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Research paper

A model describing the effect of enzymatic degradation on drug release from collagen minirods

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Abstract

A drug delivery system, named minirod, containing insoluble non-cross-linked collagen was prepared to investigate the release of model drug compounds. To characterise the complete drug release process properly, a mathematical model was developed. Previously, a mathematical model describing water penetration, matrix swelling and drug release by diffusion from dense collagen matrices has been introduced and tested. However, enzymatic matrix degradation influences the drug release as well. Based on experimental data, a model was developed which describes drug release by collagenolytic matrix degradation based on enzyme diffusion, adsorption and cleavage. Data for swelling, collagen degradation and FITC dextran release from insoluble equine collagen type I minirods were collected. Sorption studies demonstrated a tight sorption of collagenase on collagen surfaces that follows a Freundlich sorption isotherm and results in a degradation constant of 3.8×10^{-5} mol/l for the minirods. The diffusion coefficients of FITC dextran 20 and 70 (3×10^{-3} and 2.4×10^{-3} cm^2/h) in water were analyzed by fluorescence correlation spectroscopy (FCS). Using these data, the mathematical model was verified by two-dimensional simulations. The numerical results agreed well with the measurements.
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Keywords: Collagen; Mathematical model; Enzymatic degradation; Enzymatic adsorption; FCS; Numerical simulation

1. Introduction

Collagen is the major constituent of connective tissue [1]. Due to its wide distribution in the mammalian body, it is a promising material for biodegradable drug matrices. Dense collagen matrices for sustained release of higher weight drugs such as proteins or polysaccharides offer an alternative to implants based on synthetic polymers [1]. Especially drug release from insoluble collagen devices is of interest since drug liberation can be controlled by swelling and enzymatic degradation. Initially the matrix swells and drug is released by diffusion. This phenomenon was described previously in a mathematical model by assuming Fickian diffusion [2]. Subsequently, degradation by collagenolytic enzymes occurs and the release rate becomes additionally dependent on the enzyme binding and cleavage rate. So far, either matrix degradation without consideration of the drug release [3] or the drug release by diffusion [2,4] was implemented. However, a mathematical approach that describes both, matrix degradation and drug release, is still missing. In this work, such a model was developed and carefully analyzed by comparison of numerically and experimentally determined data. We focused on identifying and modelling all the relevant processes of drug release by matrix degradation. Drug release due to matrix swelling was not considered in the modelling part of this work. An overall mathematical model combining both model components – drug release by diffusion [2] and drug release by matrix degradation will be studied in a future work. Separating the two mechanisms in their mathematical modelling and numerical simulation seemed reasonable since they proceed on different time scales, as our

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experimental studies demonstrated. Moreover, this approach allowed the application of more adapted mathematical and numerical methods to the separated subprocesses.

Almost all relevant parameters required for the mathematical model were determined experimentally with a cylindrical dosage form, first mentioned by Fujioka et al. who used soluble atelocollagen as matrix material [5,6]. In our studies insoluble non-cross-linked equine collagen type I with enhanced resistance against collagenolytic cleavage was used to prepare minirods loaded with FITC dextran 20 and 70. Equine collagen was preferred against bovine atelocollagen because of a higher natural cross-linking degree and in consequence a slower degradation profile. Furthermore, equine material is a good alternative to collagen from cattle to avoid discussions concerning BSE.

In detail, the degradation constant, the immobilization capacity of the matrix, the diffusion coefficient, the in vitro drug release of FITC dextran, the sorption profiles and rates of collagenase onto collagen surface were investigated experimentally for this matrix. A small number of process parameters were fitted mathematically (see Section 4.7 and Table 1). The sensitivity of the calculated results with respect to these parameters was studied in a further work [7]. Besides these parameters, which are necessary for this part of the mathematical model, the swelling of minirods was investigated.

2. Materials and methods

2.1. Preparation of collagen devices

Minirods were prepared by using a micro extruder (MiniLab® Micro Rheology Compounder, Thermo Haake GmbH, Karlsruhe, Germany). Insoluble non-cross-linked lyophilised equine tendon collagen type I (Innocoll GmbH, Saal/Donau, Germany) was cut into pieces of approximately 0.5 cm diameter. Without pre-swelling of the collagen pieces, the extruder was loaded with approximately 7 g material by alternatively adding collagen and an aqueous solution of FITC dextran 20 or 70 (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) to yield rods containing approximately 40% (w/w) collagen and 1% (w/w) FITC dextran. FITC labelled dextrans were used as model compounds in release experiments. Loading the extruder was performed until the second pressure in the bypass could be monitored. After homogenisation for approximately 5 min at 20 °C, a strand was extruded and dried at 25 °C, 55% r.h. for 5 d. Subsequently, the rods were cut into pieces of 20 mg weight, 10 mm length and 1.7 mm diameter. For release studies rods of 20 and 40 mm length were produced as well. The residual water content, determined by Karl-Fischer titration, was approximately 5% (w/w).

For degradation studies collagen powder was prepared by re-dispersion of insoluble collagen material in water at 10% (w/w). The pH was adjusted to 7.4. After 60 min swelling at room temperature, the dispersion was centrifuged at 5000 rpm (10 °C, 10 min). The residuum was split into portions of approximately 20 mg, dried in a desiccator and finally dried under vacuum at 25 °C. The dried material was ground with a Pulverisette 14 (Fritsch GmbH, Idar-Oberstein, Germany). Particle size was determined by sieve analysis and particles smaller than 180 μm were used.

