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Increase of fibrin gel elasticity by enzymes: A kinetic approach

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ABSTRACT

Two enzymes, thrombin and transglutaminase, both participate in the formation of fibrin networks and contribute to the mechanical strength of biogels. A theoretical model built from the available kinetic data showed that a competition may take place between the two enzymes for their common substrate, fibrinogen. To evidence this phenomenon experimentally, the concentrations of the reactants were varied and the rheological properties of the resulting fibrin gels explored. The elasticity of the gels was not a singular function of the transglutaminase concentration, the optimum being also related to fibrinogen and thrombin concentrations. Thrombin concentration influenced the kinetics of gelation, but not the evolution of the mechanical properties of the gel. An indirect relationship between gel elasticity and thrombin concentration was observed upon covalent binding. The liquid phase inside the gel contained a high amount of soluble proteins when a high transglutaminase concentration was used. The impact of this competition between the two enzymes, demonstrated here for the first time, is evaluated for biomaterial elaboration.
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1. Introduction

Many biological media display gel characteristics [1,2]. Biogels are of great potential interest as biomaterials such as scaffolds or three-dimensional cell culture systems for tissue engineering. Fibrin soft gels are at the basis of wound repair; they are formed subsequent to the thrombin hydrolysis of the fibrinogen molecule and fibrin polymerization [3,4]. Fibrin gels are then stabilized by covalent cross-linking of adjacent fibrin units in the fibrin matrix [5–8] by a transglutaminase. All these components are present in blood and in wound-healing regions so that fibrin gels of patient blood can be produced and used as autologous scaffolds [9]. Fibrin gels are also used as surgical glue, and are also promising as biocompatible and biodegradable materials.

Matrix elasticity and stiffness have been proven to influence cell behavior through a response to the physical state of the matrix [10,11]. Thus, long-term stability and mechanical integrity of the biomaterial may be essential for cells that require sufficient time and stiffness to produce their tissue-specific matrix [12]. Fibrin hydrogels are weak at concentrations that support cell growth; increasing their mechanical strength may enlarge their potential use. However, during wound repair, migrating cells have to invade and degrade fibrin clots in the form of a three-dimensional lattice [13]. It is thus necessary to control the mechanical strength of fibrin gels for their further use as biomaterials. However, the dense networks which make up the biogels are not static systems; often, transient gels are formed, undergoing different phase transitions [14], all steps being catalyzed by enzymes. The mechanical properties of biogels are thus evolving with time, and these must be perfectly mastered for their technological use.

In most biological events, two different types of enzymes are responsible for the variations of the matrix properties. Usually, proteases cleave peptide bonds and weaken or dissolve the gel while transglutaminases generate isopeptide bonds [15] and contribute to the insolubilization of the protein lattice. In contrast, in fibrin gelation, thrombin, a protease and transglutaminase coexist and participate synergistically to the formation of a solid phase from macromolecules in solution [16,17]. To our knowledge, this complementary contribution of the two enzymes to the obtaining of an insoluble phase has never been explored in kinetic terms.

In vivo thrombin hydrolysis of fibrinogen leads to a soft gel and ligation due to factor XIIIa, a transglutaminase, only increased the shear storage modulus (G'), or stiffness, of the clots 2.3-fold, up to 30 Pa [8] through a well-described mechanism [18]. Various pathologies are associated with disequilibrium in fibrinogen, thrombin and transglutaminase concentrations [19,20]. Intramolecular and intermolecular cross-linkings of fibrinogen by transglutaminase have been observed on electron micrographs, leading to disorganized fibrin gels [18] and indicating various supramolecular arrangements of fibrinogen. This confirms that the history of the gel, i.e., the kinetics of the enzymes involved in its formation, is of great importance for its mechanical properties. From the data available in the literature it appears that the synergy between thrombin and transglutaminase may be disturbed by the covalent bonding of fibrinogen molecules. In this case, thrombin and

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transglutaminase are both reacting on the same substrate molecule and could compete for it.

The cross-linking of the biogel is of interest to increase it stiffness for technological applications. Genipin cross-linked fibrin has shown promise as microporous scaffold after dehydration [21] or as gel for tissue engineering of human cartilage [22]. Another possibility is to use transglutaminase as the enzyme kinetics can be controlled. The physiological enzyme, factor XIII, does not allow a large improvement of gel mechanical properties [18]. The microbial transglutaminase increases the viscoelasticity of the gel more than the mammal one (150 vs. 30 Pa) [23]. It is of interest to master the activity of this enzyme.

In the present paper, we varied theoretically, and then experimentally, the relative concentrations of fibrinogen, thrombin and transglutaminase. Using various ratios of protein-to-enzyme concentrations, both through the mathematical model and the experiments, we have evidenced that thrombin and transglutaminase may present competitive activities towards fibrinogen when high transglutaminase concentrations are used, leading to different gelation processes and various fibrin networks. The paper defines the limit of the increase of fibrin gel stiffness by transglutaminase.

