Electronic Supplementary Material (ESI) for RSC Advances.

Supplemental Information

Exploring the effect of a peptide additive on struvite formation and morphology: a high-throughput method

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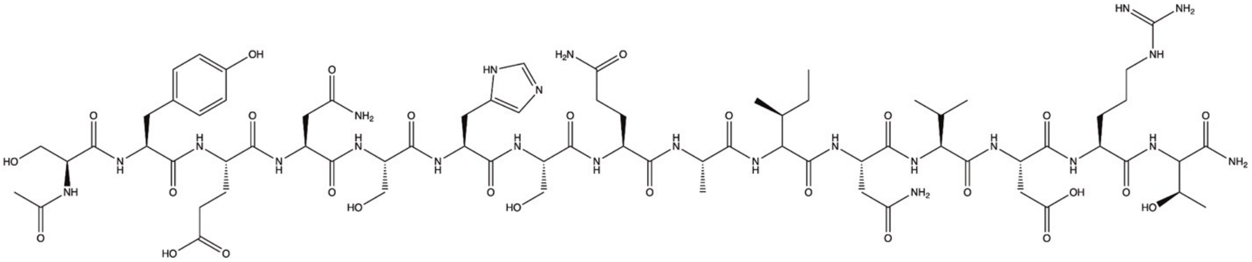


Figure S1. The primary structure of the shADP5 peptide used in this study. The N-terminus was acylated and the C-terminus was amidated to increase stability in solution. The one letter amino acid abbreviation is: Ac-SYENSHSQAINVDRT-Am.

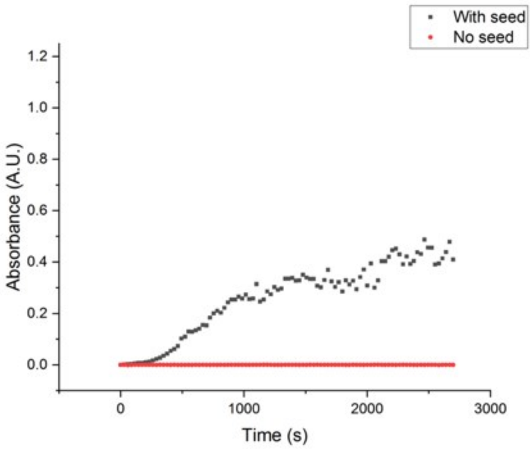


Figure S2. The effect of seeding (shown in black) on the precipitation of struvite. When no seed was present (shown in red), the absorbance did not increase in the time scale of the experiment nor were crystals visually present at the end of the run.

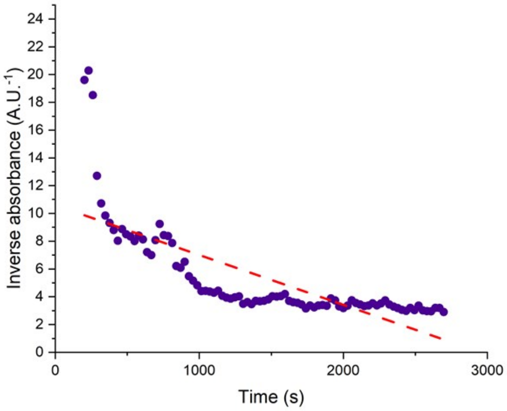
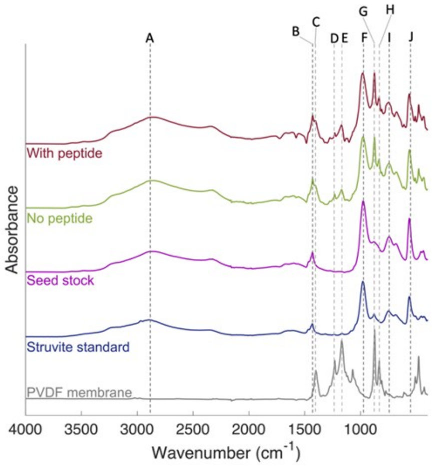


Figure S3. A representative second order fit of kinetic data. The purple circles represent the raw, transformed data and the red dashed line is the fit according to **Equation S1**.