Table 1
Overview of parameter used for the mathematical model of FITC dextran 70 release from 10 mm equine non-cross-linked minirods

Experimentally determined parameters	Fitted parameters	Fixed parameters
Ω	n	k_{act}
J	γ	β_E
C_E^{ext}	ϑ	β_A
C_K^0	–	–
C_A^0	–	–
$D_E^0 = D_P^0$	–	–
D_A^0	–	–
Sorption isotherm	–	–
k_1	–	–
k_2	–	–
σ		

2.2. Collagenase studies

Collagenase from *Clostridium histolyticum* was obtained from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany). This crude collagenase, a lyophilised mixture of all seven known forms of collagenase (α, 68 kDa; β, 115 kDa; γ, 79 kDa; η, 130 kDa; δ, 100 kDa; ε, 110 kDa and ζ: 125 kDa) [8], has an activity of 1.2 U/mg solid (1 U hydrolyzes 1 μmol of FALGPA (Furylacryloyl-Leu-Gly-Pro-Ala) per minute at pH 7.5 in the presence of calcium ions). All studies were performed in 50 mM Tris buffer, pH 7.5, containing 200 mM sodium chloride, 10 mM calcium chloride and 0.01% sodium azide.

2.2.1. Determination of enzymatic activity

The enzymatic activity over 2 weeks was determined by incubating 10 μg/ml collagenase in Tris buffer, pH 7.5, at 37 °C. The remaining enzymatic activity was determined by digestion of FITC labelled gelatine after 3 h reaction time (EnzChek® [9]) using a Varian Cary Eclipse fluorimeter (ex. 493 nm; em. 518 nm; Varian Deutschland GmbH, Darmstadt, Germany).

2.2.2. Sorption studies

Sorption studies were performed in Tris buffer, pH 7.5, at 37 °C. Minirods were incubated with 5 μg/ml collagenase. Over 24 h, the remaining enzymatic activity in the supernatants was determined by using the EnzChek®. To verify the amount of collagenase which was bound unspecifically in the matrix, samples were incubated for distinct time intervals and washed several times with buffer. Incubation media and washing solutions were investigated for the remaining enzymatic activity. Furthermore, the sorption isotherm and sorption constants were determined.

Different amounts of collagenase (2.5–30 μg/ml) were incubated with minirods for 1 h. The enzymatic activity in the supernatant was measured with the EnzChek®.

2.2.3. Determination of degradation constant

Samples, containing 5–200 mg collagen powder or 5–100 mg minirods, were incubated with 0.1 μg/ml, 0.4 μg/ml, 1.25 μg/ml and 2 μg/ml collagenase, respectively, at 37 °C. Samples were taken periodically and stored at −80 °C to stop the enzymatic degradation process. Degraded soluble collagen fragments were quantified by a BCA (bicinchoninic acid)-assay [10] using a Shimadzu densitometer (562 nm, CS 9301 PC Tokyo, Japan). The amount of soluble collagen was calculated from a standard curve obtained with gelatine A 180.

2.3. Fluorescence correlation spectroscopy measurements

Diffusion coefficients of FITC dextran 20 and 70 in water were measured by fluorescence correlation spectroscopy (FCS) with a ConfoCor 2 (Carl Zeiss, Jena, Germany). Experiments were performed with an argon laser (488 nm) at room temperature. A standard solution of 10 nM Alexa488 (diffusion time 26 μs) was used. Diffusion times were measured, analysed with autocorrelation curves and converted into diffusion coefficients [11].

2.4. Swelling studies

Minirods were incubated in Tris buffer, pH 7.5, at 37 °C. For studies with collagenase, the enzyme was added after 30 min swelling. Swelling was recorded with a digital video camera (JVC TK-C1380; JVC Professional Products GmbH, Friedberg, Germany; lens: Vivitar 55 mm f2.8 macro) and pictures were analysed with an image analysis system (Comet-Version 3.52a, Nikon GmbH) and Microsoft Photo Editor.

2.5. Release and degradation studies

Ten milimeter minirods containing FITC dextran 20 or 70 were incubated in 2 ml Tris buffer, pH 7.5, at 37 °C (20 mm:5 ml; 40 mm:7 ml). At designated time points, the complete incubation media were replaced. FITC dextran release and matrix degradation were determined simultaneously. Supernatants were analysed for FITC dextran release by fluorescence measurement (Varian Cary Eclipse; ex. 495 nm; em. 525 nm) and for soluble collagen fragments by the BCA assay [10]. Both measurements were performed in 96-well plates. With respect to the development of the mathematical model, the release due to diffusion and enzymatic matrix degradation was regarded separately. Therefore, incubation was performed for 6 d in pure Tris buffer, pH 7.5, to estimate the drug portion which can be released by diffusion. Then 0.1 μg/ml collagenase was added to determine the drug portion which is entrapped in the collagen matrix and can only be released after matrix degradation.

3. Mathematical model

The mathematical model of drug release by degradation of the carrier system is designed for a matrix of cylindrical geometry with length l and diameter d, which consists of a homogeneous polymer (=collagen). Collagen is a hydrogel and swells after contact with aqueous solutions. Only the radial degradation and release was simulated due to the assumed uniform collagen and drug distribution inside the collagen minirods. Subsequently, a fully swollen matrix is assumed in order to eliminate the effect of swelling on drug release. Separating both mechanisms of drug release in their mathematical modelling and numerical simulation seemed reasonable since they proceed on different time scales. To describe the interaction between collagenase and collagen properly, three steps have to be considered: diffusion of collagenase towards the collagen substrate, adsorption onto the collagen surface and subsequently collagen degradation (Fig. 1). The common reaction scheme for an enzymatically catalyzed degradation process was proposed by Michaelis and Menten [12] and can be summarized by:

$$\text{enzyme E} + \text{substrate S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{enzyme–substrate complex ES} \quad (1)$$

$$\text{enzyme–substrate complex ES} \xrightarrow{k_1} \text{enzyme E} + \text{product } P \quad (2)$$

where k_1, k_{-1}, k_2 are rate parameters: k_1 describes the adsorption of collagenase onto the collagen fibre, k_{-1} the dissociation of the ES complex without degradation (in general zero [3]) and k_2 the degradation process. The degradation is assumed to be the rate-limiting step, because equilibrium in adsorption was already obtained within 1 h (Fig. 2), whereas degradation lasts longer (Figs. 6b

