Supplementary Material

Main Article – Using a systems pharmacology model of the blood coagulation to predict the effect of various therapies on biomarkers

The supplementary material enclosed herewith contains the following:

1. Development of the Coagulation Network – This section describes the biology of the coagulation network included in our model.

2. Experimental methods and materials for TGA and aPTT assay

3. Figure S1-S3 show the full TGA profile when initial conditions (zymogens or inactive factors) are varied

4. Figure S4 shows a comparison between changes in TGA parameters when zymogens are varied for Normal plasma and 9DP (FIX deficient plasma).

5. Figure S5 shows changes in TGA parameters when active proteins are varied for Normal plasma, 8DP (FVIII deficient plasma) and 9DP.

6. Figure S6 – Changes in aPTT when active proteins are changed for Normal plasma, 8DP and 9DP.

7. Figure S7-S10: Figure S7 through S10 contain results of parameter optimization using simulated annealing when data for each of the different experimental conditions is used separately.

   In Figures S7 – S10, the 'Literature' refers to TGA profile obtained with unchanged parameters taken from literature, 'All expt' refers to the TGA profile obtained with parameters that were obtained when all the experiments were used for optimization simultaneously and 'This expt' refers to TGA profile obtained with parameters obtained by optimizing the current experimental dataset only.

   The four different experimental conditions are

   a. Addition of FVIIa in Normal Plasma (Figure S7)
   b. Addition of FVIIa in 8DP (Figure S8)
   c. Addition of FXa in Normal Plasma (Figure S9)
   d. Addition of FXa in 8DP (Figure S10)

Development of the Coagulation Network

A mechanistic model of the blood coagulation network was constructed by combining models from three different literature sources, i.e., models by Chatterjee *et al.*,[26] Hockin *et al.* [27] and Wajima *et al.* [28]. Initial estimates of the parameters for the extrinsic pathway were taken from publication by Hockin *et al.*, whereas parameters of the intrinsic pathway and common pathway were obtained from the publication by Chatterjee *et al.* The simulation scheme in the model by Wajima *et al.* was used to simulate aPTT. **Figure 1** shows the schematic of the network described in our model. The coagulation network can be roughly divided into 3 sections: intrinsic pathway, extrinsic pathway and common pathway. The extrinsic pathway is initiated by tissue factor (TF) binding to FVIIa to form the TF-FVIIa complex that catalyzes activation of FVII to FVIIa. The intrinsic pathway is initiated by contact activators (CA), which are a surrogate for anionic or hydrophilic solid surfaces which activate factor XII (FXII) [29]. Activation of FXII to factor XIIa (FXIIa) by CA is also catalyzed by Kallikrein (K), which is the active form of zymogen Pre-Kallikrein. Since FXIIa activates Pre-Kallikrein, there is positive feedback between FXIIa and K levels. FXIIa sets off the intrinsic pathway by activating factor XI (FXI) to factor XIa (FXIa), which subsequently activates FIX to FIXa. FIXa combines with activated FVIII (FVIIIa) to form the intrinsic tenase complex, FIXa-FVIIIa. The intrinsic and extrinsic pathways converge upon the common pathway, which starts with the activation of factor X (FX) to FXa by either TF-FVIIa, FVIIa and FIXa-FVIIIa complex. FXa alone can activate prothrombin (FII) to thrombin (FIIa) at a very slow rate.

Thrombin then activates coagulation factors FVII, FVIII and factor V (FV) forming positive feedback loops. Once FXa binds to activated FV (FVa) form thrombinase, it has a much increased catalytic activity and cleaves prothrombin to generate an intermediate product -- meizothrombin (mIIa), then to thrombin (factor IIa or FIIa). Thrombin, in turn, catalyzes soluble fibrinogen into insoluble fibrin monomers, which form the building blocks of a blood clot.

In order to prevent the network from producing runaway levels of thrombin in response to vascular damage or injury, negative feedback loops and specialized inhibitor proteins are present. The main negative feedback incorporated in the model acts via protein C (PC). Thrombomodulin (Tmod) binds with thrombin to form the FIIa-Tmod complex that can activate PC to active protein C (APC). APC can bind and sequester FVIIIa and FVa, eventually leading to their degradation. In addition, there are specialized inhibitors of the network including anti-thrombin III (ATIII) that bind to FXa, FIXa, mIIa, FIIa and TF-FVIIa complex. Tissue Factor Pathway Inhibitor (TFPI) is another important inhibitor that binds to free or FVIIa bound FXa and reduces the effect of TF on thrombin generation by the extrinsic pathway.

Experimental materials and methods

Thrombin Generation Assay (TGA)

Platelet poor, citrated normal human plasma was prepared from healthy donors and FVIII deficient plasma was purchased from GeorgeKing Biomedical (Overland Park, KS). Plasma derived FXa (pdFXa, Haematologic Technologies, Inc., Burlington VT) or recombinant FVIIa (rFVIIa) (NovoSeven, Novo Nordisk) was diluted in dilution buffer composed of 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 140 mM NaCl at pH 7.5. The thrombin generation reagents, PPP-Reagent LOW (resulting in a mixture of 4 μM phospholipids and 1 pM TF in the final reaction), Thrombin Calibrator and FluCa kit were obtained from Diagnostica Stago (Parsippany, NJ). The calibrated automated thrombography (CAT) system (Thrombinoscope BV, Maastricht, Netherlands) was used.

The thrombin generation assay was performed following the manufacturer's manual. Briefly, 20 µL of PPP-reagent low and 70 µL of plasma was initially mixed, followed by 10 µL rFVIIa, pdFXa or vehicle (dilution buffer). Plasma derived FXa was added to achieve final concentrations ranging from 0.03125 to 1 nM. rFVIIa was added to achieve final concentrations ranging from 125 to 4,000 nM. Finally, upon addition of 20 µL FluCa buffer containing calcium chloride and fluorogenic substrate, reactions (120 µL final volume) were immediately read on a Fluoroskan Ascent (Thermo Scientific) fluorescence plate reader. Fluorescence signal was read at 37°C at 20 second intervals for 60 minutes and thrombin

generation curves were analyzed to extract lag time, peak thrombin and endogenous thrombin potential (ETP, i.e. the area under the curve, AUC) using the Thrombinoscope software (Thrombinoscope BV version).