Equation S1. $\frac{1}{A} = -k_2 t + \frac{1}{A_0}$

Where A=absorbance, k_2 is the second order rate constant, t is time, and A_0 is the initial absorbance.



Peak label	Peak location (cm^{-1})	Peak assignment
A	2800	O-H/N-H stretching[1]
B	1450	$\nu_4(NH_4)$ peak[1]
C	1402	C-H stretching[2]
D	1275	C-F symmetrical stretching[2]
E	1178	C-F asymmetrical stretching[2]
F	1000	$\nu_3(PO_4)$ peak[1]
G	870	CF_2 peak[3]
H	840	CH_2 peak[3]
I	800	H_2O vibrational band[1]
J	570	PO_4 bending[1]

Figure S4. Complete FTIR assignments including PVDF membrane peaks.[1-3] Peaks C, D, E, G, and H are from the PVDF membrane used as a backing during spectra collection.

Table S1. Results of the ANOVA on the linear fit in **Figure 1a**.

Source	**DF**	**SS**	**MS**	**F**	**P**
Regression	1	3.10036	3.10036	177.81	0.000
Error	31	0.54053	0.01744		
Total	32	3.64089			

Table S2. Representative first order R^2 values obtained from fitting **Equation 1** and representative R^2 values obtained from fitting **Equation S1**. These values were obtained with 57 μM peptide added and varying amount of seed addition.

Treatment	First order R^2	Second order R^2
5 μL seed	0.939	0.685
	0.951	0.567
	0.953	0.408
	0.746	0.240
	0.865	0.632
	0.550	0.179
27.9 μL seed	0.843	0.576
	0.955	0.495
	0.929	0.633
	0.923	0.521
	0.934	0.374
	0.872	0.313

Table S3. Results of ANOVA from **Figures 2** and **4**.

Source	DF numerator	DF denominator	F-value	P-value
Figure 2a	3	28.8	49.3	<0.001
Figure 2b	3	36.9	9.6	<0.001
Figure 2c	3	37.4	9.2	<0.001
Figure 4a	5	45.8	37.9	<0.001
Figure 4b	5	53.6	8.2	<0.001
Figure 4c	5	53.7	6.8	<0.001

Table S4. Statistical groupings determined at 95% confidence by the Games-Howell *post hoc* test.

Data	Factor	N	Mean	Grouping
Figure 2a (yield)	113 µM peptide	17	0.43	A
	57 µM peptide	13	0.36	A
	28 µM peptide	27	0.33	A
	No peptide	57	0.16	B
Figure 2b (formation constant)	No peptide	57	0.0046	A
	113 µM peptide	17	0.0024	B
	57 µM peptide	12	0.0023	B
	28 µM peptide	27	0.0022	B
Figure 2c (induction time)	113 µM peptide	16	490	A
	No peptide	56	170	B
	28 µM peptide	27	130	BC
	57 µM peptide	12	56	C
Figure 4a (yield)	27.9 µL seed, with peptide	17	0.38	A
	12.9 µL seed, with peptide	27	0.33	A
	27.9 µL seed, no peptide	18	0.24	B
	5 µL seed, with peptide	16	0.23	B
	5 µL seed, no peptide	17	0.20	BC
	12.9 µL seed, no peptide	46	0.16	C
Figure 4b (formation constant)	12.9 µL seed, no peptide	46	0.0042	A
	27.9 µL seed, no peptide	18	0.0033	AB
	12.9 µL seed, with peptide	27	0.0022	B
	5 µL seed, with peptide	17	0.0019	B
	27.9 µL seed, with peptide	16	0.0019	B
	5 µL seed, no peptide	17	0.0013	B
Figure 4c (induction time)	12.9 µL seed, no peptide	45	190	A
	5 µL seed, with peptide	17	190	A
	12.9 µL seed, with peptide	27	130	AB
	27.9 µL seed, with peptide	17	110	AB
	5 µL seed, no peptide	16	65	B
	27.9 µL seed, no peptide	18	54	B

Table S5. The results of Levene's test on various data sets to determine if an equal variance assumption was justified. The null hypothesis is the variance is equal.