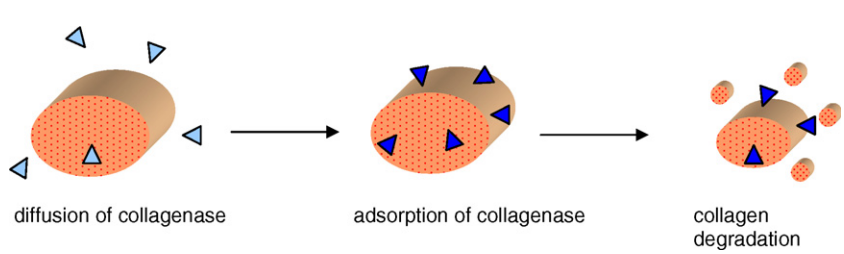

Fig. 1. Schematic illustration of the degradation of collagen minirods (△ free collagenase, ▲ adsorbed collagenase).

and 7b). This is in agreement with Tzafriri et al., who modelled the degradation of sparsely fibrillar collagen gels by human skin fibroblast collagenase [3]. Hydrolytic erosion of a solid polymer matrix can occur by two extreme mechanisms. Heterogeneous erosion is restricted to the surface of the device and the non-degraded carrier remains chemically intact during degradation. Homogeneous erosion, such as erosion of non-cross-linked collagen matrices, involves random cleavage at a uniform rate throughout the complete matrix. While the molecular weight of the polymer decreases steadily, the matrix can remain essentially mechanically intact until 90% of the polymer is degraded. Then mass loss and disintegration of the device start. In the model for the release of chlorhexidine gluconate from a cross-linked gelatine chip, Tzafriri assumed solely matrix bulk erosion [4]. Due to enzyme binding at distinct reaction sites and the dense matrix structure, which hinders diffusion of enzymes and degradation products, it would be incorrect to consider pure bulk erosion. A pure surface erosion, as was proposed by Tzafriri et al. for the degradation of dense collagen fibres [3], would also not reflect the complete digestion process properly, because we found that a fully swollen matrix showed a higher degradation rate than a still swelling device [40]. From these observations it was concluded that a limited collagenase penetration into the collagen matrix is possible. Consequently, for our dense collagen matrices both types of erosion were combined: surface erosion of the tropocollagens [13] and bulk erosion of the complete collagen extrudate. Further basic assumptions in the model are that the diffusion of collagenase towards the collagen surface is Fickian. Based on our experimental investigations we concluded that the Michaelis–Menten scheme in its standard form does not describe the degradation in sufficient detail. The degradation rate k_2 is dependent on the concentration of collagen and collagenase and increases with higher concentration of collagenase. Because the enzymatic reaction of an insoluble substrate is modelled, degradation is limited by the enzyme reaction sites on the surface of collagen. These effects are taken into account by Eq. (3).

$$\partial_t C_E - \nabla \cdot (D_E(C_C)\nabla C_E) + k_{act} C_E = -k_1 (C_E)^n C_C + k_2 C_{ES}^{\gamma} \text{ in } J \times \Omega \quad (3)$$

$$\partial_t C_{ES} = k_1 (C_E)^n C_C - k_2 C_{ES}^{\gamma} \quad \text{in } J \times \Omega \quad (4)$$

$$C_E = C_E^{ext} \quad \text{in } J \times \Gamma \quad (5)$$

$$C_E = 0 \quad \text{on } 0 \times \Omega \quad (6)$$

$$C_{ES} = 0 \quad \text{on } 0 \times \Omega \quad (7)$$

$$\partial_t C_C = -k_1 (C_E)^n C_C \quad \text{in } J \times \Omega \quad (8)$$

$$C_C = C_C^0 \quad \text{on } 0 \times \Omega \quad (9)$$

The concentrations C_E, C_{ES}, C_C of free enzyme E, enzyme–collagen complex ES and collagen C, respectively, are expressed in mole per volume. Ω is a bounded domain in IR^2 with a sufficiently smooth boundary $\Gamma = \partial\Omega \cdot J = (0, T)$ denotes a finite interval with final time T. In (3) k_{act} represents a constant rate modelling the loss in enzymatic activity over time in the incubation medium. In contrast to Tzafriri et al. [3], a Freundlich isotherm is implemented (see (8)), based on experimental results (see Section 4.2) which demonstrated that the Langmuir adsorption isotherm does not describe the adsorption process adequately. Further, n represents the index of the adsorption reaction, which is often set as a positive number (e.g. 2/3 [14]). The parameter γ describes the dependency of the degradation rate on the concentration of the ES complex. This relationship was investigated by determination of k_2 in dependency of the enzyme concentration (Fig. 5). The initial concentration of the enzyme and of the ES complex in the matrix is zero and at the boundary $C_E = C_E^{ext}$ is prescribed, where C_E^{ext} denotes the enzymatic concentration in the ambient medium. For the diffusion coefficient of the enzyme in the matrix (D_E) a Fujita like dependence on the concentration of collagen C_C is assumed (free volume theory [15]), i.e. $D_E = D_E^0 \exp(-\beta_E \frac{C_C}{C_C^0})$, where D_E^0 is the diffusion coefficient of the enzyme in water, C_C^0 is the initial concentration of collagen and β_E is a dimensionless parameter [16]. An almost free diffusion of enzyme inside the completely swollen matrix was assumed [4,17].