2. Materials and methods

2.1. Chemicals

All chemicals including thrombin (T-4648), bovine fibrinogen (Fg) type IV (F-4753), were purchased from Sigma. Ajinomoto transglutaminase Activa WM 100 U g^{-1} from *Streptoverticillium* sp. (UNIPEX, France) was used without further purification, except for filtration through a 0.2 μm membrane as no significant protease activity is contained in the transglutaminase preparation in the used conditions. All proteins were solubilized in a 50 mM Tris–HCl buffer pH 7.4. In absence of transglutaminase, no ligation products from fibrin or fibrinogen due to possible traces of factor XIII in fibrinogen were detected on SDS electrophoresis.

2.2. Gelation procedure

All reactants were incubated at 37 °C for 15 min prior to each experiment. Gels were formed in a 1.5 ml plastic microvial or in a glass tube by mixing fibrinogen (3 or 4.8 mg ml^{-1}) and thrombin (from 0.2 to 1.6 U ml^{-1}) in 50 mM Tris–HCl buffer, pH 7.4, containing 0.02 mol l^{-1} $CaCl_2$ and 0.15 mol l^{-1} NaCl. 0.5 U ml^{-1} transglutaminase (from 0 to 1 U ml^{-1}) was added to the mixture immediately after the thrombin or after a controlled time. All indicated concentrations are the final concentrations.

2.3. Dynamic rheological measurements

Rheological measurements were performed with a Rheostress150 (Haake) operating in the oscillatory mode, with an imposed amplitude of deformation of 3% and a frequency of 0.1 Hz, both monitored using a stress-controlled rheometer. Deformation, storage modulus G' and loss modulus G'' were recorded as a function of time. Cone/plate geometry with a cone of 35 mm/2° was used for the analysis of 500 μl samples. A trap filled with 3 ml water was added to limit evaporation. The gelation process was followed at 37 °C.

2.4. Analysis of the gel liquid phase

Absorbance at 280 nm was also used to detect the presence of protein in the liquid phase of the gel. 1 h after gelation, the gels were shaken for 48 h in the presence of equivalent volumes of buffer and the absorbance of the solution was measured.

All experiments were realized at least three times. The values presented are ±8%.

3. Results

3.1. Elaboration of the kinetic model

The interest in building a mathematical model is to point out the parameters which are theoretically pertinent for a given effect; this theoretical approach allows to vary parameters in large scales, even over physiological ranges, in order to determine parameter thresholds possibly leading to peculiar behaviors. Here, we wish to evidence, if they exist, the values over which a competition for a single substrate can occur between thrombin and transglutaminase. Assuming such a reaction of both thrombin and transglutaminase on fibrinogen, a kinetic model was constructed.

The following hypotheses are built on reactions well described in literature. Fibrinogen is mostly hydrolyzed into fibrin by thrombin [3] but may also be partly bound by transglutaminase [24]. Fibrin is bound by transglutaminase with a high efficiency [5].

In the proposed model, fibrinogen is called A, fibrin is B, C is covalently bound A (bound fibrinogen), D is covalently bound B (bound fibrin), thrombin is E_1, transglutaminase is E_2. K_{M1A} is the K_M of thrombin for fibrinogen, K_{M2A} is the K_M of transglutaminase for fibrinogen, K_{M2B} is the K_M of transglutaminase for fibrin.

From the data available in the literature, we assumed that the affinity of thrombin (E_1) for fibrinogen (A) is higher than that of transglutaminase (E_2) for this substrate [14,18]: $K_{M1A} < K_{M2A}$. The affinity of E_2 for fibrin (B) is considered either equal or lower than that of E_1 for A: $K_{M2B} \geqslant K_{M1A}$. The ratio between E_1 and E_2 activity on A was varied by four orders of magnitude; this range is greater than biological activities, but this parameter is the one which can be largely modulated in vitro. The relative affinities of transglutaminase for fibrin and that of thrombin for fibrinogen were varied only from 0.1 to 1 and the relative affinities of transglutaminase and thrombin for fibrinogen were varied only from 0.01 to 0.1 to stay in a reasonable range in good accordance with literature data.

The equations obey simple Michaelis models.

The mechanism is summarized as:

$A \rightarrow B$ through E_1: low K_{M1A}, high V_{max1A}: the first natural reaction, very efficient.

$A \rightarrow C$ through E_2: high K_{M2A}, various V_{max2A}: a parallel and unefficient reaction.

$B \rightarrow D$ through E_2: low or medium K_{M2B}, $V_{max2B} = V_{max1A}$: the second natural reaction, efficient.

Equations become:

$$\frac{dA}{dt} = -\frac{V_{max1A} \cdot A}{K_{M1A} + A} - \frac{V_{max2A} \cdot A}{K_{M2A} + A}$$

$$\frac{dB}{dt} = +\frac{V_{max1A} \cdot A}{K_{M1A} + A} - \frac{V_{max2B} \cdot B}{K_{M2B} + B}$$

$$\frac{dC}{dt} = \frac{V_{max2A} \cdot A}{K_{M2A} + A}$$

$$\frac{dD}{dt} = \frac{V_{max2B} \cdot B}{K_{M2B} + B}.$$

Biologically relevant variation ranges for some parameters can be stated.

$K_{M2A} = 10$ to $100\ K_{M1A}$

$K_{M2B} = 1$ to $10\ K_{M1A}$

V_{max2A}/V_{max1A} ratio $= 0.1$ to 100.

Using this range of parameter values, evolution upon time was calculated for each compound (A, B, C, D), for the total products inside

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