Data set	**Levene's test p-value**
Figure 2a	< 0.001
Figure 2b	0.005
Figure 2c	< 0.001
Figure 4a	0.001
Figure 4b	0.001
Figure 4c	0.029

Table S6. Coefficients in the regression equation for the factorial design and empirical model. The p-value found via ANOVA is used to test the null hypothesis that the coefficient is zero.

Term	Coded unit	Coefficient	P-value
Constant		0.220	<0.001
Magnesium dose	A	0.029	<0.001
Mixing time	B	0.005	0.497
Seed dose	C	0.024	0.002
pH	D	0.034	<0.001
Temperature	E	-0.055	<0.001
Magnesium dose*Mixing time	AB	-0.014	0.060
Magnesium dose*Seed dose	AC	-0.012	0.096
Magnesium dose*pH	AD	0.018	0.016
Magnesium dose*Temperature	AE	-0.025	0.001
Mixing time*Seed dose	BC	0.004	0.552
Mixing time*pH	BD	-0.007	0.360
Mixing time*Temperature	BE	-0.014	0.056
Seed dose*pH	CD	0.010	0.185
Seed dose*Temperature	CE	-0.024	0.001
pH*Temperature	DE	-0.038	<0.001

$$\begin{aligned}
&\text{Yield} = 0.220 + 0.029\mathbf{A} + 0.005\mathbf{B} + 0.024\mathbf{C} + 0.034\mathbf{D} - 0.055\mathbf{E} \\
&- 0.014\mathbf{AB} - 0.012\mathbf{AC} + 0.018\mathbf{AD} - 0.025\mathbf{AE} + 0.004\mathbf{BC} \\
&- 0.007\mathbf{BD} - 0.014\mathbf{BE} + 0.01\mathbf{CD} - 0.024\mathbf{CE} - 0.038\mathbf{DE}
\end{aligned}$$

Supporting Methods

Circular dichroism

Circular dichroism (CD) spectroscopy was used to determine the secondary structure of the peptide with and without ions present in the precipitation reaction at pH 9.2 in borate buffer. Scans were performed on a Jasco J-815 circular dichroism spectrometer at 20°C using a 0.1 cm path length, with 0.5 nm data pitch, a bandwidth of 1.0 nm, scanning speed of 10 nm min^{-1} and 3 accumulations. The baseline was borate buffer.

Quartz crystal microbalance with dissipation

Quartz crystal microbalance with dissipation (QCM-D, Q-Sense Explorer, operated by Q-Soft integrated software from Biolin Scientific) was used to investigate the mass adsorption of different salt ions to surface-bound peptide. Frequency shifts and dissipation changes were monitored simultaneously versus time, and all experiments were operated in aqueous solutions. Details about the gold-coated crystal sensors (QSX 301, 5 MHz, Biolin Scientific), module cleaning, and data analysis can be found in our previous work.[4] In general, QCM-D experiments were operated in a flow module at 18°C and at a flow rate of 150 µL/min. The frequency decrease and increase reflect mass accumulation and removal on the surface respectively, and dissipation changes show the viscoelastic characteristics of the adsorbed material. Five different solutions were studied: 7 mM NaH_2PO_4, 7 mM $MgCl_2$, 7 mM NH_4Cl, a mixture of NaH_2PO_4 and $MgCl_2$ at 3.5 mM each, and a mixture of NaH_2PO_4 and NH_4Cl at 3.5 mM each. Peptide was introduced to the system at 10 µg/mL in DI water prior to the introduction of salt solutions. Deionized (DI) water served as the baseline and the solvent for all solutions and was also utilized to rinse the loosely bound molecules between different solutions in our study. All ion solutions were adjusted to pH 9.2 prior to introduction. QCM-D data were analyzed by QSense Dfind software (Biolin scientific) and the seventh overtone (35MHz) was used in data analysis.

