Enhancing conjugation rate of antibodies to carboxylates: Numerical modeling of conjugation kinetics in microfluidic channels and characterization of chemical over-exposure in conventional protocols by quartz crystal microbalance

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This research reports an improved conjugation process for immobilization of antibodies on carboxyl ended self-assembled monolayers (SAMs). The kinetics of antibody/SAM binding in microfluidic heterogeneous immunoassays has been studied through numerical simulation and experiments. Through numerical simulations, the mass transport of reacting species, namely, antibodies and crosslinking reagent, is related to the available surface concentration of carboxyl ended SAMs in a microchannel. In the bulk flow, the mass transport equation (diffusion and convection) is coupled to the surface reaction between the antibodies and SAM. The model developed is employed to study the effect of the flow rate, conjugating reagents concentration, and height of the microchannel. Dimensionless groups, such as the Damköhler number, are used to compare the reaction and fluidic phenomena present and justify the kinetic trends observed. Based on the model predictions, the conventional conjugation protocol is modified to increase the yield of conjugation reaction. A quartz crystal microbalance device is implemented to examine the resulting surface density of antibodies. As a result, an increase in surface density from 321 ng/cm², in the conventional protocol, to 617 ng/cm² in the modified protocol is observed, which is quite promising for (bio-) sensing applications. © 2015 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4937929>]

Microfluidics have been implemented in various bio-medical diagnostic applications, such as immunosensors and molecular diagnostic devices.¹ In the last decade, a vast number of biochemical species has been detected by microfluidic-based immunosensors. Immunosensors are sensitive transducers which translate the antibody-antigen reaction to physical signals. The detection in an immunosensor is performed through immobilization of an antibody that is specific to the analyte of interest.² The antibody is often bound to the transducing surface of the sensor covered by self-assembled monolayers (SAMs). SAMs are organic materials that form a thin, packed and robust interface on the surface of noble metals like that of gold, suitable for biosensing applications.³ Thiolic SAMs have a “head” group that shows a high affinity to being chemisorbed onto a substrate, typically gold. The SAMs’ carboxylic functional group of the “tail” end can be linked to an amine terminal of an antibody to form a SAM/antibody conjugation.^{3,4} The conjugation process is usually accomplished in the presence of carbodiimides, such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). A yield increasing additive, *N*-Hydroxysuccinimide (NHS), is often used to enhance the surface loading density of the antibody.^{4,5}

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A typical reaction for coupling the carboxylic acid groups of SAMs with the amine residue of antibodies in the presence of EDC/NHS is depicted in Figure 1.⁴ NHS promotes the generation of an active NHS ester (k_2 reaction path). The NHS ester is capable of efficient acylation of amines, including antibodies (k_3 reaction path). As a result, the amide bond formation reaction, which typically does not progress efficiently, can be enhanced using NHS as a catalyst.⁴

A number of groups have studied EDC/NHS mediated conjugation reactions such as the ones depicted in Figure 1. The general stoichiometry of the reaction involves a carboxylic acid (SAM), an amine (antibody), and EDC to produce the final amide (antibody conjugated SAM) and urea. However, the recommended concentration ratio of the crosslinking reagents inside the buffer, i.e., the ratio of EDC and NHS with respect to adsorbates and each other, varies from one study to another.⁶ The kinetics of the reactions outlined in Figure 1 have also been investigated,^{4,6-8} but only in the absence of NHS for EDC or carboxylic acids in aqueous solutions.⁸ A relatively recent experimental study verified the catalytic role of the yield-increasing reagent *N*-hydroxybenzotriazole (HOBt), which acts similarly to NHS.⁷ In this study, the amide formation rate (k_3 reaction path, Figure 1) was found to be dependent on the concentration of the carboxylic acid and EDC in the buffer solution, and independent of the amine and catalyst reagent concentration. The same group also showed that the amide bond formation kinetics is controlled by the reaction between the carboxylic acid and the EDC to give the O-acylisourea, which they marked as the rate-determining step (k_1 reaction path, Figure 1).

