

## Microaligned collagen matrices by hydrodynamic focusing: Controlling the pH-induced self-assembly

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### ABSTRACT

The hierarchical structure of type I collagen fibrils is a key contributor to the mechanical properties of the extracellular matrix (ECM). It is known that the process of *in vitro* fibrillogenesis strongly depends on the pH of the collagen solution. To date, there are few methods available for precisely controlling and investigating the dependence of collagen fibril assembly on the local pH. The objective of this work was to create highly defined pH gradients to systematically determine the effects of local pH on microscale collagen fibrillogenesis and alignment. We use a microfluidic mixing device to create a diffusion controlled pH gradient, which in turn initiates the self-assembly and concurrent flow-alignment of soluble collagen. Finite element method simulations of the hydrodynamic and diffusive phenomena are used to calculate the local concentrations of the components involved in the reaction. We develop a model to analytically calculate the local pH in the microfluidic device from these concentrations. A comparison with the experimental results from polarized light microscopy are in good agreement with the simulations.

### INTRODUCTION

Collagen I is the most common type of all classified collageneous proteins. In contrast to other types which form networks, collagen I is a fibril-forming protein that self-assembles hierarchically at the nano-, micro-, and macroscales. It is mainly found in bone, skin and tendon but also in ligaments, the cornea and internal organs. Besides this noticeable biological importance collagen I plays a key role as a component for engineered functional tissue replacements [1-3]. While the pH dependence of collagen fibrillogenesis is known in principle [4,5], its understanding is complicated by the fact that existing methods cannot precisely predict the pH within the system. However, these pH conditions are crucial for the comprehension of the process of collagen assembly into its unique hierarchical organization. We are particularly interested in controlling structural properties such as the degree of alignment and at the same time investigating the dynamics of the assembly process *in situ*. Microfluidics has proven to be a powerful tool for the study of reactions on small length scales [6]. Here, we present a method which is used to orient and concurrently self-assemble collagen I. We use a cross geometry microfluidic diffusive mixing device containing three inlets and one outlet to align soluble collagen under hydrodynamic flow and initiate collagen self-assembly and fibril formation by creating a defined pH gradient in the outlet microchannel. Finite element method (FEM) simulations are used to model the conditions within the microchannels

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and relate the pH to the local state of fibril formation. It can be shown that the simulations confirm the experimental findings.

## MODELING

To obtain predictions of the pH profile within the microchannels, FEM simulations are carried out using the commercial software Femlab (Comsol, Inc., Burlington, MA). Using about 20,000 elements the incompressible Navier-Stokes equation is solved in two dimensions to obtain the stationary solution (low Reynolds number, i.e.,  $< 2$ ) for the diffusive mixing of sodium hydroxide (NaOH) and acetic acid (AcH). The diffusion of collagen molecules in aqueous solution is much slower than the diffusion of the relatively smaller hydroxide ions ( $\text{OH}^-$ ), and AcH molecules ( $D = 6.9 \cdot 10^{-12} \text{ m}^2/\text{s}$  for single collagen molecules in water versus  $D \sim 10^{-9} \text{ m}^2/\text{s}$  for  $\text{OH}^-$  or AcH in water [7]). Thus, the diffusion of collagen from the center outlet stream into the parallel NaOH streams is disregarded in these simulations. However, we did consider the increased viscosity within the collagen flow. Because the system is governed by laminar flow, the collagen, NaOH, and AcH distribution is stationary. In figure 1a) and b) the spatial distribution of NaOH and AcH, respectively, is shown. In this example, a channel width of  $100 \mu\text{m}$  was chosen. Since we model the system in two dimensions, the channel depth is disregarded here. The fluid velocity in the main channel was set to  $1.35 \text{ mm/s}$ , in the side channels to  $40.5 \text{ mm/s}$ . The flow rate ratio was  $v_{\text{collagen}} : v_{\text{NaOH}} = 1:30$ . Experimental findings have shown that the diffusion of  $\text{OH}^-$  ions into a collagen solution is comparable to the diffusion into an aqueous solution. Therefore we assumed a diffusion constant of  $D_{\text{H}_2\text{O}} = 5 \cdot 10^{-10} \text{ m}^2/\text{s}$ .