Isothermal titration calorimetry

A Microcal ITC 200 was used to assess the binding of phosphate to the peptide in solution. A 0.0475 mg/mL solution (500 µM) phosphate solution was titrated into 250 µL of a 0.0441 mg/mL peptide solution (25 µM) in 30 injections of 1.6 µL injections at 25°C.

Supporting Results

Both the CD (**Figure S5**) and the QCM data (**Figure S6**) show no interaction between the peptide and various ions in solution. The CD spectra show that the peptide's secondary structure is conserved in the presence of Mg^{2+}, NH_4^+, and PO_4^{3-} which suggests there is no binding occurring. The QCM data show that after the immobilized peptide is exposed to the various salt solutions, upon rinsing the frequency shift returns to baseline levels. Therefore, the ions did not strongly adsorb to the peptide layer on gold, suggesting there is no strong interactions between the peptide and ions in solution. The ions had similar behavior on a gold sensor without peptide present, implying that the frequency changes are due to nonspecific adsorption. The ITC data (**Figure S7**) suggest that phosphate, the only common ion between this study and the study the peptide originated,[5] does not interact with the peptide in solution. The heat supplied after each injection does not change in magnitude, indicating that the temperature change is due to dilution rather than a binding event.

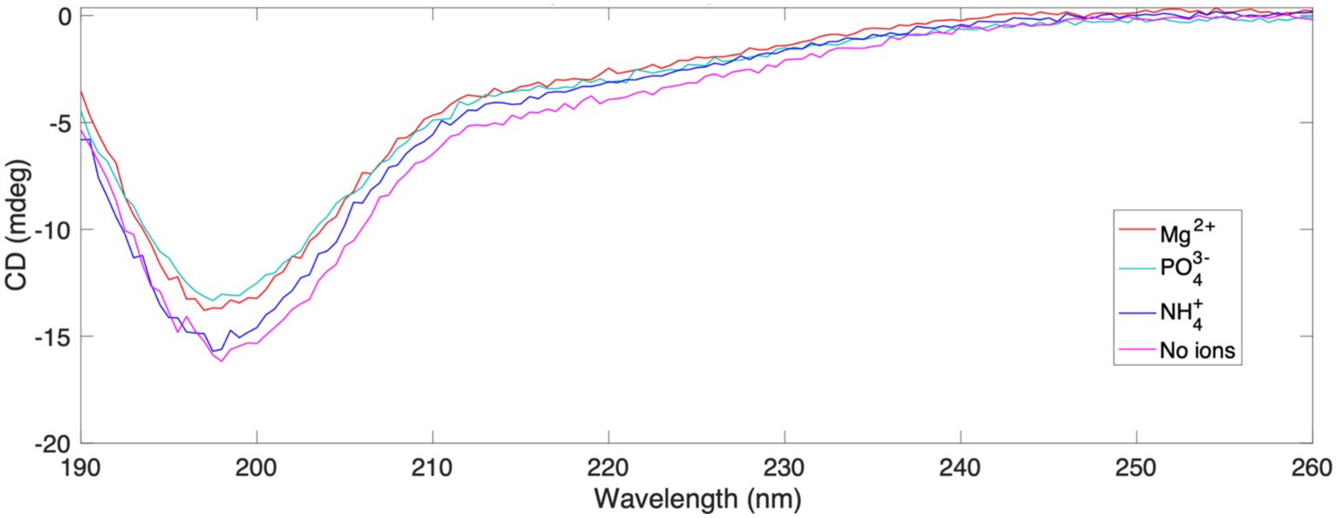


Figure S5. CD spectra of only peptide (magenta), peptide with magnesium ions (red), peptide with phosphate ions (green), and peptide with ammonium ions (blue). No changes in secondary structure are seen.