The k_1 reaction path, or the conjugation reaction, is usually a lengthy process and takes between 1 and 3 h.^{4,9} Compared to k_1 , the k_2 and k_3 reactions are considerably faster. Microfluidics has the potential to enhance the kinetics of these reactions using the flow-through mode.^{10,11} This improvement occurs because while conventional methods rely only on diffusion as the primary reagent transport mode, microfluidics adds convection to better replenish the reagents to the reaction surfaces. However, there are many fundamental fluidic and geometrical parameters that might affect the process time and reagents consumption in a microfluidics environment, such as concentration of antibodies and reagents, flow rate, channel height, and final

FIG. 1. NHS catalyzed conjugation of antibodies to carboxylic-acid ended SAMs through EDC mediation (Adapted from G. T. Hermanson, *Bioconjugate Techniques*, 2nd. Edition. Copyright 2008 by Elsevier⁴). EDC reacts with the carboxylic acid and forms o-acylisourea, a highly reactive chemical that reacts with NHS and forms an NHS ester, which quickly reacts with an amine (i.e., antibody) to form an amide.

surface density of antibodies. A model that studies the kinetics of conjugation reaction against all these parameters would therefore be helpful for the optimization of this enhanced kinetics.

There are a number of reports on numerical examination of the kinetics of binding reactions in microfluidic immunoassays.^{12–15} All these models developed so far couple the transport of reagents, by diffusion and convection, to the binding on the reaction surface. Myszkowski's model assumes a spatially homogeneous constant concentration of reagents throughout the reaction chamber, thus fails to describe highly transport-limited conditions due to the presence of spatial heterogeneity and depletion of the bulk fluid from reagents.^{16,17} In transport-limited conditions, the strength of reaction is superior to the rate of transport of reagents to the reaction surface.^{18,19} More recently, the convection effects were included in a number of studies, describing the whole kinetic spectrum from reaction-limited conditions to transport-limited reactions.^{20–22} Immunoreaction kinetics has also been examined with a variety of fluid driving forces, from capillary-driven flows,²⁰ to electrokinetic flows in micro-reaction patches,²¹ pressure-driven flows in a variety of geometric designs.²² Despite these comprehensive numerical investigations, the fundamental interrelations between the constitutive kinetic parameters, such as concentration, flow velocity, microchannel height, and antibody loading density, have not been studied in detail. In addition, the conjugation kinetics has not yet been exclusively examined.

In this paper, a previous model for immunoreaction is modified to study the antibody/SAM conjugation reaction in a microfluidic system. Model findings are used to examine the process times recommended in the literature and possible modification scenarios are proposed. The new model connects the convective and diffusive transport of reagents in the bulk fluid to their surface reaction. The conjugation reaction is studied against fluidic and geometrical parameters such as flow rate, concentration, microchannel height and surface density of antibodies. Damköhler number is used to compare the reaction and fluidic phenomena present and justify the kinetic trends observed. Model predictions are discussed and the main finding on possible overexposure of carboxylates to crosslinking reagents, in conventional protocols, is verified by comparing the resultant antibody loading densities obtained using a quartz crystal microbalance (QCM) set up. The results demonstrate an improved receptor (antibody) loading density which is quite promising for a number of (bio-) sensing applications.^{23,24} Major application areas include antibody-based sensors for on-site, rapid, and sensitive analysis of pathogens such as *Bacillus anthracis*,²³ *Escherichia coli*, and *Listeria monocytogenes*, and toxins such as fungal pathogens, viruses, mycotoxins, marine toxins, and parasites.²⁴

MATHEMATICAL MODEL

The rectangular microchannel used for the model is shown in Figure 2. The height (h) of the microchannel is much smaller than its width, so the variation in concentration across the width can be neglected and the flow can be considered two-dimensional. A fully developed laminar flow inside the microchannel is expected due to the low Reynolds numbers encountered in microfluidic applications.^{25–27} The effect of electrical forces and gravity are neglected. It is assumed that the temperature variation during the reactions is not significant so variations in physical properties due to temperature can be reasonably neglected. SAMs form a packed,

FIG. 2. Schematic of the fluid flow pattern and the heterogeneous conjugation medium inside the microchannel under study (not to scale). Reagents enter the channel from the left inlet with an initial concentration of C_0 and an average cross-sectional velocity of u_{avg} by applying an appropriate pressure difference across the length of the microchannel. X is the distance of the reaction surface from the inlet and is equal to $80\ \mu\text{m}$, and h is the microchannel height. Conjugation takes place on the activated surface within a width $w = 1500\ \mu\text{m}$.

uniformly distributed monolayer over metal surfaces with minimum pin-holes or defects.^{3,28} In this work, the surface distribution of reaction sites was controlled by maintaining a 3:1 ratio between thiols and by minimizing the steric hindrance that can prevent proper receptor/SAM conjugation,²⁸ as detailed in the “Experimental Setup and Results” section). As a result, the heterogeneity of the conjugation sites was assumed to be negligible.