After matrix digestion, degraded collagen fragments P and drug molecules A are released by diffusion. Diffusion is instantaneously compared to the time scale of degradation [3]. This is described by the following set of equations:

$$\partial_t C_P - \nabla \cdot (D_P \nabla C_P) = k_2 C_{ES}^{\gamma} \quad \text{in } J \times \Omega \quad (10)$$

$$C_P = 0 \quad \text{in } J \times \Gamma \quad (11)$$

$$C_P = 0 \quad \text{on } 0 \times \Omega \quad (12)$$

Here, C_P denotes the concentration of the hydrolyzed collagen fragments which can diffuse out of the matrix. A constant diffusion coefficient of the collagen fragments is assumed and the initial concentration of the product in the matrix is zero.

According to Tzafriri [4], the drug load is composed of two pools: one part which can diffuse freely inside the swollen matrix and the other part which is immobilized by the polymer due to physical or chemical entrapment and can only diffuse after matrix degradation. Weadock drew a similar conclusion from adsorption studies [18] and assumed that drugs in collagen membranes exhibit a dual sorption behaviour; one drug fraction can diffuse freely inside the collagen membrane, whereas the diffusion of the other part is restricted because of reversible interactions with the collagen device. Since no chemical interactions with collagen could be detected for FITC dextrans, it was assumed that the drugs were only immobilized physically and drug liberation is proportional to the number of cross-links. Drug delivery is governed by a diffusion equation with a source term due to liberation of the immobilized drug by matrix degradation. Thus, writing a mass balance the following equation is obtained.

$$\partial_t C_A - \nabla \cdot (D_A(C_C)\nabla C_A) = -\partial_t C_{Ai} \quad \text{in } J \times \Omega \quad (13)$$

The concentrations of free (A) and immobilized drug (Ai), respectively, are denoted by C_A, C_{Ai}. The incorporated drug is released by a diffusion mechanism due to the con-

centration gradient. The diffusion is restricted because of the physical entrapment. This effect is represented by the source term $\partial_t C_{Ai}$. Due to simultaneously occurring matrix degradation, the matrix phase through which the diffusion takes place, changes continuously as a function of the extent of collagen hydrolysis. The diffusion coefficient of the drug within the matrix cannot be considered as constant, but as a function of the fluid and/or collagen concentration. According to the free volume theory [15] the diffusion coefficient is given by $D_A = D_A^0 \exp(-\beta_A \frac{C_C}{C_C^0})$, where D_A^0 denotes the diffusion coefficient of the drug in water and β_A a dimensionless constant [16].

To complete the model, a relation between the concentrations of free and immobilized drug is needed. A general functional dependency $C_{Ai} = \sigma C_C^{\vartheta}$ is assumed. The immobilizing capacity σ is a dimensionless constant, which was determined by release studies and corresponds to the number of hindering cross-links or entanglements per mole of the fully swollen substrate. A correlation factor ϑ is incorporated, because in contrast to Tzafriri [4], a non-linear relationship between the immobilized drug and substrate concentration was valid. The model is completed by implementing homogeneous Dirichlet boundary conditions. An overview of relevant parameters is given in Table 1. In [7], the mathematical model is studied in more detail, e.g. the sensitivity of the calculated results with respect to the model parameters is analysed carefully.

4. Results and discussion

4.1. Determination of enzymatic activity

For describing the in vitro collagenolytic degradation process properly, the loss in activity of collagenase during incubation was needed. After 24 h incubation at 37 °C in Tris buffer, pH 7.5, the enzymatic activity was reduced to 90% of the initial activity. Subsequently, the activity decreased to 80% after 48 h and remained constant at this level over 2 weeks. This was in agreement with Olde Damink who determined a loss in enzymatic activity of 10% after incubation of 24 h and 30% loss after 48 h incubation at 37 °C [19]. This degradation kinetic was considered in the data interpretation of sorption studies.

4.2. Sorption studies

The sorption isotherm of collagenase on collagen surface had to be determined in an equilibrium state. Therefore, the time dependency of adsorption was investigated. After a fast binding of collagenase, the adsorbed enzyme amount remained constant over 24 h (Fig. 2). It was assumed that within 1 h all accessible reaction sites were reached and subsequently the number of enzyme–substrate complexes was almost constant due to simultaneously occurring sorption and desorption processes. After cutting the collagen helix, collagenase diffuses on the collagen surfaces [20] towards binding sites within the matrix which becomes accessible due to matrix swelling and degradation. Collagenase will not be released before the number of binding sites became less than the enzyme molecules [20,21]. The amount of free collagenase in the medium was compared to the amount which could not be washed off the minirods with buffer. As can be seen in Fig. 2, part of collagenase can be removed and it was concluded that this amount was only entrapped in the collagen matrix, but not bound specifically onto the collagen surface.

Both Freundlich and Langmuir sorption isotherms could be used to describe the enzymatic adsorption fairly well. For degradation of gelatine by trypsin, both isotherms were described in the literature [14]. McLaren et al. postulated a Langmuir sorption isotherm based on the general assumptions that adsorption is faster than the enzymatic reaction, that the adsorbed enzyme does not interact with itself on the surface and that the affinity to all reaction sites is the same. On the other hand, with data from Tsuk et al. [22], McLaren suggested a Freundlich isotherm for gelatine degradation by trypsin because it was observed that a higher surface area was covered with enzyme than the actual surface area of the substrate [23,24]. For collagen degradation by collagenase the more appropriate approach seemed to be the Freundlich isotherm, as was described by Rubingh et al. for the degradation of Azocoll, a collagen-dye conjugate, by three different enzymes [25]. This is in agreement with Gaspers et al. [20] who postulated a non-uniformity of adsorption states of enzymes during degradation. For our data adsorption was also described best by a Freundlich sorption isotherm [40] (Fig. 3).