Activated Partial Thromboplastin Time (aPTT) Assay

Platelet poor plasmas were obtained from the same sources as in TGA assay. An aPTT clotting assay was slightly modified from manufacturer's manual (aPTT STA C.K.Prest Diagonostica Stago, Parsippany, NJ). Briefly, normal pooled plasma (50 µL) was added to 50 µL of aPTT reagent and incubated for 3 minutes at 37°C. Plasma derived FXa (pdFXa) was diluted into assay buffer (20 mM HEPES, 0.15M sodium chloride, 2 mM calcium chloride, 0.1% polyethylene glycol 8000, pH 7.4). To limit the exposure of pdFXa to plasma inhibitors, right before adding $CaCl_2$, 5 µL of the various concentration of pdFXa was added to the plasma and aPTT reagent mixture at final concentrations ranging from 0.001 to 100 nM and the reaction was immediately initiated with the addition of 50 µL 25 mM $CaCl_2$. The time to clot was measured using a STart4 Hemostasis Analyzer (Diagnostica Stago, Parsippany, NJ). It is important to note that, in both aPTT and TGA assay, phospholipid was part of the reagent to reproduce the partial function of platelets.

Figure S1-S3

Full TGA profiles are shown for Normal Human Plasma (S1), FVIII deficient plasma (S2) and FIX deficient plasma (S3), when the zymogens are varied from 0.1x to 10x their nominal values.

