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Modeling of the fibrin agarose plate assay and its application for thrombolytic analysis

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The fibrin agarose plate assay is widely used in the detection of thrombolysis efficacy. However, a rigorous mathematical model for analyzing data or comparing activities of different thrombolytics has been absent. This study investigated the relationship between thrombolysis radius, R, and diffusion time, t, of molecular medicines in an agarose hydrogel system by deriving a model based on Fick's law and experimental verification by the fibrin agarose plate assay method. The theoretical results showed that a plot of $\log(R)$ versus $\log(t)$ has a linear curve with the slope of 1/2 and this was verified by experimental results using urokinase as a modeling agent. Moreover, it was found that $\frac{R}{\sqrt{t}}$ is constant for a specific thrombolytic and can be used as a parameter for evaluating activities of different thrombolytics. The theoretical model has potential for improving the understanding of mechanisms involved in molecular medicine diffusion and offers benefits for thrombolytic therapy.

thrombosis, thrombolysis, fibrin agarose plate assay, Fick's law

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Thrombosis, such as acute myocardial infarction [1], pulmonary embolism [2], ischemic stroke [3] and peripheral arterial occlusions [4], is a severe clinical condition in which thrombus forms in a critical blood vessel [5]. Thrombolytic therapy has been a major treatment of thrombosis to date, using thrombolytics such as streptokinase (SK) [6], urokinase (UK) [7] or its mutant [8] and tissue type plasminogen activator (t-PA) [3,7] to dissolve thrombi. The assessment of thrombolytic activity, therefore, is of fundamental concern for the quality control of existing drugs and the development of new drugs.

To date, the fibrin agarose plate assay (FAPA) has been widely used to determine the fibrinolytic activity of thrombolytics, due to its good reproducibility, specificity, low cost and high sensitivity [9]. The general procedure of FAPA is to inject thrombolytic agents such as SK, UK or t-PA, into a fibrin agarose plate. The thrombolytics then directly catalyze the conversion of plasminogen into plasmin that, in turn, degrades fibrin and subsequently leads to the appearance of fibrinolytic discs in the agarose gel plate. By measuring the diameters of the fibrinolytic discs at a certain time point, activities of the tested thrombolytic agents are obtained.

However, interpretation of the data for fibrinolytic discs and activity comparisons between drugs cannot be properly conducted because of the absence of a rigorous mathematical theory or standard. Therefore, establishing a mathematical computing model is of critical importance.

In this paper, we describe a new mathematical model for the thrombolytic activity assay, based on Fick's law. The solution of the model generated theoretical predictions for the diameters of the fibrinolytic discs, which were then con-

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firmed by experiments using UK as the modeling agent. Based on these theoretical analyses, a general rule was proposed to compare the activities of different thrombolytics by the FAPA method. This study may provide a theoretical reference for thrombolytic therapy and is expected to be applicable for the evaluation of drugs in the treatment of thrombosis.

1 Theoretical analysis of the FAPA process

1.1 Physical description

Figure 1 depicts the physical model of the FAPA process. Figure 1(a) is the planform of the agarose plate. The thrombolytic agent is injected at initial concentration (C_0) into the small hole located at the center of the plate (denoted as the sample pool), where the concentration of the thrombolytic remains highest during the whole measurement process. The concentration difference in and around the sample pool will generate a driving force for the diffusion of the drug along the plate. If the diffusion channels within the plate are uniformly distributed, a net diffusion flux will be generated only along the radial direction. For every small unit volume (Figure 1(b)), the difference between the amount of drug flowing in and out must be used to increase the drug concentration within the volume. This will cause a change in concentration and a shift in the concentration gradient in the radial direction with time. The fibrinolytic disc (the shaded part in Figure 1(a) and (c)) front corresponds to the position where fibrin can be dissolved by the minimum drug concentration ($C_{\min}$) (Figure 1(a) and (c)). The distance from the disc front to the center of the sample pool is the radius (R) of the fibrinolytic disc.

1.2 Mathematical model

The concentration (C) of drug in the plate is expected to be a function of diffusion time (t) and the distance from the sample pool (x). Several assumptions are made, as follows, to establish the mathematical model:

(1) the agarose plate medium (Figure 1(a)) is homogeneous;

(2) the diffusion of drugs in plate is symmetrical;

(3) the sample pool is indefinitely small;

(4) the initial highest concentration (C_0) does not vary with t; and

(5) drug diffusion follows Fick's second law.