The model is based on binding antibodies from the bulk fluid to the activated self-assembled monolayer formed on the reaction surface. Reagents are released into the microchannel from the left inlet, with a concentration of c_0 . The velocity field is described by the two dimensional Navier-Stokes and the continuity equations as

$$\rho(\vec{u} \cdot \nabla)\vec{u} = -\nabla p + \mu \nabla^2 \vec{u}, \quad (1)$$

$$\nabla \cdot \vec{u} = 0, \quad (2)$$

where ρ denotes the density ($\rho = 1 \text{ g cm}^{-3}$), $\vec{u}$ is the velocity vector, p is the pressure, and μ is the dynamic viscosity of the carrier fluid ($\mu = 8.9 \times 10^{-4} \text{ Pa s}$). The Navier-Stokes equations reasonably simplify to the Stokes equations because of the low Reynolds numbers.²⁹ A net constant velocity of the fluid, with a constant cross-sectional average velocity (u_{avg}) is applied at the inlet, and the outlet is under atmospheric pressure. An appropriate pressure difference across the two ends drives the fluid in the microchannel, and all walls are assumed to be in no-slip condition.^{19,30}

The transport of reagents inside the microchannel can be described by the convection-diffusion equation as follows:

$$\frac{\partial c}{\partial t} + \vec{u} \cdot \nabla c = \nabla \cdot (D \nabla c), \quad (3)$$

where c is the concentration of EDC, and D is the diffusion coefficient ($D = 10^{-11} \text{ m}^2 \text{ s}^{-1}$).^{21,31} The convection-diffusion equation is assumed decoupled from the Navier-Stokes equation. This assumption is reasonable since solutes have negligible effect on flow pattern dilute solutions.²²

The time evolution of the antibodies' surface concentration on the reaction surface (c_s) is related to the reaction rate term (R_s), taking into account the surface diffusion of the antibodies, described by the following equation:

$$\frac{\partial c_s}{\partial t} + \nabla \cdot (-D_s \nabla c_s) = R_s, \quad (4)$$

where D_s is the surface diffusion coefficient of the antibodies ($D_s = 1 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$).^{21,32}

The reaction rate follows the equation⁷

$$R_s = k_e [\text{EDC}] [\text{RCO}_2\text{H}]_s, \quad (5)$$

where k_e is the kinetics rate constant, and $[\text{EDC}]$ denotes the molar concentration of EDC. $[\text{RCO}_2\text{H}]_s$ is the instantaneous surface concentration of the carboxylic groups available for conjugation with antibodies. $[\text{RCO}_2\text{H}]_s$ equals the initial surface concentration of the carboxylic acids (θ_0), minus the amount bound with antibodies (c_s): $[\text{RCO}_2\text{H}]_s = \theta_0 - c_s$. For the case of antibody/SAM conjugation, the parameter c in Equation (3) is the EDC concentration, $[\text{EDC}]$. Equation (4) can then be re-written to describe the antibody/SAM conjugation reaction as

$$\frac{\partial c_s}{\partial t} = \nabla \cdot (D_s \nabla c_s) + k_e c (\theta_0 - c_s). \quad (6)$$

Equation (6), or the surface concentration equation, includes the bulk concentration field of the EDC (c) and must be solved in combination with the mass transport equation, Equation (3). The mass flux from bulk toward the reaction surface couples the bulk and surface concentration equations as

$$\vec{n} \cdot (-D\nabla c + c\vec{u}) = -R_s, \quad (7)$$

where $\vec{n}$ is the unit normal vector to the surface. In the last equation, the sum of the convective, $-D\nabla c$, and diffusive terms, $c\vec{u}$, should be zero at the non-reacting surfaces (i.e., $\vec{n} \cdot (-D\nabla c + c\vec{u}) = 0$).²² The inlet is held at a constant concentration ($c = c_0$), and the mass flux at the outlet is zero (i.e., $\vec{n} \cdot (-D\nabla c) = 0$). Equations (1)–(3) and (6) are solved via Equation (7) for c_s , which is here the instantaneous surface concentration of the antibodies bound. The reaction progress can be assessed at each moment by comparing the c_s/θ_0 ratio, which approaches unity upon reaction completion. Figure 3(a) depicts a close-up of the Poiseuille flow inside the microchannel calculated according to Ref. 29. Figure 3(b) illustrates the concentration fields of the reagents at the early stages of the conjugation process, which closely exceeds the velocity profile due to the diffusion. Figure 3(c) portrays the concentration profile of the reacting species above the reaction site around the conjugation completion time. It can be observed that there is no steep concentration gradient. However, the reaction surface still affects the bulk concentration profile.