Figure S1 – TGA profiles on varying inactive coagulation factors in NHP

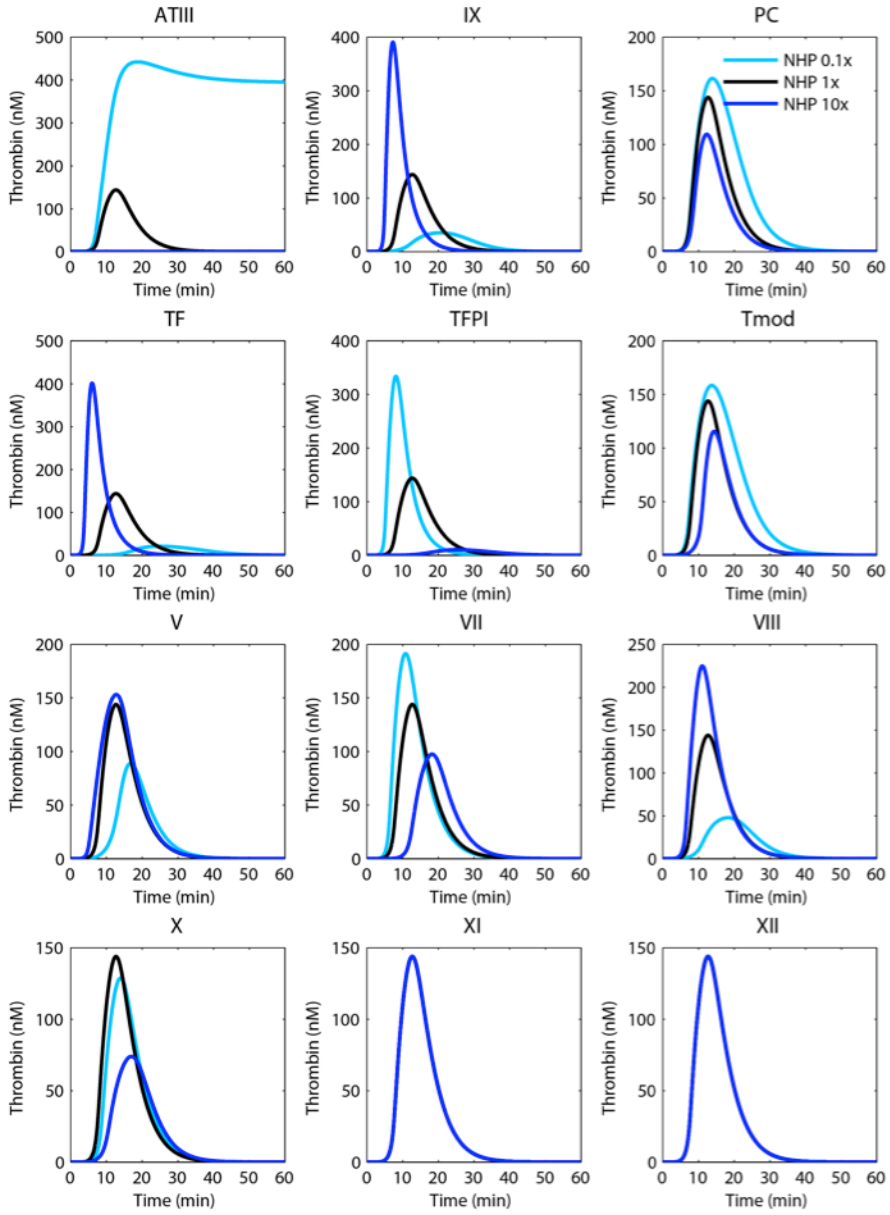


Figure S2 – TGA profiles on varying inactive coagulation factors in 8DP

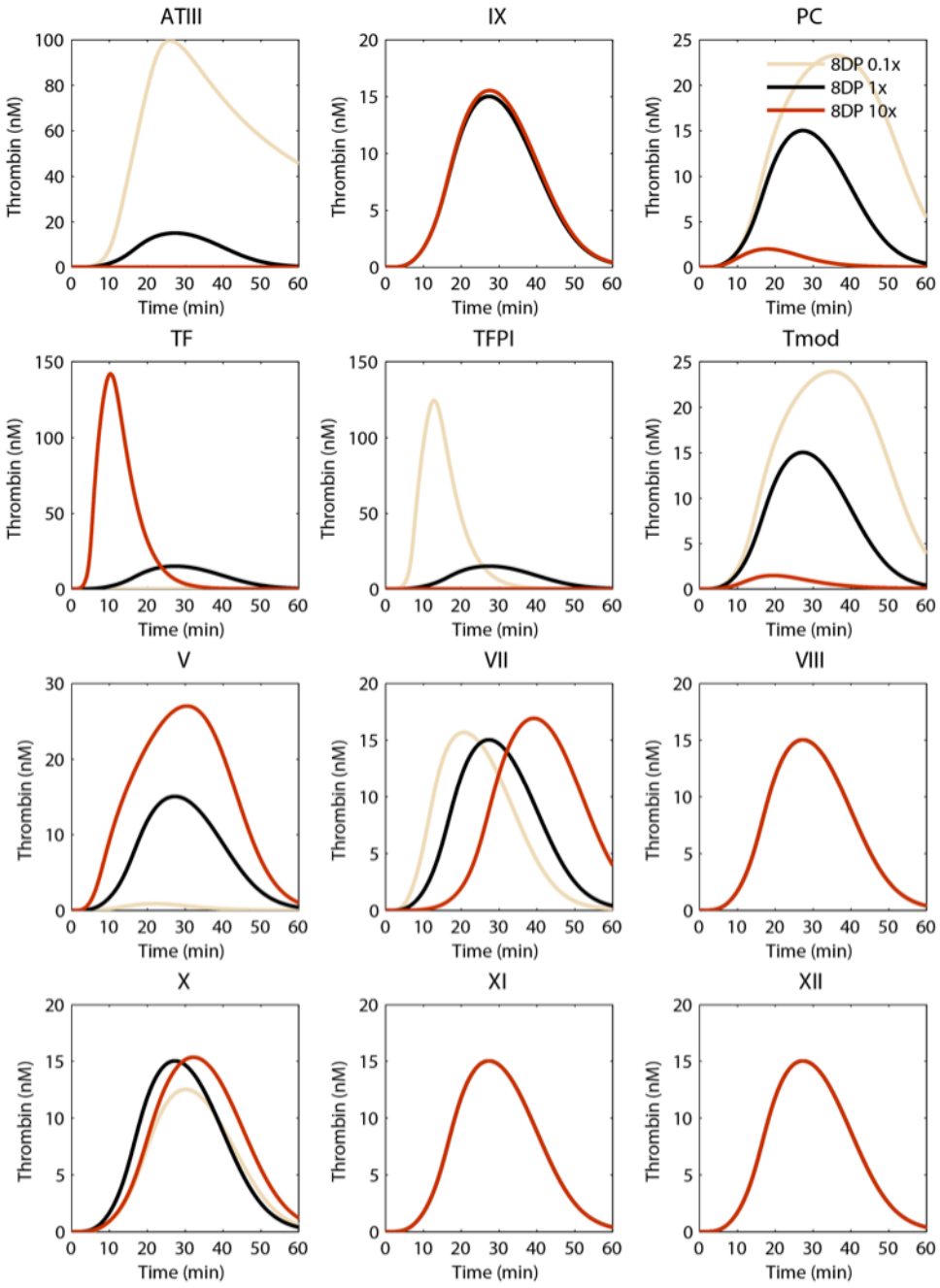


Figure S3 – TGA profiles on varying inactive coagulation factors in 9DP

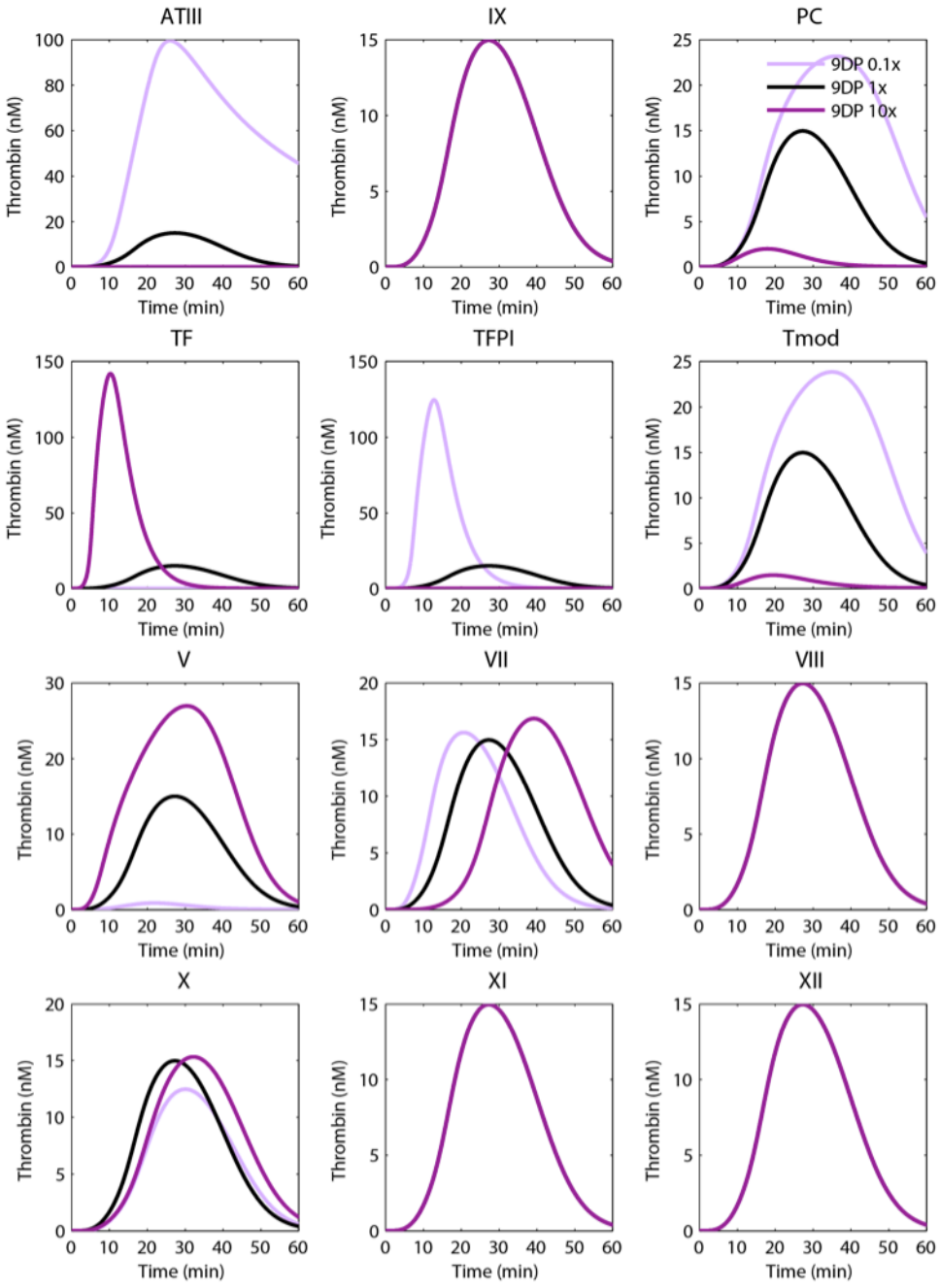