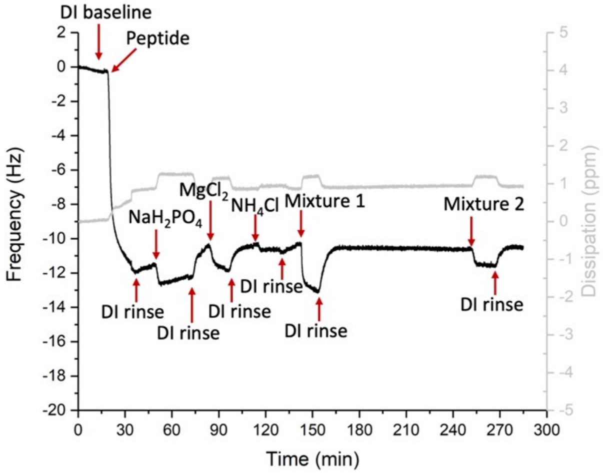


Figure S6. QCM-D experiment monitoring frequency and dissipation shifts as a function of time. Frequency is shown in black, and dissipation is shown in gray. The red arrows correspond to when the solution was changed. Mixture 1 was magnesium and phosphate ions, while mixture 2 was ammonium and phosphate ions.

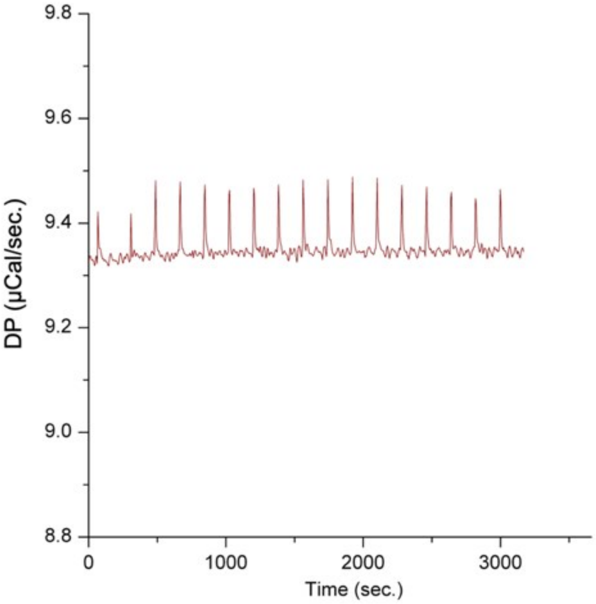


Figure S7. ITC experiment monitoring differential power (DP) as a function of time. The large spikes correspond to injections of phosphate into peptide solution.

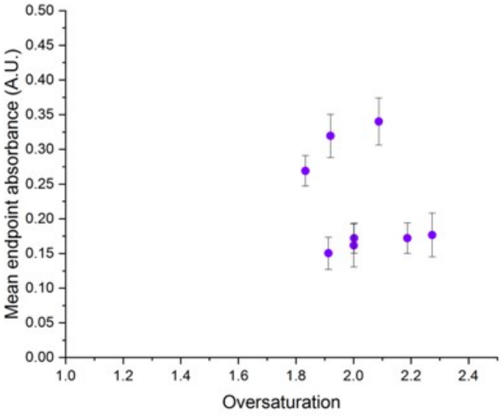


Figure S8. The effect of oversaturation on the final absorbance for the factorial design experiment. Error bars represent standard error. Oversaturations were computed using Visual MINTEQ 3.1.