MODELING RESULTS AND DISCUSSION

The model was used to find conjugation completion times. The conjugation kinetics was studied against the variation of the major kinetics parameters such as EDC concentration, flow velocity, and microchannel height. NHS has a catalytic role;⁷ therefore, its effects were excluded from the kinetic simulations. The rate constant in Equation (5) is $k_e = 4.6 \times 10^{-3} \text{m}^3(\text{mol s})^{-1}$, corresponding to a pH of around 7.4.⁷ The number of carboxyl-ended thiols in each $1.4 \times 10^{-10} \text{cm}^2$ substrate that could be conjugated with the antibody of interest is around 6000 sites.³³ This number corresponds to $7.3 \times 10^{-11} \text{mol m}^{-2}$ of conjugation sites for antibodies. A maximum of 60% of these carboxylic groups can be conjugated to the antibodies, corresponding to a surface concentration of $\theta_0 = 4.38 \times 10^{-11} \text{mol m}^{-2}$.³⁴ The simulated conjugation reaction was considered completed when the surface concentration of conjugated antibodies (c_s) passed 99% of the total surface concentration of the carboxylic groups on the reaction surface, i.e., $c_s \cdot \theta_0^{-1} > 0.99$.

Figure 4 shows the predicted antibody/SAM conjugation completion times for an input concentration of EDC between 0.1 and 100 mM. This figure depicts the time period required to

FIG. 3. (a) Poiseuille flow profile and distribution of reacting species in the microchannel (b) a few seconds after the reaction initiation and (c) close to the reaction completion. Time is non-dimensionalized by the corresponding process time of $17' : t^* = t/t_{comp}$, inlet flow velocity is $10 \mu\text{m s}^{-1}$, and inlet EDC concentration is 10 mM.

FIG. 4. Calculated antibody/SAM conjugation completion times predicted by the model for different EDC concentrations and plotted in the log scale for two different flow velocities.

conjugate the antibody molecules from their amine group to the carboxylic head groups of the thiolic SAM, as a function of the initial concentration of the EDC molecules in the bulk fluid. Results are presented for zero-flow mode (traditional incubation or pipetting) and flow-through conjugation. The parameters under consideration are the concentration of EDC and the flow velocity. Simulation results show a nearly exponential reduction of the process time via increasing the EDC concentration. Note that process time decreases from 605 min to 11 min upon increasing the EDC concentration from 0.1 to 100 mM, for the zero-flow mode. Similar results can be seen for the flow-through mode. Upon conjugation initiation from a 5 mM EDC sample, the reaction is predicted to become complete in around 1 h ($T_{\text{comp}} = 63$ min) for the zero-flow case. This process time is in agreement with the 1-h conjugation process that is recommended in conventional protocols. However, these protocols use higher concentrations of EDC, such as 50 or 100 mM. According to the figure, the times needed for antibody conjugation at these higher EDC concentrations are 18 and 11 min, respectively, which are considerably lower than the process times used in the literature, usually between 1 and 3 h.^{4,9} On the other hand, the outcomes agree with experimental results available in the literature,⁷ for an EDC concentration of 0.7 mM with less than a 3% difference.