The sorption is incorporated in the mathematical model by equations Eqs. (3), (4) and (8). The sorption coefficient n was fitted and k_1 was calculated using $K_M = \frac{k_{-1}+k_2}{k_1}$ [3], with $k_{-1} = 0$.

4.3. Collagenolytic degradation rate

In general, the Michaelis–Menten kinetic describes reactions with saturation phenomena such as enzymatic degradation of soluble substrates [26]. Based on the original

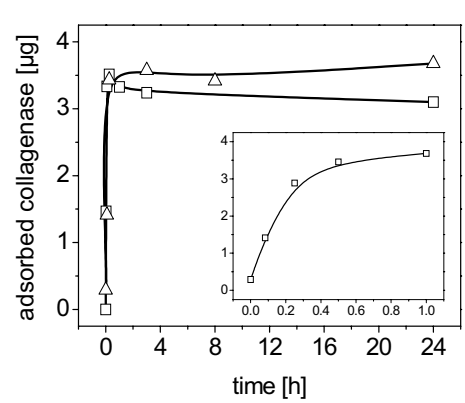

Fig. 2. Sorption of 5 µg/ml collagenase at 37 °C on 20 mg equine non-cross-linked collagen minirods (△ sorption on minirods; □ sorption on minirods after washing; $n = 3$; inset: sorption during the first hour).

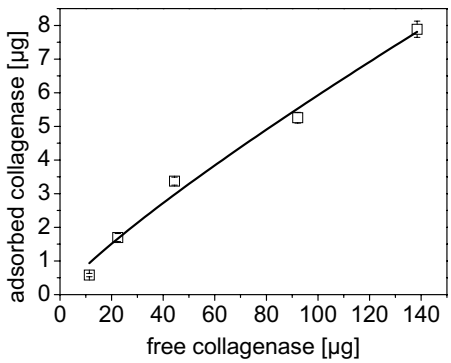

Fig. 3. Freundlich sorption isotherm fit for collagenase on 20 mg collagen minirods after 1 h incubation at 37 °C (average ± SD; $n = 3$).

Michaelis–Menten equation, McLaren proposed different approaches to describe the digestion of insoluble substrates properly. Assuming that the reaction rate is not only dependent on the initial concentration of enzyme, but on the concentration of adsorbed enzyme, the adsorption of enzyme on the surface of the substrate was inserted into the Michaelis–Menten equation. Different proposals for Langmuir and Freundlich sorption isotherms were made [14,23]. Huang developed a similar kinetic model for the degradation of cellulose [27]. Another approach was to extend the Michaelis–Menten equation by adding diffusion equations for the enzyme and released substrate fragments [28]. In the literature, several Michaels–Menten constants for bacterial collagenase digesting a soluble rat collagen type I gel were given (3.1–5.5 µM; $k_2 = 900$–$2100\ h^{-1}$ [29], 1.9 µM and $k_2 = 1000\ h^{-1}$ [30]). However, when fibrillar collagen was investigated, the enzymatic activity decreased [31,32].

To investigate whether it is sufficient to describe the degradation of insoluble collagen by using the Michaelis–Menten kinetic only, the degradation constants of collagen powder and minirods were determined. Typical Michaelis–Menten and Lineweaver–Burk diagrams were used to assess the degradation constants (Fig. 4). K_M for insoluble equine collagen powder and minirods were 2.55×10^{-5} mol/l and 3.8×10^{-5} mol/l, respectively. For comparison k_2 was used. The degradation rate of the collagen powder ($k_2 = 750\ h^{-1}$) was almost in the range of the k_2 values of soluble rat collagen type I gels [29,30]. Comparison with the reaction rate of the minirods ($k_2 = 174\ h^{-1}$) showed that the collagen powder was degraded faster than the minirods due to the less compact matrix structure, the higher relative surface area and consequently the higher amount of reaction sites directly accessible for collagenase. This assumption could be manifested by Bicsak et al. who investigated degradation of soluble and fibrillar collagen by tadpole back-skin collagenase [31]. McLaren also observed a higher k_2 for trypsin degradation of a gelatine solution than for a gelatine gel [14] and attributed this phenomenon to differences in the tertiary structure of the fibrils in solution and in the gel which hampers enzyme binding. According to these results, it was assumed that the enzymatic degradation of insoluble collagen is not described sufficiently by using the Michaelis–Menten equation only, because the enzymatic reaction rate of insoluble substrates is also dependent on the matrix surface. Consequently, the adsorption process of the enzyme (see Section 4.2) and the reaction rate of the cleavage itself had to be considered to characterise the enzymatic reaction properly. Furthermore, the digestion was also dependent on the added enzyme concentration. In contrast to soluble substrates [25,33], a non-linear relationship was determined (Fig. 5). In the mathematical model, these observations were incorporated by the term $k_2 C_{ES}^{\gamma}$ in Eqs. (3) and (8). The degradation constant k_2 was determined experimentally and set as $174\ h^{-1}$. γ was fitted. Beyond this, $K_M = 3.8 \times 10^{-5}$ mol/l was used to determine k_1.