Figure S4 – Effect on TGA parameters (lag time, AUC and peak thrombin) is shown when the various zymogens are varied (10x – 0.1x) times their nominal value in Normal Plasma and FIX deficient Plasma (9DP). A comparison between figure above and Figure 4 (main text) shows that for most TGA parameters, the effect on 9DP and 8DP is same or close in magnitude.

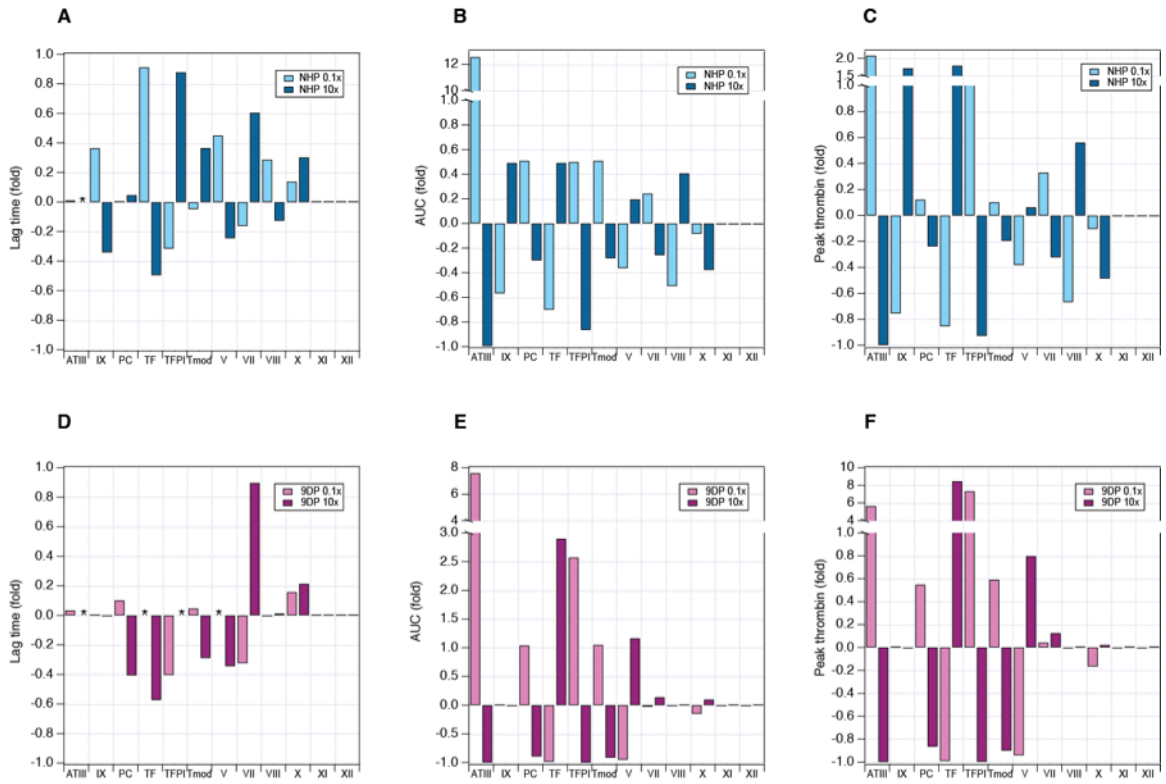
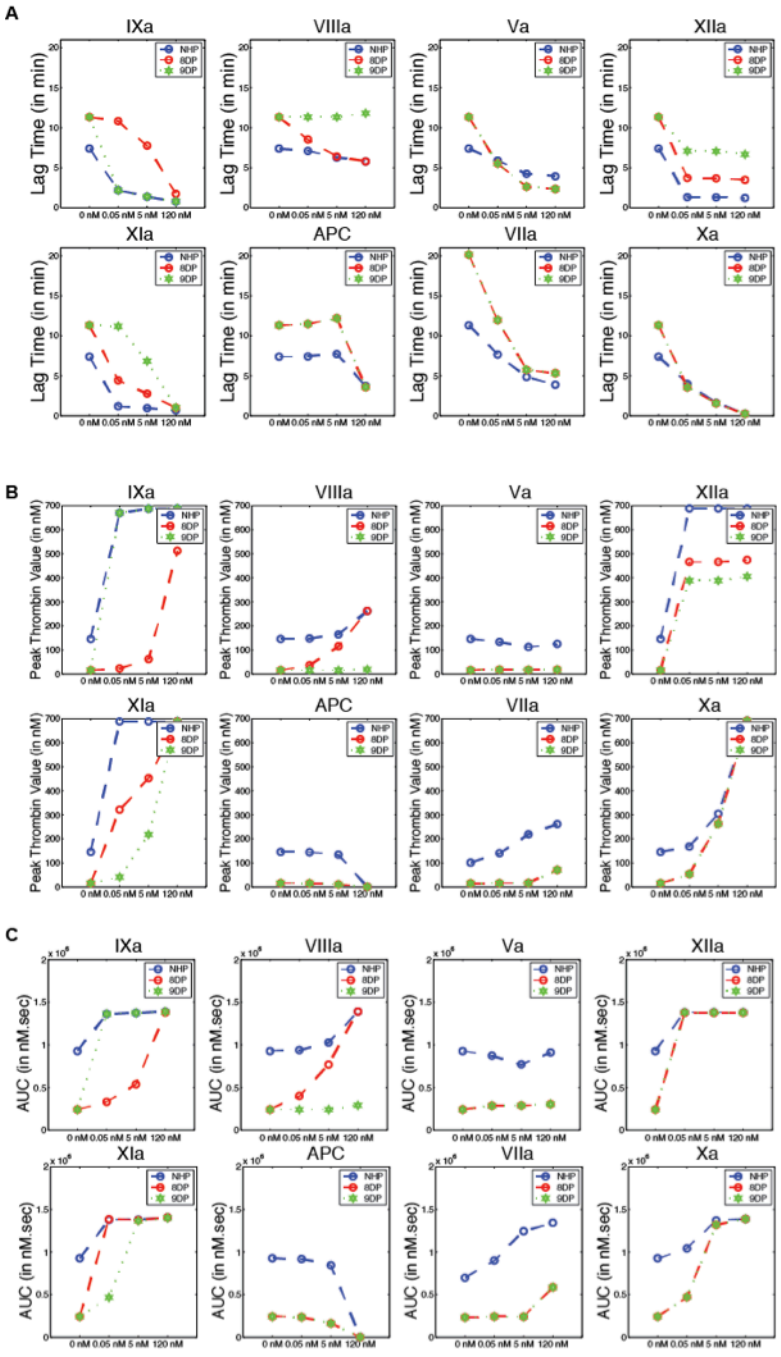