As depicted in Figure 1(b) (J is the diffusion flux, kg m^{-2} s^{-1}), Fick's second law of diffusion in one dimension is initially [10,11]

$$\frac{\partial C(x,t)}{\partial t} = D\left[\frac{\partial^2 C(x,t)}{\partial x^2}\right], \tag{1}$$

where D is the diffusion coefficient (m^2/s); C (x, t) is concentration (kg/m^3); t is time (s); and x is the distance (m) from the center of the disc or sample pool.

The solution of this equation is in the form (see Appendix 1 for the detailed derivation of the formula):

$$C(x,t) = f\left(\frac{x}{2\sqrt{Dt}}\right). \tag{2}$$

The initial condition for our drug diffusion agarose gel system is,

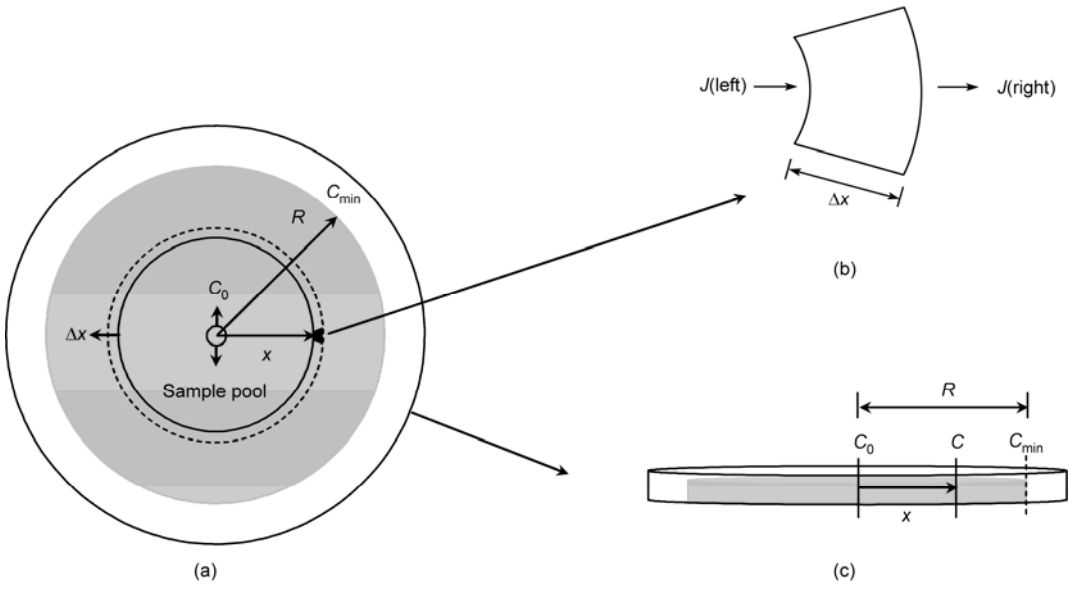

Figure 1 Schematic diagram of thrombolytic diffusion in an agarose plate. (a) Planform; (b) a small unit volume and (c) one-dimensional view of the diffusion system. The shaded part indicates the fibrinolytic disc, C_0 and $C_{\min}$ are the initial concentration and the minimum fibrin dissolution concentration of the drug, respectively. J is the diffusion flux.

$$\frac{x}{2\sqrt{Dt}} = \pm\infty (t = 0, x > 0),\ C = 0\,, \tag{3}$$

and the boundary condition is

$$\frac{x}{2\sqrt{Dt}} = 0 (t \geqslant 0, x = 0),\ C = C_0\,. \tag{4}$$

We then finally obtain

$$\frac{C(x,t)}{C_0} = 1 - erf\left(\frac{x}{2\sqrt{Dt}}\right), \tag{5}$$

as the concentration distribution of thrombolytics with radial distance and time, as depicted in Figure 2(a) and (b).

Clearly, when C_0 is constant, the graph shows that $C(x, t)$ decreases with distance x (Figure 2(a)) but increases with incubation time (Figure 2(b)).