4.4. Diffusion coefficients of FITC dextran 20 and 70 in water

The final parameters that were needed to close the mathematical model are the diffusion coefficients. The diffusion coefficients of FITC dextran 20 and 70 in water were determined by FCS. In these experiments, FITC dextran 70 models both, collagenase and a model protein drug. The diffusion coefficient at 23 °C was $1.7 \times 10^{-3}\ cm^2/h$. For

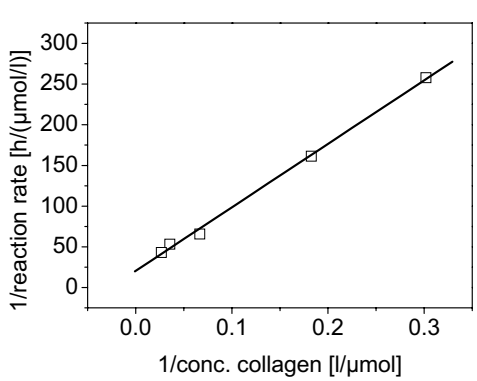

Fig. 4. Lineweaver–Burk plots of 2 µg/ml collagenase acting on collagen matrices ($n = 2$).

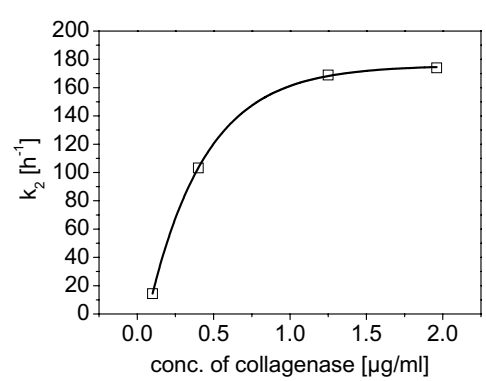

Fig. 5. Dependency of the degradation rate of collagen minirods on the concentration of collagenase.

FITC dextran 20, simulating a protein drug, 2.1×10^{-3} cm^2/h was obtained. The diffusion coefficient is particularly dependent on the temperature and the molecular size of the investigated drug. By applying the Stokes–Einstein equation the diffusion coefficient can be converted to 37 °C (FITC dextran 70: 2.4×10^{-3} cm^2/h; FITC dextran 20: 3×10^{-3} cm^2/h). These values were used in the mathematical model. To verify whether the diffusion coefficient of FITC dextran 70 could be used for modelling collagenase, measured data were compared with data published in the literature. Seifter et al. measured a diffusion coefficient of collagenase A (MW: 105 kDa) of 1.55×10^{-3} cm^2/h at 20 °C in water [34]. By conversion with the Stokes–Einstein equation a diffusion coefficient of 2.2×10^{-3}cm^2/h at 37 °C was derived. By two different methods, Gaspers et al. determined a diffusion coefficient of collagenase of 2.4×10^{-3} cm^2/h in solution [20]. Since the diffusion coefficients for collagenase, given in the literature, were in good agreement with the measured FITC dextran 70 diffusion coefficient, the results of FITC dextran 70 were used for modelling collagenase. Furthermore, the diffusion coefficient of the degradation products was needed. According to French et al. [35], degradation of the collagen triple helix results in fragments smaller than 70 kDa. Therefore, the diffusion coefficient of FITC dextran 70 was used as upper limit for the diffusion coefficient of the products as well.

4.5. Swelling of collagen minirods

Swelling of minirods was investigated with and without addition of collagenase. As can be seen in Fig. 6, the swelling of minirods incubated without collagenase could be divided into two phases. After a fast fluid uptake over approximately 5 h a swelling factor of 1.5 was reached. Over the next days the diameter increased by approximately 0.008/h. In comparison, minipellets containing atelocollagen [36] showed a fast swelling during the first 3 h, before the swelling was nearly constant over the next 24 h (increase in diameter 1.5–2, maximal change in weight approximately 300%). The change in weight was almost identical for our minirods (approximately 320% after 24 h). However, the swelling was delayed compared to minipellets. As was demonstrated by Fujioka et al. [5] and Maeda et al. [36], the swelling rate is influenced by both the manufacturing and drying method and decreased with higher initial collagen concentration. Both devices were produced by extrusion and were air-dried, but differ in the used collagen, insoluble equine collagen type I and soluble atelocollagen for minirods and minipellets, respectively, and the collagen concentration (40% (w/w) versus 30% (w/w) collagen). Subsequently, the delayed swelling of the minirods could be explained by the higher degree in natural cross-linking and the higher concentration of collagen.

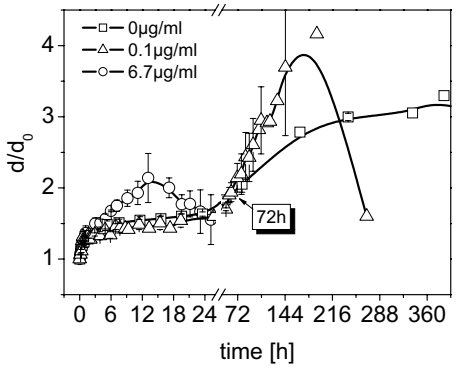

Fig. 6. Swelling profiles of collagen minirods in the presence of 0, 0.1 and 6.7 µg/ml bacterial collagenase, respectively, at 37 °C (72 h time point indicates point of separation in swelling profiles between 0 and 0.1 µg/ml enzyme; average ± SD; $n = 3$).