Figure S5

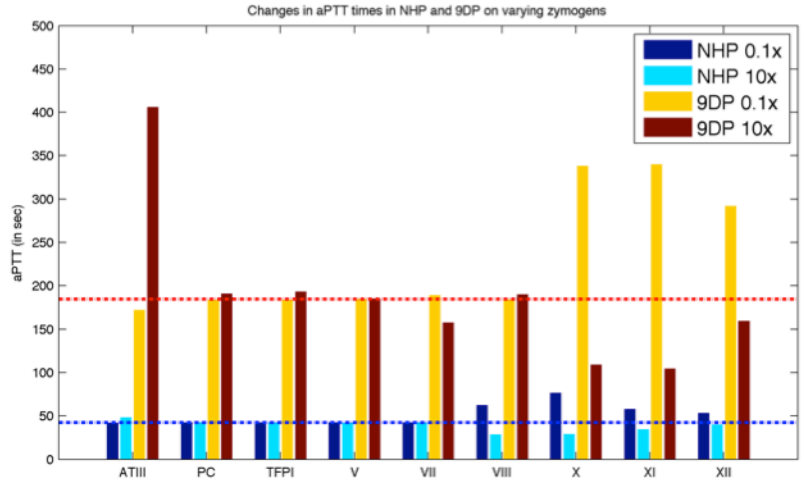
A comparison between the TGA parameters lag time, peak thrombin and AUC when the different **active** proteins in the coagulation network in NHP, 8DP and 9DP are varied is given below:



The figure above shows that the behavior of FIX deficient plasma (9DP) and FVIII deficient plasma (8DP) is similar in when FVa, APC, FVIIa and FXa are varied according to our simulations. However, key differences emerge when active

proteins in the intrinsic pathway are varied, e.g., FXIa, FXIIa, FIXa and FVIIIa. Since, FVIIIa is downstream of FIXa in the intrinsic pathway, varying FVIIIa in 9DP has no difference whatsoever in any of the TGA parameters. On the contrary, varying FIXa in 8DP has measurable effect on all the three parameters, but the effect is blunted when compared to adding FIXa in 9DP plasma.

Figure S6 – Change in aPTT when zymogens and active concentrations protein in normal plasma and 9DP are varied is shown below:



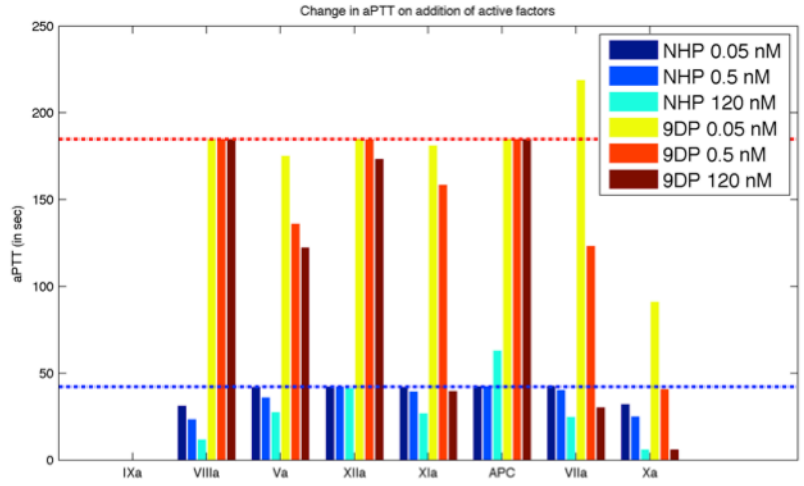


Figure S7 – Figure shows the optimization results when **only the TGA with varying levels of FVIIa experiment in Normal plasma was optimized** using Simulated Annealing. Here 'Literature' refers to TGA profile obtained with unchanged parameters taken from literature, 'All expt' refers to the TGA profile obtained with parameters that were obtained when all the experiments were used for optimization simulataneously and 'This expt' refers to TGA profile obtained with parameters obtained by optimizing the current experimental dataset only.