The impact of process time on conjugation efficiency can be investigated through the analysis of the by-product composition on the reaction surface to reveal the possible reactions paths. The findings of this new model along with published experimental data can illuminate the general trend. Sam *et al.* investigated the formation or absence of chemical by-products at varying EDC concentrations, using a constant 90-min process time using infrared spectroscopy.⁶ They reported the presence of acid and anhydride by-products for the 2 mM EDC samples, which is a product of the reaction between the o-acylisourea and an additional carboxylic acid, indicative of unbound carboxylic groups on the reaction surface, suggesting an incomplete reaction. This finding is in agreement with the simulation results here, as the conjugation completion time for a 2 mM EDC concentration was expected to be 100 min, instead of 90 min (Figure 4). According to our model predictions, a sample with 50 mM–100 mM EDC concentration only needs between 10 and 20 min for conjugation completion. At the 50–100 mM EDC concentrations, urea derivatives were detected in the experiments performed by Sam *et al.* Since urea is the main conjugation product, this implies that the use of longer times at these concentrations (i.e., 90 min) might result in the presence of urea derivatives,⁶ thus suggesting overexposure to the crosslinking reagents. At the 5 mM EDC concentration, no by-products

were observed experimentally and the reaction was considered complete.⁶ At this concentration, our model also predicts a conjugation reaction process of around 63 min, which closely matches our experimental findings. The absence of by-products is due to the low concentration of EDC used, in at least one order of magnitude less than 50 or 100 mM samples. Thus, almost all the crosslinking reagents were presumably consumed in the conjugation process, and despite a prolonged process, not enough reagents were available for reaction with urea.

Change of velocity can considerably alter the ratio of reaction progress rate with respect to the mass transport strength, which can be quantified by the Damköhler number, Da .¹⁸ For instance, in the case of zero velocity, the $Da = 8.1 \times 10^{-7} (\sim 10^{-6})$, i.e., around three orders of magnitude more than the case of flow-through mode ($Da = 1.34 \times 10^{-9}$), implying a considerable potential for kinetics enhancement in the flow-through mode. The value of these Damköhler numbers indicates that the kinetics for both of these modes is reaction-limited; thus, an increase of flow velocity is not expected to cause a considerable kinetics enhancement. However, the latter condition does not correspond to a complete independency of the reaction-progress rate from the flow rate. Hence, there is always a possibility to increase the rate of reaction by augmentation of the mass transport rate (though not necessarily significant). Figure 4 shows that the conjugation kinetics gains a higher flow rate dependency via an increase in the EDC concentration. Additionally, binding capacities ($\epsilon = c_0 h \cdot \theta_0^{-1}$) are relatively high in the conjugation reaction.¹⁸ For our case, the binding capacity ranges between $9.13 \times 10^4 < \epsilon < 9.13 \times 10^7$ for $0.1 \text{ mM} < c_0 < 100 \text{ mM}$, correspondingly. Hence, upon elevation of the EDC concentration, the binding capacity (ϵ) is linearly magnified, which implies fewer binding sites available for the analytes binding, assuming that a fixed number of analytes is present in the bulk fluid. Therefore, binding sites are expected to be occupied faster, resulting in enhanced kinetics at higher concentrations of EDC.

Flow velocity may also affect transient processes, as illustrated in Figure 5, where the instantaneous dimensionless surface concentration of antibodies ($c_s^* = c_s \cdot \theta_0^{-1}$) over time was calculated and plotted for a variety of flow velocities. The velocity and time are non-dimensionalized by a typical flow velocity of $75 \mu\text{m s}^{-1}$ ($u^* = u_{\text{avg}} / (75 \mu\text{m s}^{-1})$) and its corresponding process time (T_{comp}) of 3 min and 34 s ($t^* = t / T_{\text{comp}}$). It can be seen that there is no further significant improvement in the transient kinetics after the flow velocity is increased beyond $u^* = 1$ ($75 \mu\text{m s}^{-1}$), as the c_s^* values differ by less than 1% between curves at all times. Bearing in mind that the kinetics has a reaction-limited nature, this observation may be attributed to the use of a maximum transport capacity for kinetic enhancement. Therefore, beyond this flow velocity, increasing the flow rate does not result in a noticeable transient kinetics improvement. However, the reagent consumption may increase, leading to additional costs.

FIG. 5. Simulated transient curves of the dimensionless surface concentration of conjugated antibodies (c_s^*) at some typical flow velocities and an EDC concentration of 5 mM.