The concentration profiles from the FEM simulation are used to calculate the local pH distribution using Matlab (The Mathworks, Inc., Natick, MA). These definitions and abbreviations are used in the following discussion:

$K_s$ : acidity constant ( $1.78 \cdot 10^{-5}$  for AcH)

$K_w$ : dissociation constant of water ( $1 \cdot 10^{-14}$ )

$[\text{H}^+]$ : hydronium ion concentration

$[\text{OH}^-]$ : hydroxide concentration, which consists of input hydroxide and additionally forming hydroxide from dissociation of water (this is the case before the stoichiometric point is reached) and the formation of AcH (this is the case after the stoichiometric points reached)

$[\text{Na}^+]$ : local concentration of sodium ions

$[\text{Ac}^-]$ : effective local acetate concentration

$[\text{Ac}^-]_{\text{free}}$ : free acetate without addition of NaOH (pure AcH in water)

$[\text{AcH}]_{\text{free}}$ : effective concentration of free (not dissociated) AcH

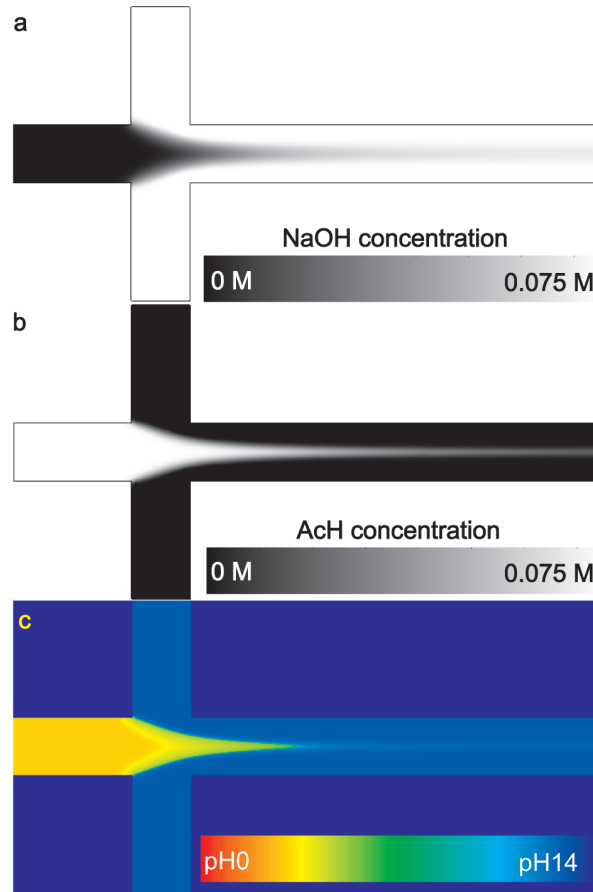
$[\text{AcH}]_{\text{input}}$ : local input concentration of AcH, which is diminished by dissociation of the weak acid

$[\text{AcH}]_0$ : input AcH concentration which flows into the main channel ( $0.075 \text{ M}$  in our case)

$e = [\text{AcH}]_{\text{input}}$ : AcH concentration as determined by FEM simulations

$n = \frac{[\text{Na}^+]}{e}$ : the sodium ion concentration corresponds to the added hydroxide concentration; since there is additional hydronium present in the system it is not possible to use  $[\text{OH}^-]$  for the calculations here.