Because the dissolution front of the fibrinolytic disc corresponds to the position where the drug concentration equals $C_{\min}$, we can obtain the exact value of the R by replacing C/C_0 with $C_{\min}/C_0$ in the simulation curve of C/C_0~x in Figure 2(a). That is to say, when C=$C_{\min}$, x=R. Different relationships between R and t can be seen for different $C_{\min}$ of thrombolytics (Figure 2(c)). A more rapid increase in R with time corresponds to a lower $C_{\min}$, which indicates a higher thrombolytic activity. This is consistent with the FAPA measurement and the model clearly provides a theoretical explanation.

1.3 Predictions of the mathematical model

By substituting C=$C_{\min}$, x=R, eq. (5) can be transformed to

$$\frac{C_{\min}}{C_0} = 1 - erf\left(\frac{R}{2\sqrt{Dt}}\right). \tag{6}$$

For a given C_0 and specific thrombolytics, $C_{\min}$ is a constant. Thus, from eq. (6), we can see that $erf\left(\frac{R}{2\sqrt{Dt}}\right)$ and $\frac{R}{\sqrt{Dt}}$ are also constants.

Suppose the constant $\frac{R}{\sqrt{Dt}}$ is K, the relation between R and t is

$$\log(R) = \frac{1}{2}\log(t) + M\,, \tag{7}$$

where M=$\log(K) + \log(D^{1/2})$. Eq. (7) predicts that $\log(R)$ versus $\log(t)$ has a linear relationship with a slope of 1/2 and an intercept of M, as shown in Figure 3(a).

From eq. (6), we further predicted that, first, for the same drug with the same $C_{\min}$, a sample with a higher initial concentration will show the higher value of K=$\frac{R}{\sqrt{Dt}}$ and a larger intercept, M, i.e.,

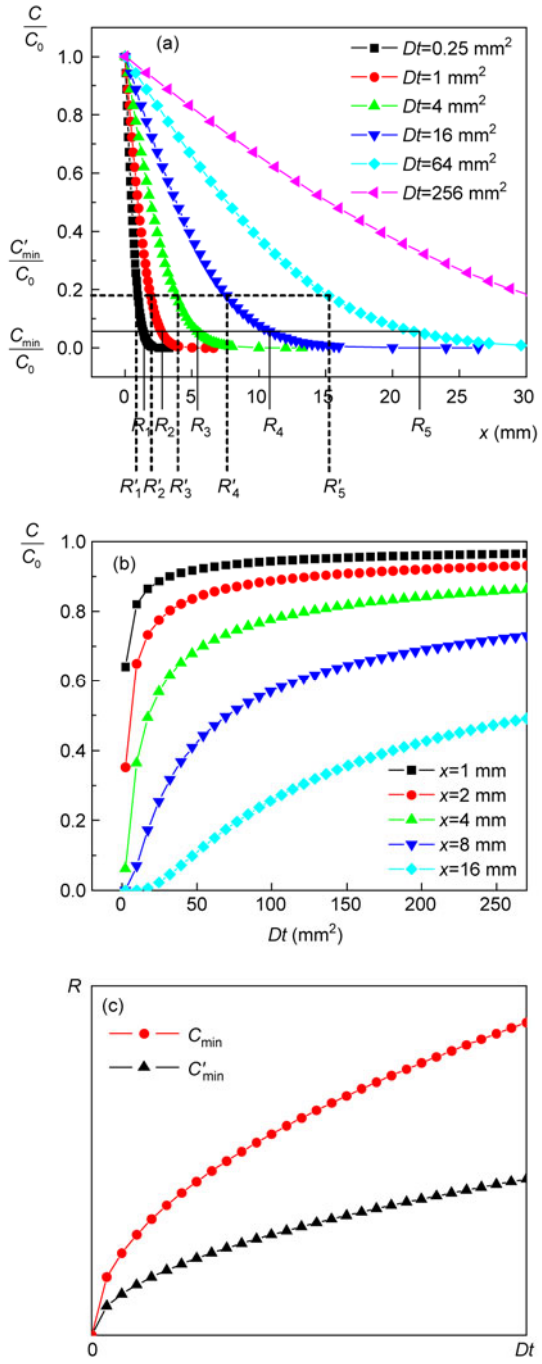

Figure 2 (Color online) Theoretical concentration, C, distribution of thrombolytics with (a) radial distance x and (b) diffusion time t, as well as (c) the theoretical values of the radii, R, of fibrinolytic discs using drugs with different activities ($C_{\min}$<$C'_{\min}$).