Figure 6 summarizes the simulated antibody conjugation completion times for flow velocities between zero and $1500 \mu\text{m s}^{-1}$, corresponding to $0 \leq u^* \leq 20$ in Figure 5. The completion time is reduced from around 63 to 12 min for an increase in velocity from zero to $1.5 \mu\text{m s}^{-1}$ ($u^* = 0.02$), showing the sensitivity toward mass transport augmentation. This can be attributed to a high binding capacity ($\epsilon \sim 10^7$) and a considerable change in the Damköhler number, that reduces for around one order of magnitude from 8.1×10^{-7} to 1.15×10^{-7} , for this same velocity range. Comparison of Figures 5 and 6 reveals that flow rate affects transient processes more than long-term runs. While transient curves are “saturated” at the nominal flow velocity of $75 \mu\text{m s}^{-1}$ ($u^* = 1$), the conjugation completion time saturates at a considerably lower velocity, $15 \mu\text{m s}^{-1}$ ($u^* = 0.2$). This implies that the antibody-conjugation kinetics may be easily enhanced at flow velocities approximately equal or below this saturation value.

To observe the effect of microchannel height, the transient conjugation process was plotted for a number of microchannel heights with respect to a dimensionless time (see Figure 7). For this plot, the EDC input concentration and the flow velocity are once again 5 mM and $150 \mu\text{m s}^{-1}$, respectively. The height is non-dimensionalized by a reference height of $40 \mu\text{m}$: $h^* = h/(40 \mu\text{m})$, and time is non-dimensionalized by the corresponding process time ($T_{\text{comp}} = 3' : 27 \text{ in.}$): $t^* = t \cdot T_{\text{comp}}^{-1}$. For all heights, the receptor surface loading density (SAMs' carboxylic group) is kept at its nominal value of $\theta_0 = 4.38 \times 10^{-11} \text{ mol} \cdot \text{m}^{-2}$. It can be observed that increasing the microchannel height does not contribute to better kinetics, as the resulting kinetic curves differ by less than 1%. The zero-flow mode demonstrated the same trend as the flow-through mode, showing a weak impact in the conjugation kinetics with respect to the microchannel height variations. This finding is counter-intuitive, since deeper microchannels should have intrinsically more reagents available in the reaction environment, and as a result are expected to have a faster reaction. However, due to the reaction-limited kinetics ($\text{Da} \ll 1$), the thickness of the depletion zone, or minimum height that contains the total amount of antibodies in the bulk needed for the completion of the reaction, is much less than the microchannel height ($\delta_c \ll h$). Therefore, the change in microchannel height does not affect the reaction progress rate.

Simulation results included in Figure 7 for θ^* , the non-dimensionalized value of θ_0 , given by $\theta^* = \theta_0/(4.38 \times 10^{-11} \text{ mol m}^{-2})$, also show that the number of available conjugation sites, have minimal effect on the conjugation kinetics. This implies that due to the reaction limitation, there is an abundance of reactants available for each reaction site. Therefore, possible surface heterogeneity of the conjugation sites, even for conjugation sites up to five orders of magnitude ($0.01 < \theta^* < 1000$), would result in minimal impact on the conjugation kinetics. This also

FIG. 6. Calculated antibody conjugation completion times, in minutes, corresponding to a 5 mM EDC concentration and plotted in log-scale for various inlet flow velocities. Data points are connected by straight-line segments for better visualization.

FIG. 7. Simulated transient evolution of dimensionless surface concentration of conjugated antibodies (c_s^*), for a number of microchannel heights (h^*) and for a number of receptor surface loading densities (θ^*). The EDC input concentration is 5 mM, and the flow velocity is $150 \mu\text{m s}^{-1}$. The height and time were non-dimensionalized by a reference height of $40 \mu\text{m}$ and by the corresponding process time of $3' : 27 \text{ in.}$, respectively. θ_0 was non-dimensionalized by a reference value of $4.38 \times 10^{-11} \text{ mol m}^{-2}$.

implies that the antibody concentration or number of receptor sites per unit surface does not have a significant impact on the kinetics and that the reaction of EDC and the carboxylic group of thiols is the rate-determining step.⁷

EXPERIMENTAL SETUP AND RESULTS

The immobilization protocol to assess the performance of the EDC/NHS conjugation process uses a QCM in a real time configuration to quantify the loading density of antibodies. The process involves step-by-step binding of a 11-mercaptoundecanoic acid (11-MUA) and 1-octanethiol (1-OT) SAM with a concentration of $1 \mu\text{g/ml}$ (dilution factor of 1 : 1000) to a gold coated quartz crystal, followed by carboxyl head group modification using different EDC concentrations (0, 2, 5, 50, and 100 mM) and 1 h of modification. Synthetic rabbit anti-HSP70 (SPC-313D) antibody, with a concentration of $1 \mu\text{g/ml}$ (dilution factor of 1 : 1000), is then bound to the modified SAM, which can selectively capture Salmon HSP70.