In principle, a pH titration of a weak acid (AcH) with a strong base (NaOH) is conducted



**Figure 1:** a) NaOH concentration b) AcH concentration as modeled by finite element simulations. c) Calculated pH within the microfluidic device.

in the microfluidic device. However, the situation is somewhat more complicated since both the concentration of AcH and NaOH change constantly. Therefore we have to calculate the pH from the actual concentrations in each point of the device. For a few very specified points on a titration curve an analytical expression exists, such as for pure weak acid (AcH in our case), i.e.  $n = 0$

$$pH = \frac{1}{2} \left( pK_s - \log_{10}([AcH]_0) \right), \quad (1)$$

pure strong base (NaOH in our case), i.e.  $e = 0$

$$pH = pK_w + \log_{10}([Na^+]), \quad (2)$$

and the stoichiometric point, where the molarity of the weak acid equals the molarity of the strong base, i.e.  $n = 1$

$$pH = \frac{1}{2} \left( pK_s + pK_w + \log_{10} \left( \frac{1}{2} [AcH]_0 \right) \right). \quad (3)$$

For the remaining points on the titration curve before the stoichiometric point is reached the pH is usually approximated with the Henderson-Hasselbalch equation and, even more inconveniently, there is no such approximation available after the stoichiometric point is reached.

In order to find an analytical expression for the whole titration curve we determined the equations which define the system and solved this system of equations using Mathematica (Wolfram Research, Inc., Champaign, IL). Since the collagen we used was dissolved in 0.075 M AcH and 0.075 M NaOH was used to neutralize the solution and initiate the assembly process we used the corresponding numbers for our calculations. However, they can be adjusted for any titration of a weak acid with a strong base.

### System of Equations

It is necessary to perform a case differentiation and to set up a system of equations for the conditions before and after the stoichiometric point is reached, respectively. Before the stoichiometric point is reached we have to consider the definition of the acidity constant

$$K_s = \frac{[H^+][Ac^-]}{[AcH]_{free}}, \quad (4)$$

and the definition of dissociation constant of water

$$K_w = [H^+][OH^-]. \quad (5)$$

When the AcH dissociates the total number of acetate ions has to be kept constant. The input AcH dissociates partially to acetate and the free AcH remains in the system:

$$[AcH]_{free} = [AcH]_{input} - [Ac^-]. \quad (6)$$

The available number of acetate ions consists of the acetate owing to the dissociation of pure AcH and the acetate which forms because  $OH^-$  ions are added (corresponds to  $[Na^+]$ ):

$$[Ac^-] = [Ac^-]_{free} + [Na^+]. \quad (7)$$

We also have to assure that the number of charges is conserved:

$$[H^+] + [Na^+] = [Ac^-] + [OH^-]. \quad (8)$$

After the stoichiometric point is reached, equations 4-6 hold as well. In addition, we have to take into account that enough NaOH is added to dissociate the input AcH. However, the resulting acetate ions react with water molecules which in turn leads to additional formation of  $OH^-$  according to



Therefore the effective  $OH^-$  concentration in the system stems from this newly formed  $OH^-$  (corresponds to  $[AcH]_{free}$ ), and the input  $OH^-$  (corresponds to  $[Na^+]$ ), diminished by the  $OH^-$  which reacts with the dissociating input AcH. This leads to the following relation:

$$[OH]^- = [AcH]_{free} + [Na^+] - [AcH]_{input}. \quad (10)$$

### Solution of the system of equations

The equations can be analytically solved using Mathematica. For  $0 < n < 1$ , i.e. before the

stoichiometric point is reached, we obtain the following solution:

$$pH = -\log_{10} \left[ \frac{\frac{1}{3}(-K_s - en) + 2^{1/3}\zeta}{3 \left( \zeta + \sqrt{4\xi^3 + \zeta^2} \right)^{1/3}} - \frac{1}{3 \cdot 2^{1/3}} \left( \zeta + \sqrt{4\xi^3 + \zeta^2} \right)^{1/3} \right] \quad (11)$$

$$\xi = -K_s^2 - 3K_s e + K_s en - 3K_w - e^2 n^2 \quad (12)$$

$$\zeta = 2K_s^3 - 18K_s K_w + 9K_s^2 e - (3K_s^2 - 9K_w) en + 9K_s e^2 n - 3K_s e^2 n^2 + 2e^3 n^3, \quad (13)$$