$$M > M'(C_{\min} = C'_{\min}; C_0 > C'_0)\,, \tag{8}$$

as shown in Figure 3(b). Second, a drug with a higher specific activity (lower $C_{\min}$ value) will present a higher constant value of $n = \frac{R}{\sqrt{t}}$ when C_0 and D remain constant, i.e.,

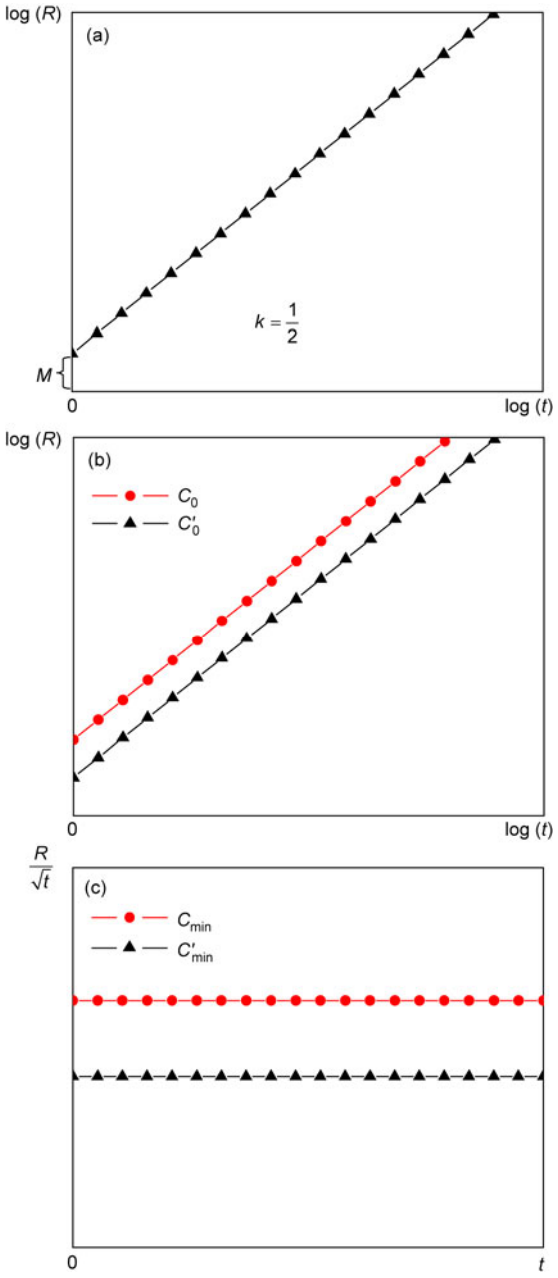

Figure 3 (Color online) Theoretical curves of the relationships between the radius R of the fibrinolytic discs and diffusion time t. (a) log(R) versus log(t) of a drug; (b) log(R) versus log(t) of a drug with two different initial concentrations (C_0>C'_0); (c) $\frac{R}{\sqrt{t}} \sim t$ of two drugs with different activities (C_{min}<C'_{min}).

$$n = \frac{R}{\sqrt{t}} > \frac{R'}{\sqrt{t}} = n'(C_{min} < C'_{min};\ C_0 = C'_0), \tag{9}$$

as shown in Figure 3(c).

It is shown in eq. (9) that the constant value of $\frac{R}{\sqrt{t}}$ can be directly used to evaluate the activity of thrombolytics. The higher value of $\frac{R}{\sqrt{t}}$, the higher the activity of the drug. This prediction is significant because it can be used to set up a new and general standard for FAPA testing.