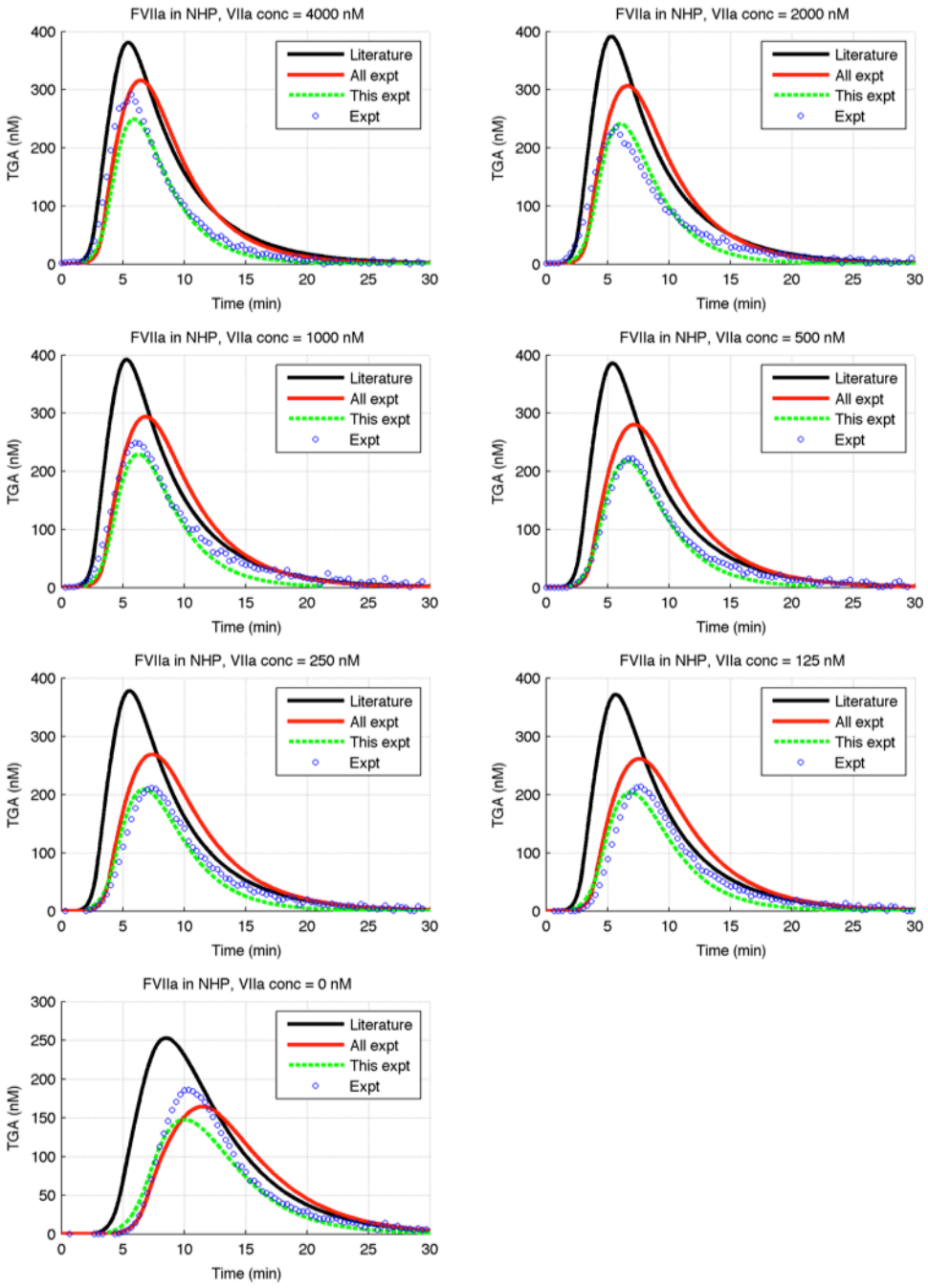


Figure S8 – Figure shows the optimization results when **only the TGA with varying levels of FVIIa experiment in FVIII deficient plasma was optimized** using Simulated Annealing. Here 'Literature' refers to TGA profile obtained with unchanged parameters taken from literature, 'All expt' refers to the TGA profile obtained with parameters that were obtained when all the experiments were used for optimization simulataneously and 'This expt' refers to TGA profile obtained with parameters obtained by optimizing the current experimental dataset only.