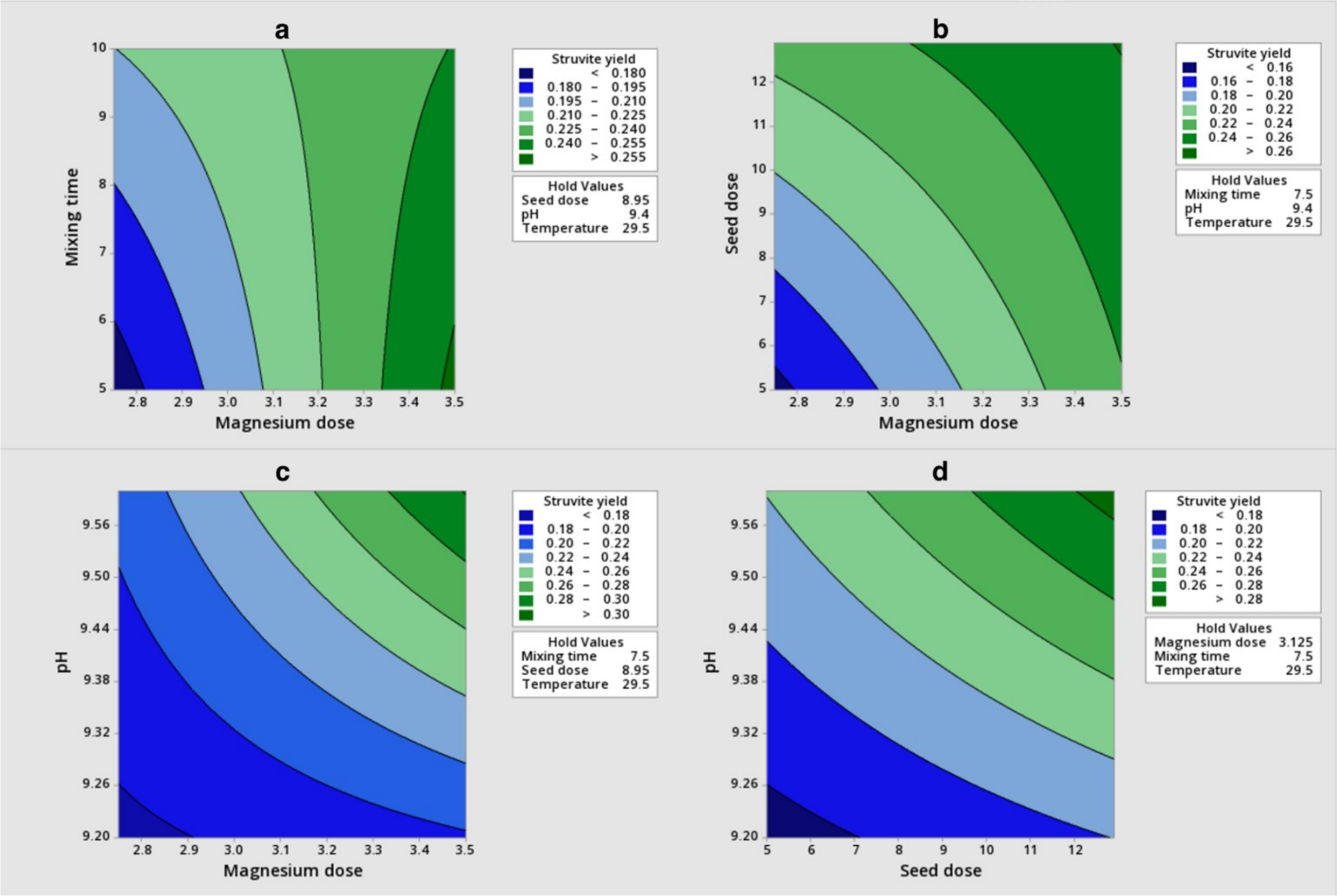


Figure S9. Contour maps generated from the factorial design experiment. (a) The result of changing mixing time (s) and magnesium dose (mM). (b) The result of changing seed dose (μL) and magnesium dose (mM). (c) The result of changing magnesium dose (mM) and pH. (d) The result of changing seed dose (μL) and pH.