To investigate the influence of degradation on the swelling behaviour, minirods were incubated without enzyme, with 0.1 µg/ml (0.04 Mandl U/ml) and with 6.7 µg/ml (2.06 Mandl U/ml) collagenase. In general, the in vivo concentration of collagenase is not constant in all tissues and changes with disease. For example, intestinal collagenase is increased 65-fold in chronic foot ulcers compared to traumatic wounds [37]. In the literature, in vivo/in vitro – correlation is described sparsely. Okada et al. investigated the degradation of different kinds of Catgut in vitro and in vivo [38]. A correlation was found (in vitro concentration of bacterial collagenase: 2.5 µg/ml), but data for enzymatic activity were missing. Therefore, adaptation of this concentration had to be handled with care. Friess set 2.5 µg/ml (1.15 Mandl U/ml) as in vivo/in vitro concentration and concluded after subcutaneous implantation of dense collagen matrices in mice that this concentration was chosen too high [1]. Another approach was made by Yannas et al., who investigated the degradation of bovine tendon collagen tapes [39]. Good in vivo/in vitro – correlation was found by using 3.5 Mandl U/ml bacterial collagenase. Nevertheless, this enzymatic concentration appeared to be extremely high. According to own in vivo studies [40], a very slow degradation was observed, indicating an in vivo/in vitro – concentration of collagenase below 1.15 Mandl U/ml. To bracken the range of possible concentrations of collagenase, two extreme enzymatic concentrations were used to investigate the in vitro degradation of collagen minirods [40] and to observe the influence of enzymatic degradation on swelling. 6.7 µg/ml collagenase was set as the high end enzymatic concentration due to good analytical handling and fast matrix degradation and 0.1 µg/ml collagenase as the low end because of the detection limit of the EnzCheck®.

In general, the penetration of collagenase was very slow due to adsorption phenomena (see Section 4.2) and the dense matrix structure. Hence, the influence of the enzymatic degradation on swelling behaviour could be neglected for the first 3 d for low enzymatic concentrations which were used in release and degradation studies (Fig. 6). If incubation was performed with high concentrations of

collagenase, swelling was more pronounced during the first 12 h. The matrix became less dense because the pronounced cleavage of collagen fibrils led to faster disintegration. Since swelling is assumed to be a faster process than matrix degradation, 0.1 μg/ml collagenase was found to simulate in vivo circumstances best.

4.6. Release and degradation studies

Release was monitored for 6 d in absence of collagenase. On day 6 0.1 μg/ml collagenase was added, and release and degradation were determined in parallel. This experimental setup was applied to eliminate the influence of drug release by diffusion due to matrix swelling, to guarantee a fully swollen matrix and to obtain the parameters which were necessary to fit the part of the mathematical model dealing exclusively with the drug release due to matrix degradation. In order to investigate the influence of the molecular size of a drug on its release, FITC dextran 20 and FITC dextran 70 were studied. Looking at the complete release behaviour, approximately 80% of FITC dextran 20% and 70% of FITC dextran 70 were released during the first 48 h followed by no further significant release over the next 4 d for both model compounds when no collagenase was added (Fig. 7a). Consequently, the molecular size of the drug only influenced the extent of the initial release by diffusion due to physical hindrances within the minirod. After that time interval, swelling was completed (see Section 4.5) and the molecular size had no further significant influence on the release kinetic. This was in agreement with the measured diffusion coefficients in water which were nearly identical for both compounds (see Section 4.4). Furthermore, Fujioka et al. postulated that there must not be a relationship between the molecular size of proteins and their in vitro release rate observed in this phase of release [6]. After 6 d, the freely diffusible drug portion was released and, after addition of collagenase, the remaining drug portion was released over 5 h (FITC dextran 20) and 6 d (FITC dextran 70), respectively. This portion was entrapped within the matrix and could only be released after matrix degradation. Again, the first drug portion was released fast. The more prolonged release of FITC dextran 70 could be explained by the larger size of the compound. Due to the naturally cross-linking of collagen, which also limited the FITC dextran 70 release without enzyme to 70%, the release in presence of collagenase was more hampered than the release of FITC dextran 20. This could be demonstrated by comparing the degradation grade at the point of complete drug release. For FITC dextran 70, the matrix had to be degraded for approximately 85%, whereas for minirods loaded with FITC dextran 20 a degradation of 40% was sufficient. Degradation started after a short lag phase of approximately 0.5 h which was necessary to reach equilibrium in adsorption of collagenase on the matrix surfaces (see Section 4.2). Subsequently, the minirods were degraded in two steps. During the first 8 h a rapid linear degradation occurred. Afterwards, the degradation rate slowed down due to a decrease in enzymatic binding sites on the collagen surface (Fig. 7b). Since both minirods were prepared from the same collagen material, a similar degradation profile was observed. Matrices were degraded completely after 6 d. A similar "two step degradation" was described by Etherington for the degradation of insoluble collagens by cathepsins and pepsin [41]. Furthermore, the influence of different aspect ratios (length/diameter) was investigated. Minirods with a higher aspect ratio exhibited a slower release of FITC dextran 70 (Fig. 8a). The release during the first 48 h could be decreased from approximately 70% towards 45%. Additionally, the entrapped drug portion after 6 d can be increased from 25% to 50%. Due to extrusion, collagen fibres were highly orientated in the drug device. With elongating the device, the portion of the lateral side surface increased relatively. This was in agreement with Maeda et al. [36] who postulated that the decrease in delivery was a result of a slower release from the smoother lateral side surface of the minipellet than from the more porous cross-sections. Consequently, for adaptation of minirods for in vivo usage, the release kinetic could be controlled by adjusting the dimensions of the minirods. Degradation was independent of the amount of collagen or surface of the minirods for all three aspect ratios (Fig. 8b). Again, a two step degradation as was described by Etherington was observed [41].