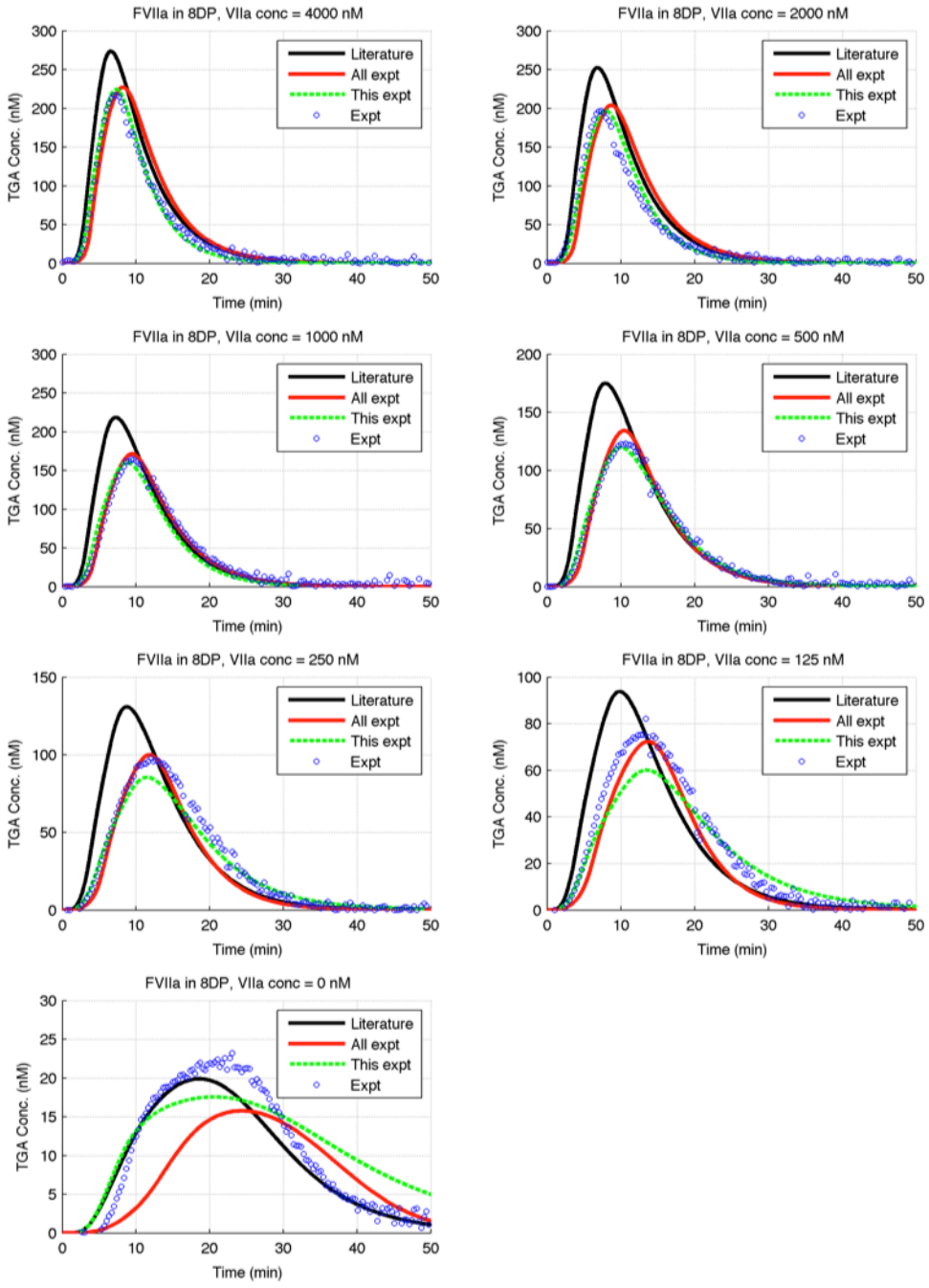


Figure S9 – Figure shows the optimization results when **only the TGA with varying levels of FXa experiment in Normal Plasma plasma was optimized** using Simulated Annealing. Here 'Literature' refers to TGA profile obtained with unchanged parameters taken from literature, 'All expt' refers to the TGA profile obtained with parameters that were obtained when all the experiments were used for optimization simulataneously and 'This expt' refers to TGA profile obtained with parameters obtained by optimizing the current experimental dataset only.