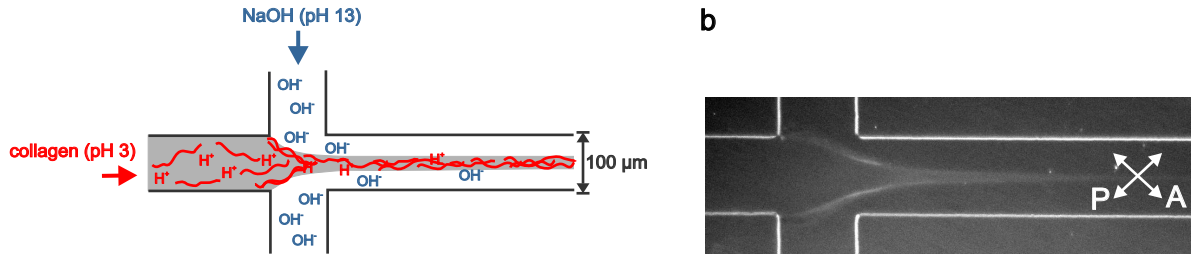
whereas for  $n > 1$ , i.e. after the stoichiometric point is reached, the solution is:

$$pH = -\log_{10} \left[ \frac{\eta + \sqrt{4enK_s K_w + (\eta)^2}}{2en} \right] \quad (14)$$

$$\eta = eK_s + K_w - enK_s. \quad (15)$$

In figure 1c the graphical representation of the pH distribution within the microfluidic device we used is shown.

## EXPERIMENT



**Figure 2:** a) Schematic representation of the experimental setup. b) Experimental findings for a flow rate ratio of  $v_{collagen} : v_{NaOH} = 1:30$ .

The results of the simulations were used to verify our experimental findings. The setup is shown in figure 2a and is described in more detail elsewhere [8]. Briefly, A 'cross' configuration of poly(dimethylsiloxane) (PDMS) microchannels (35  $\mu\text{m}$  deep, 100  $\mu\text{m}$  wide) is fabricated using standard soft lithography techniques [9,10]. The flow velocities in the channels are determined by LabView (National Instruments Corporation, Austin, TX) controlled custom-made syringe pumps. A 10 mg/mL solution of type I collagen (calf skin, USB Corporation, Cleveland, OH) in 0.075 M AcH (pH 3.7) is injected into the main channel. The collagen triplehelical monomers are stable at acidic pH and assemble into fibrils at  $\sim$ pH 6. This process is often referred to as "fibrillogenesis" [4] or "gelation" [5]. In order to stabilize the collagen flow before initiating the collagen self-assembly, we first inject ultra pure water into the side channels. Then, the flow is switched to a 0.075 M NaOH solution (pH 13) using a T-valve. The fluid velocity in the main channel (1.35 mm/s) is slower than the side channels (40.5 mm/s), leading to a hydrodynamically focused collagen stream [8,11]. In this configuration, the pH of the collagen solution gradually increases along the length of the

outlet channel owing to diffusive mixing with the NaOH. An Olympus BX61 microscope (Hamburg, Germany) equipped with crossed polarizers, a 10x objective, and a halogen lamp is used to obtain polarization images during the collagen assembly process in the microfluidic device. Images of the flowing collagen stream are captured using a SensiCam CCD camera (PCO, Kelheim, Germany). In figure 2 b) the polarized light micrograph is shown for a flow rate ratio of  $v_{collagen} : v_{NaOH}=1:30$ . The regions within the stream where the collagen is assembled can be identified by a strong increase of the birefringence signal.

## DISCUSSION AND CONCLUSIONS

Comparing the result of the FEM modeling (figure 1c) and the experimental findings (figure 2b) it can clearly be seen that with our system it is possible to predict diffusive and hydrodynamic phenomena and taking these into account we are able to relate the local pH to the process of fibrillogenesis. The onset of fibrillogenesis at  $\sim$ pH 6 confirms results from bulk experiments. We have shown that microfluidics is extremely well suited to create defined pH gradients which in turn can be used to tune biochemical reactions. Collagen I is an important and well known example for the dependence on pH gradients and there are many more biological systems which could be investigated by these means such as the gelation of mucin [12] and many reactions which involve electrostatic interactions.

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