The schematic and photograph of the experimental setup is shown in Figure 8. It consisted of a Pump 11 Pico Plus syringe pump (Harvard Apparatus, Holliston, USA), which pumped the

FIG. 8. (a) Schematic and (b) photograph of the QCM flow injection analysis setup for real time monitoring of protein binding. A syringe pump was used to pump fluid through an injection valve and into a sealed flow cell, which contained the gold coated quartz crystal. Samples were injected via a sample loop on the injection valve and changes in frequency monitored and recorded using a PC.

carrier fluid at a constant rate from a 5 ml gas tight syringe into a six port, two position selector valve (C22Z, Valco, Houston, USA) via Polyetheretherketone (PEEK) tubing. The fluid was pumped through the valve and into an axial flow cell made of Kynar[®], which seals against a QCM200 crystal holder (Stanford Research Systems, Sunnyvale, USA). Inside the crystal holder, 1 in. diameter AT-cut chrome/gold quartz crystals (standard 5 MHz resonant frequency) were installed (Stanford Research Systems, Sunnyvale, USA). Electrical contact is made via the backside of the crystal, while fluid flows over the front side via the flow cell, which has a volume of approximately 150 μ l. A QCM25 crystal oscillator was connected to the crystal holder and controlled via a QCM200 digital controller (Stanford Research Systems, Sunnyvale, USA), which outputs the frequency and resistance measurements to a personal computer (PC). Prior to the start of the experiment, capacitance cancellation was performed while the crystal was in solution to reduce error caused by interfacial and stray capacitances. After exiting the flow cell, the fluid was pumped into a waste beaker. The samples were injected by loading them into a sample loop through a fill port (Valco, Houston, USA) on the six port valve.

Clean chrome/gold coated quartz crystals were first incubated for 15 min in ethanol solutions containing a 10 mM mixture of 3:1 1-OT:11-MUA. The latter ratio is maintained to avoid steric hindrance²⁸ (1-OT acts as spacer). Following this incubation, the crystal was rinsed gently with ethanol and deionized water (DI) water, and inserted into an aqueous solution of EDC and NHS (equal concentrations, as recommended⁴) for 1 h to allow for chemical modification of the 11-MUA head groups. The crystal was then rinsed in DI, dried with N₂, and inserted into the QCM flow cell, allowing minimal exposure to air. A 13 mM phosphate buffered saline (PBS) solution (pH = 7.4) was used as a carrier fluid and was pumped over the crystal at a rate of 7 μ l/min, until a stable frequency baseline was obtained. HSP70 antibody (320 μ l, various dilutions) was then injected into the sample loop through the injection port of the valve, and allowed to flow over the functionalized crystal for 45 min at 7 μ l/min. After antibody binding was complete, the crystal was rinsed and allowed to stabilize in buffer, after which HSP70 (4.6 mg/l, 320 μ l) was injected into the system at 7 μ l/min for 45 min. After the binding was complete, the crystal was again allowed to stabilize in flowing buffer solution. Each experiment was done in triplicate. The entire procedure was carried out at room temperature. The adsorption of biomolecules to the surface of the QCM crystal results in a change in the oscillation frequency (Δf). The mass change per area (Δm) due to the adsorption of biomolecules onto the crystal surface can be calculated via the simple relationship $\Delta f = -\Delta m/C$,³⁵ where C is the mass sensitivity constant (17.7 ng cm⁻² Hz⁻¹ for the 5 MHz crystal used). Figure 9 presents the results of the protein immobilization experiment with a number of initial EDC concentrations. Table I list QCM frequency shifts and the corresponding mass loading densities calculated for both HSP70 antibody and HSP70 at each concentration of EDC. The results show that optimal

FIG. 9. QCM frequency response plots of anti-HSP70 conjugation and HSP70 binding for different EDC concentrations. The first drop in frequency corresponds to the anti-HSP70/SAM conjugation reaction, and the second drop is due to the immunoreaction of HSP70 with anti-HSP70.

TABLE I. QCM frequency response and calculated load density of the binding of HSP70 Antibody (Ab) to the SAM, followed by HSP70 binding at different concentrations of EDC.