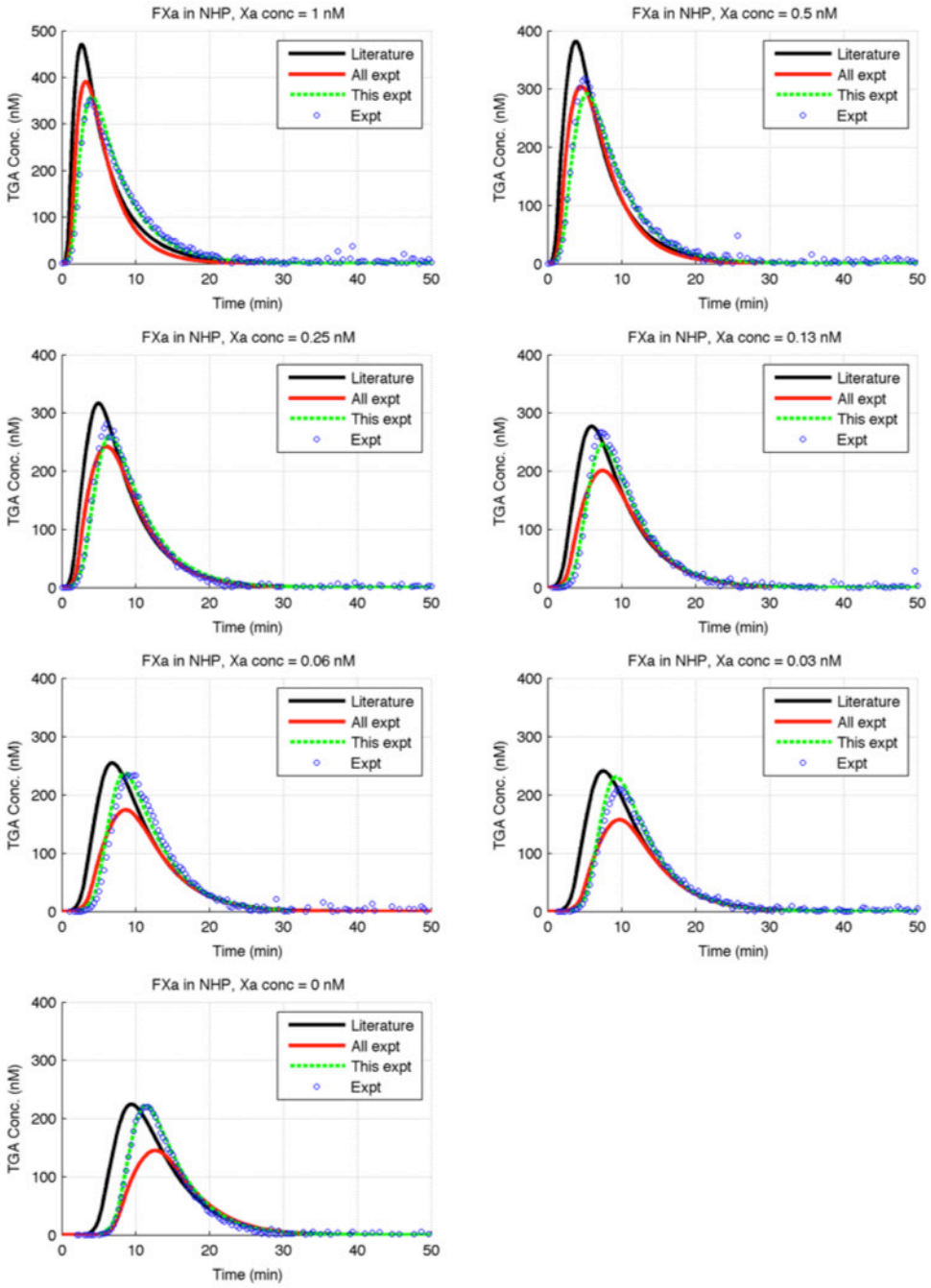
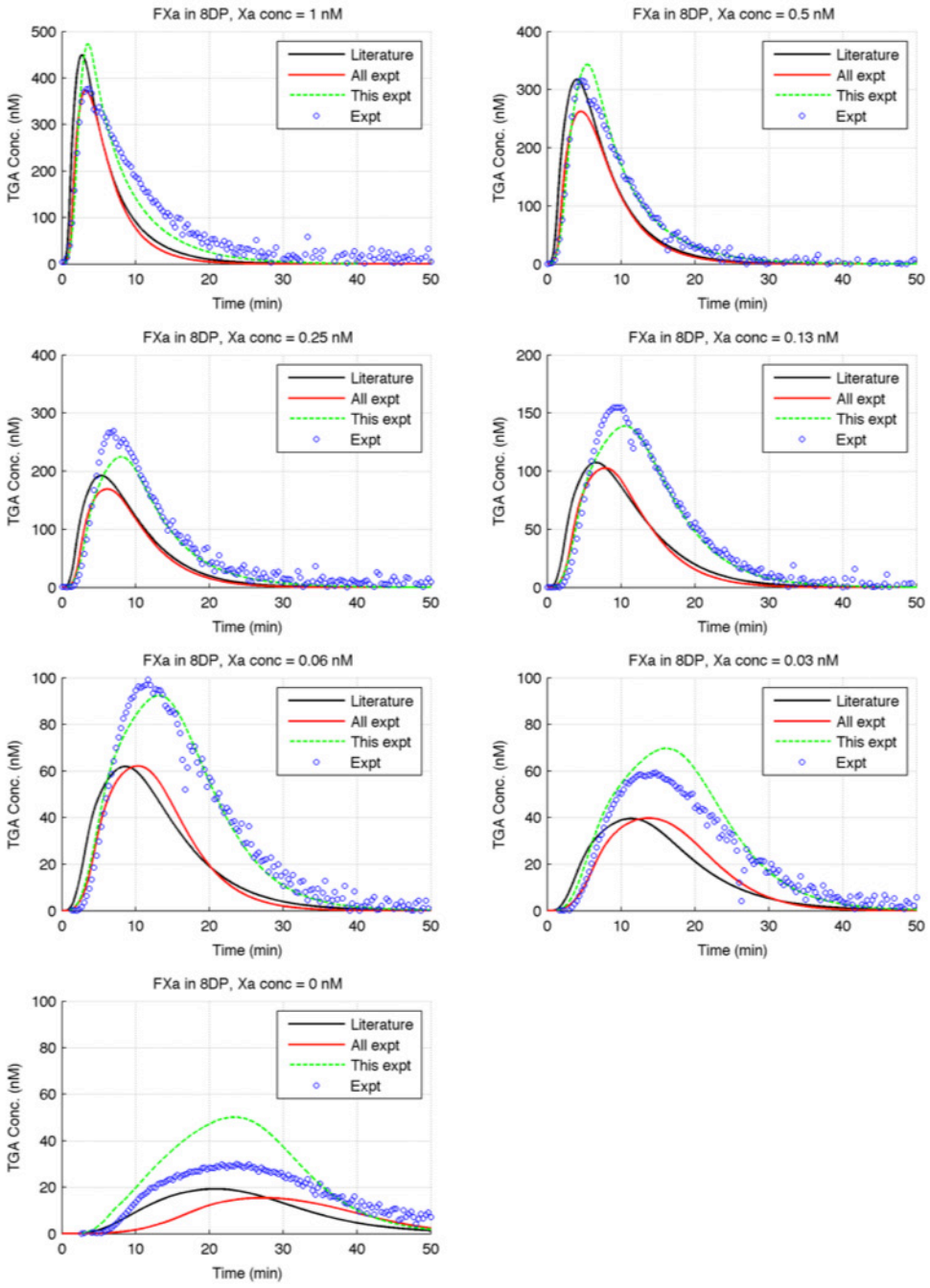


Figure S10 – Figure shows the optimization results when **only the TGA with varying levels of FXa experiment in FVIII deficient plasma was optimized** using Simulated Annealing. Here 'Literature' refers to TGA profile obtained with unchanged parameters taken from literature, 'All expt' refers to the TGA profile obtained with parameters that were obtained when all the experiments were used for optimization simulataneously and 'This expt' refers to TGA profile obtained with parameters obtained by optimizing the current experimental dataset only.



As seen from the figure S6 - S10 above, simulation results obtained by optimizing with each experimental condition separately provide better results than the parameter set which is obtained by approximating all the four experimental conditions simultaneously. This underscores the difficulty in simulating different experimental conditions simultaneously using a single parameter set and boosts the confidence in the simulation algorithm used.