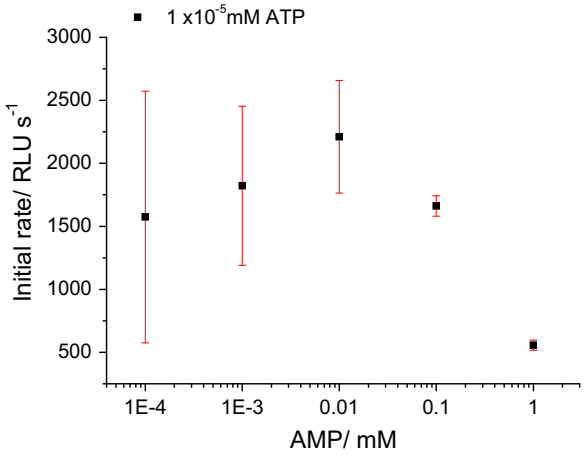



**Fig. 6.** Effect of AMP concentration on initial rate of reaction in the luciferase catalysed turnover of ATP.

luciferase catalyses can also be self-inhibiting, that is to say that two of the primary products formed in the production of light from ATP (oxyluciferin and dehydroluciferyl-adenylate) are both competitive inhibitors of luciferase [21]. Table 2 shows a summary of the calculated kinetic parameters.

The results presented here show that in terms of activity and catalytic efficiency tAK performs best when adsorbed to polypropylene. The data shows a large increase in the $k_{cat}$ with 6 and 4 fold increase for tAK immobilised to polypropylene and maleic anhydride derivatised polypropylene respectively. A small increase in the $K_M$ is also observed for the immobilised enzyme. This is consistent with an increase in the turnover number observed in other enzymes immobilised onto surfaces [22,23].

### 3.5. Removal of tAK during simple wash experiment

Simple wash experiments were conducted to evaluate whether the maleic anhydride treatment of the polypropylene altered the retention of the enzyme. For tAK absorbed onto polypropylene, even simple washing removed significant amounts of bound enzyme, between 97.4 and 97.5% of the surface bound protein was removed with water or PBS. This is consistent with previous studies using the tAK enzyme as a model for assessing washing performance. In these studies, tAK removal is biphasic with the majority of the enzyme being rapidly ( < 5 min) removed by water or cleaning formulations and subsequent protein removal being very slow ( > 2 h). The ability of the assay system to measure protein removal over a greater than 5-log dynamic range [5]

**Table 2**
Summary of calculated kinetic parameters for tAK in solution and bound to activated and non-activated surfaces. The previously highlighted deviations from the hyperbola in the initial rate data (e.g. when using 0.05 mM ADP) were omitted from the calculations.

| | $k_{cat}$ ($s^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ ($mM^{-1}\ s^{-1}$) |
|---|---|---|---|
| Solution | **0.138** ± 0.040 | **0.017** ± 0.003 | **8.45** ± 3.04 |
| Adsorbed to polypropylene | **0.855** ± 0.029 | **0.027** ± 0.003 | **32.23** ± 3.20 |
| Plasma activation of polypropylene | **0.558** ± 0.182 | **0.024** ± 0.011 | **24.46** ± 4.41 |

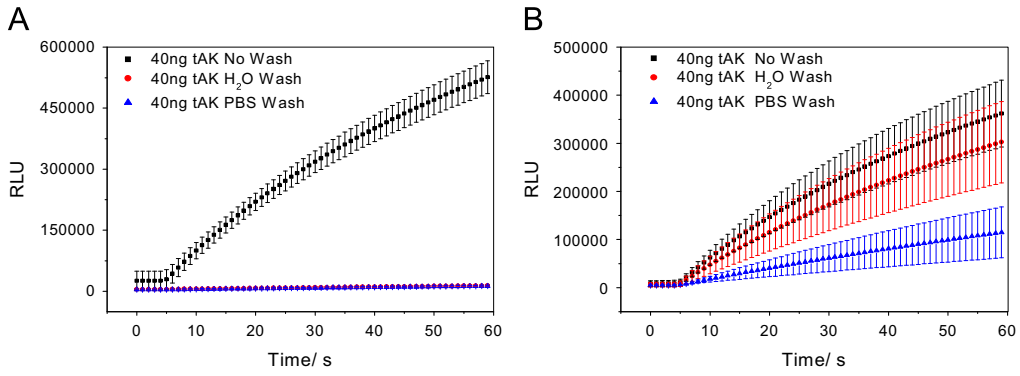



**Fig. 7.** Effect of different washing strategies on the surface removal of tAK physisorbed to a polypropylene surface (A) and covalently bound to a polypropylene surface (B).

means that it is possible to model such removal with formulations that affect the slow-phase of tAK removal. With tAK covalently bound to maleic anhydride modified polypropylene which had been plasma deposited onto the polymer support, only 16.5 and 68% of the surface bound protein was removed when washed with water and PBS respectively, thus demonstrating a higher degree of surface binding. This is shown in Fig. 7.

Enhancing the binding capacity of the surface for retention of the tAK enzyme provides a more challenging comparison for evaluating cleaning products or approaches which have improved protein removal.

## 4. Conclusions

In conclusion this study has highlighted the importance of methods for the evaluation of hospital cleaning procedures related to reusable surgical equipment. Whilst the current system in use (WASHtAK) provides a reasonable method of assessing washing efficacy, it has been shown that modification of the surface contamination model with tAK may provide a model with greater ability to differentiate between effective cleaning and decontamination processes such as those required to remove exceptionally adherent molecules such as prions. There may be scope to develop this system based on optimising the immobilisation of tAK through the use of plasma activation of surfaces, or by improving the detection reagents in order to remove any inhibition of signal output.

The use of maleic anhydride plasma modification of polypropylene to improve the binding of proteins and to reduce desorption form materials, may have additional value in the study of biofilm communities which adhere to surfaces and for a variety of biotechnology processes that depend on immobilised enzymes for biocatalysis or other applications.

## Acknowledgements

The authors wish to acknowledge the BBSRC and Public Health England for funding an iCASE studentship.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.03.011.

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