EDC Conc. (mM)	HSP70Ab binding (Hz)	HSP70Ab LD ^a (ng/cm ²)	HSP70 Binding (Hz)	HSP70 LD ^a (ng/cm ²)
0	26.1 ± 3.04	461 ± 54	0 ± 0	0 ± 0
2	19.5 ± 1.41	345 ± 25	2.3 ± 3.3	40 ± 60
5	34.9 ± 0.49	617 ± 9	5.8 ± 0.83	100 ± 20
50	18.2 ± 1.63	321 ± 29	4.4 ± 0.99	80 ± 20
100	14.9 ± 0.35	263 ± 6	4.9 ± 1.8	90 ± 30

^aLoad density.

antibody and protein mass density was achieved using a 5 mM EDC solution within 60 min of 11-MUA activation, with densities of $617 \pm 9 \text{ ng/cm}^2$ and $100 \pm 20 \text{ ng/cm}^2$, respectively. At a density of 617 ng/cm^2 , the HSP70 antibody appears to have formed a complete monolayer on the SAM, as similar immunoglobulin G (IgG) proteins have reported monolayer a $200\text{--}550 \text{ ng/cm}^2$ mass density.³⁶ The mass density depends on the orientation of the antibody, with flat-on orientation resulting in lower densities and end-on resulting in higher densities.³⁶ This indicates that the HSP70 antibody layer is densely packed with an end-on orientation, and the large protein binding density indicates that the antibody remains highly active while bound to the sensor surface.

Antibody binding performed with no chemical modification (EDC: 0 mM) resulted in a relatively high mass density of $461 \pm 54 \text{ ng/cm}^2$. However, no HSP70 binding was observed in the subsequent binding step. This indicates that for the 0 mM case, HSP70 antibodies are still able to bind to the SAM, but may do so through a combination of non-covalent means, such as hydrophobic, electrostatic, or hydrogen bonding forces. However, the lack of protein binding in the subsequent step indicates that the antibodies do not remain active once bound to the surface. The authors believe that this is possibly due to denaturing or poor orientation leading to steric hindrance. The other EDC concentrations tested (2 mM, 50 mM, and 100 mM) resulted in non-optimal antibody mass densities, but in all cases, protein binding occurred in the subsequent step. The 50 mM ($321 \pm 29 \text{ ng/cm}^2$) and 100 mM ($263 \pm 6 \text{ ng/cm}^2$) EDC concentrations resulted in significant protein mass densities of $80 \pm 20 \text{ ng/cm}^2$ and $90 \pm 30 \text{ ng/cm}^2$, respectively. The 2 mM EDC sample showed improved antibody binding ($345 \pm 25 \text{ ng/cm}^2$), but inferior protein binding ($40 \pm 60 \text{ ng/cm}^2$). The results for the 50 mM and 100 mM EDC concentrations agree with the results from Sam *et al.*⁶ confirming the presence of uric chemical by-products produced. Supported by our kinetic modeling results, the conjugation at these concentrations leads to overexposures to conjugation chemicals and has a negative effect on antibody binding. The 5 mM sample displayed the most effective antibody and protein binding of all samples, verifying that optimal binding occurs when the EDC chemical modification is allowed to occur in its optimal reaction-time, and thus, in the absence of other chemical by-products.

CONCLUSIONS

A finite element model to study the kinetics of amide bond formation between antibodies and carboxyl ended SAMs (conjugation) in microfluidic structures has been presented. Simulations and experimental verification performed using an HSP70 binding platform revealed that a 5 mM concentration of EDC is enough to complete the conjugation reaction within 1 h, while the corresponding concentrations used by conventional protocols are considerably higher. Based on the model predictions and our experimental studies, higher concentrations in a 1-h protocol will lead to overexposure to conjugation chemicals. The antibody/SAM conjugation kinetics shows a significant dependency on the flow rate. Simulation results demonstrate that using the flow-through mode, even for $15 \mu\text{m s}^{-1}$ of flow velocity, effectively enhances the transient process of the conjugation kinetics by around one order of magnitude. This observation is promising for the preparation of biosensors. The microchannel height and the surface packing

density of antibodies were observed to have minor effects on the conjugation kinetics. The Damköhler number was used to compare the reaction and fluidic phenomena present and justify the kinetic trends observed. These observations were all attributed to the reaction-limited nature of the conjugation kinetics, bearing a considerably low Damköhler number ($Da \sim 10^{-7}$), but at the same time a relatively high binding capacity ($\epsilon \sim 10^5$).

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