Note 1. MATLAB® function used to fit the absorbance data.

```
%% An attempt to fit

% assuming your data is a matrix with the first column assumed to be time,

% and subsequent columns are absorbance at those times. Each column should

% correspond to a single well


% The output is a vector composed of (in order): yield parameter, formation

% constant, induction time, sum of squared error, R2, degrees of freedom,

% adjusted R2, and root mean square error.


function parameters=kineticslag(data,plots)

% Set plots as 1 if you want all the graphs

% Set plots as 0 if you only want the fitting parameters


%% How big is the data set

dimens=size(data);

num=dimens(2);

%% Deconstruct and fit


for i=2:num

    [fitresult,statistics]=kineticfit(data(:,1),data(:,i)-data(1,i),plots);

    if plots==1

     legend off

    end

% Fitting parameters

    A(i-1)=fitresult.a;

    B(i-1)=fitresult.b;

    C(i-1)=fitresult.c;
```

```
% Statistical parameters

    sse(i-1)=statistics.sse;

    rsquare(i-1)=statistics.rsquare;

    dfe(i-1)=statistics.dfe;

    adjrsquare(i-1)=statistics.adjrsquare;

    rmse(i-1)=statistics.rmse;

    clear fitresult statistics

end


parameters=[A; B; C; sse; rsquare; dfe; adjrsquare; rmse]';

end


function [fitresult, gof] = kineticfit(time, two, p)


%% Fit: 'Kinetic model'.


[xData, yData] = prepareCurveData( time, two );


% Set up fittype and options.

ft = fittype( 'Explag(x,a,b,c)', 'independent', 'x', 'dependent', 'y' );

opts = fitoptions( 'Method', 'NonlinearLeastSquares' );

opts.DiffMaxChange = 0.001;

opts.DiffMinChange = 1e-09;

opts.Display = 'Off';

opts.Lower = [0 1e-07 0];

opts.MaxFunEvals = 6000;

opts.MaxIter = 4000;

opts.StartPoint = [0.1 1e-05 0];
```

```
opts.TolFun = 1e-09;
opts.Upper = [1 1 Inf];

% Fit model to data.
[fitresult, gof] = fit( xData, yData, ft, opts );
if p==1
% Plot fit with data.
figure( 'Name', 'Kinetic Fit' );
plot( fitresult,'--r', xData, yData,'.k');
% Label axes
xlabel 'Time (s)'
ylabel 'Normalized Absorbance'
grid off
else
end
end
```

Note 2. MATLAB function used to describe the model used.

```
function y = Explag(x,a,b,c)
y = zeros(size(x));

for i = 1:length(x)
    % The first part is if you have residual baseline in your fit
    if x(i) < c
        y(i) = 0;
    % After the baseline
    else
        y(i) = a.*(1-exp(-(x(i)-c).*b));
    end
```

```
end

end
```

References

1. V. Stefov, B. Soptrajanov, I. Kuzmanovski, H. D. Lutz and B. Engelen, *Journal of Molecular Structure*, 2005, **752**, 60-67.
2. Z. P. Zeng, D. S. Yu, Z. M. He, J. Liu, F. X. Xiao, Y. Zhang, R. Wang, D. Bhattacharyya and T. T. Y. Tan, *Scientific Reports*, 2016, **6**, 1-11.
3. R. M. Silverstein, F. X. Webster and D. Kiemle, *Spectrometric Identification of Organic Compounds, 7th Edition*, Wiley, 2005.
4. Z. H. Su, N. Pramounmat, S. T. Watson and J. N. Renner, *Soft Matter*, 2018, **14**, 3528-3535.
5. S. Dogan, H. Fong, D. T. Yucesoy, T. Cousin, C. Gresswell, S. Dag, G. Huang and M. Sarikaya, *Acs Biomaterials Science & Engineering*, 2018, **4**, 1788-1796.