2 Experimental verification and discussion

2.1 Materials and methods

The fibrin plate assay with some modifications was used to determine the UK activity [12]. For the preparation of fibrin plates, a 0.6% (wt/vol) agarose solution (21 mL), 5% (wt/vol) bovine fibrinogen (2 mL; Institute of Food and Drug Test), 1.2 units/mL of bovine plasminogen (1 mL; Institute of Food and Drug Test), and 15 units/mL of thrombin (1 mL; Sigma) in 100 mmol/L PBS buffer (pH 7.4) were mixed in a Petri dish (diameter, 90 mm). Serial dilutions of the UK sample (2.5, 5, 12.5, 25 and 50 mg/L in 0.9% NaCl solution, approximately 10 μL) were injected into sample pools in the fibrin plate and incubated at 37°C for 1, 2, 4, 6, 8, 12 and 24 h. To compare the activities of different drugs, we chose two kinds of UK from different producers (Dillon Pharmaceutical Co., Ltd. in Heilongjiang, China with a batch number 20090517 and Livzon Pharmaceutical Group Inc. in Guangdong, China with a batch number 100804) which we determined to have different activities (Dillon UK displayed the higher specific activity, data not shown). Diameters of the fibrinolytic discs were measured by Vernier caliper. To eliminate the influence of temperature during the testing process, we completed the measurement in an incubator (Yiheng Technology Co., Ltd., Shanghai, China) which provided a constant temperature of 37°C. Images of fibrinolytic discs at each time point (1, 2, 4, 6, 8, 12 and 24 h) were collected within several seconds.

2.2 Verification of equations (7)–(9)

Images of the fibrinolytic discs in the agarose hydrogel system are displayed in Figure 4. It is shown that the areas of the fibrinolytic discs for both UKs increased with incubation time and were proportional to the initial concentration of UK. Figure 5(a) and (b) depicts the correlations between log(R) and log(t) according to the experimental results. Comparing these with the predictions using eqs. (7) and (8), the experimental curves were in good agreement with the theoretical curves shown in Figure 3(a) and (b) for both UKs with different specific activities. The slopes of the curves in Figure 5(a) and (b) were between 0.503 and 0.551, which are very close to 1/2. Deviations in the experimentally determined slopes from the theoretical prediction may be caused by the assumption in the model that the concentration in the sample pool remains unchanged throughout the FAPA process. Figure 5(a) and (b) also verified the prediction of eq. (8), where the intercepts of the curves increased

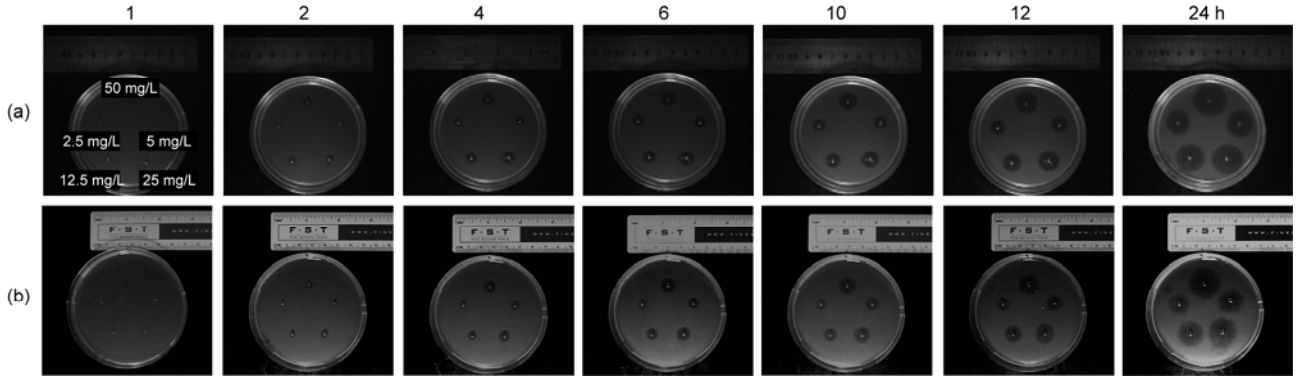

Figure 4 Thrombolysis efficacy images of (a) Dillon and (b) Livzon UKs at different incubation times. Dillon UK displayed a higher specific activity, as determined by the BCA method.

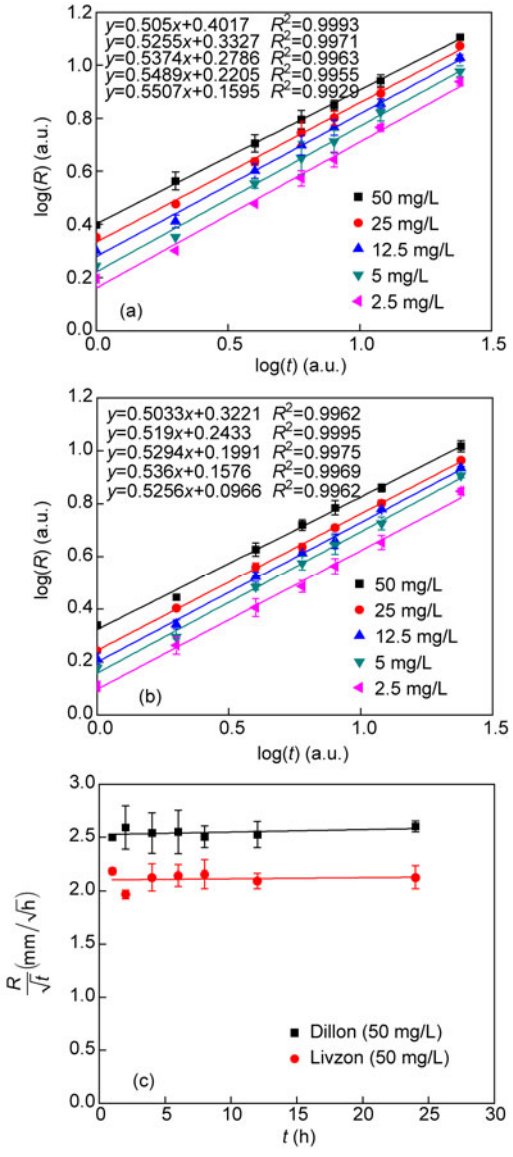

Figure 5 (Color online) Experimental curves of the relationships between the radius, R, of fibrinolytic discs and incubation time, t. (a) log(R) versus log(t) for Dillon UK; (b) log(R) versus log(t) for Livzon UK; (c) $\frac{R}{\sqrt{t}} \sim t$ for Dillon and Livzon UKs.

with the initial UK concentrations.

Figure 5(c) displays the experimental results for $\frac{R}{\sqrt{t}}$ with time for the two UKs. For both drugs, with the same initial concentration, the values of $\frac{R}{\sqrt{t}}$ are constants, which is in good agreement with the theoretical eq. (9) and the theoretical curves in Figure 3(c). Furthermore, by comparing the two drugs, we conclude that the UK with higher activity (Dillon) presented a higher value of $n = \frac{R}{\sqrt{t}}$, which agrees with the significant prediction from eq. (9). Therefore, $n = \frac{R}{\sqrt{t}}$ is confirmed as a useful parameter for evaluating activities of different enzymes.

2.3 Discussion

We developed theoretical relationships between the fibrinolysis radius and the diffusion time of drugs in the FAPA process using Fick's second law. Experimental results in Figure 5 successfully verified these relationships. These findings provide a theoretical foundation for FAPA testing and have significance in its application. First, the linearity of the curve of log(R) versus log(t) can be used to check the fidelity of FAPA testing. The linear correlation factors for all the curves in Figure 5 were over 0.99, indicating a high credibility of the test.

Second, if the value of $C_{\min}$ is measured, the diffusion coefficient D can be calculated more conveniently using eq. (6), circumventing complicated calculations using formulae involved in molecular diffusion models such as the steric effect model [13–15] and more comprehensive models [16–18].

Third, our theoretical and experimental results show that $\frac{R}{\sqrt{t}}$ can be used as a parameter for comparing the activities of different thrombolytics. This finding greatly improves the

power of the FAPA method for thrombolytic activity assessment. It may be used for the quality control of existing drugs and the development of new drugs.

3 Conclusion

A theoretical model providing the relationship between thrombolysis radius, R, and UK diffusion time, t, was established based on the Fick's second law and verified by experimental results. It is revealed that there is a linear log(R) versus log(t) curve with a slope close to 1/2 and a constant $\frac{R}{\sqrt{t}}$ value for a specific thrombolytic. Moreover, we found that the $\frac{R}{\sqrt{t}}$ value can be used as a parameter for the evaluation of activities of different thrombolytics. These new findings may provide insights into thrombolytic testing and improve FAPA measurements.

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Supporting Information

Appendix 1 Detailed derivation of the formula

The supporting information is available online at csb.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.