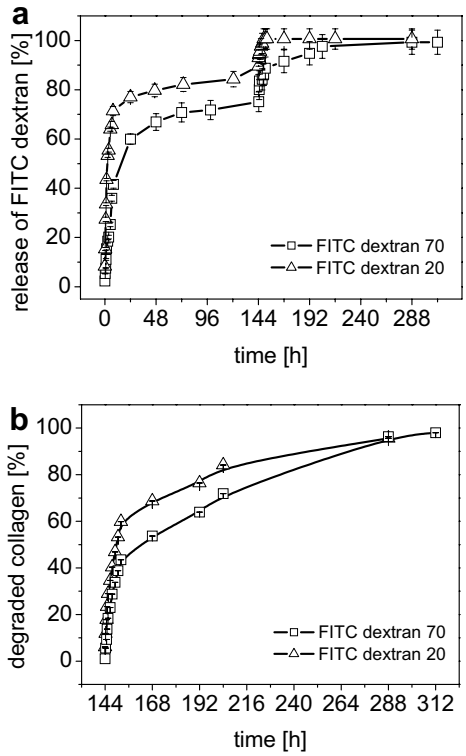

Fig. 7. Effect of the molecular weight of FITC dextran on release (a) and collagen degradation (b) of 10 mm equine non-cross-linked minirods (0.1 μg/ml enzyme was added after 6 d; average ± SD; $n = 3$).

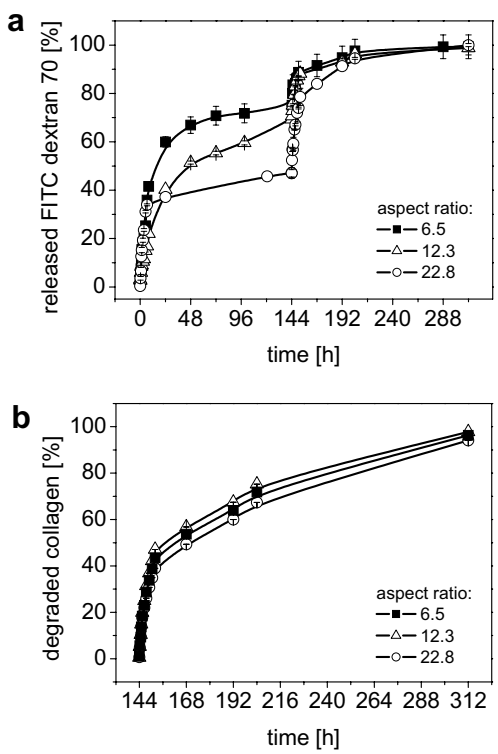

Fig. 8. Effect of the minirods' length on FITC dextran 70 release (a) and collagen degradation (b) of collagen minirods (0.1 μg/ml enzyme was added after 6 d; 10 mm (aspect ratio of 6.5); 20 mm (aspect ratio of 12.3); 40 mm (aspect ratio of 22.8); average ± SD; $n = 3$).

4.7. Mathematical model

In Section 3, a mathematical model to describe drug release from collagen matrices undergoing enzymatic degradation was developed (parameters see Table 1). In order to perform computer simulation based on this model, the equations were discretized using in space the mixed finite element method and an implicit scheme in time (backward Euler). More precisely, the lowest order finite elements of Raviart–Thomas type were used (for details see [7,42]). The mixed finite element method locally preserves mass which is an appreciable advantage of this approach. The Eqs. (3)–(12) are fully coupled and therefore solved simultaneously by a damped version of Newton's method. The linear systems of the Newton iteration are solved by a multigrid algorithm. Having determined the concentration of the collagen at the nth discrete time point, the drug release equation can be solved and it can be proceed to the next time step. The algorithm was implemented in the software package (UG, version 3.8, see also [43]), and two- or three-dimensional computations can be performed. Two-dimensional simulations were performed on a SUN BLADE 1000 workstation.

To validate the mathematical model a relevant test was developed to compare the experimental data with the numerical simulations. A cylindrical collagen matrix with the length l and diameter d was used. For the two dimensional simulation the cross section Ω of a disc with diameter $d = 0.278$ cm is taken, but very similar results are obtained by simulating a vertical section. The initial concentration of collagen in the matrix was $C_C^0 = 1.12$ μmol/cm^3, while the concentration of enzyme at time $t = 0$ was set to zero. The diffusion coefficient of enzyme in the polymer matrix, measured by FCS (see Section 4.4), was determined to be $D_E^0 = 2.4 \times 10^{-3}$ cm^2/h. In this experimental setup, the dimensionless parameter β_E was set as 1.0. The second part of the experiment (Fig. 7, FITC dextran 70) was mathematically simulated, in order to guarantee a fully swollen matrix and to plot exclusively the drug release by matrix degradation. At the beginning, the enzyme concentration in the ambient fluid was $C_E^{ext} = 1.4 \times 10^{-6}$ μmol/cm^3. It was observed that after approximately seven minutes the enzyme was absorbed at the polymer matrix. In a first stage, every 30 min the matrix was placed in a new solution containing again enzyme at $C_E^{ext} = 1.4 \times 10^{-6}$ μmol/cm^3 and the released mass of collagen was measured. After approximately 8 h the solution was changed only every 12 h. In the model these features are captured by implementing time depending Dirichlet boundary conditions. The catalysis rate was experimentally determined to be $k_2 = 174$ h^{-1}. k_1 was calculated according to Tzafriri [3] by the relation $k_1 = \frac{k_2}{K_M}$. A value of $k_1 = 4579$ cm^3/(h × μmol) was obtained. The parameters $n = 0.681$ and $\gamma = 0.44$ were mathematically fitted. The simulation of the mass of degraded collagen versus the experimental data are plotted in Fig. 9a. The experimentally measured and numerically calculated data are in a

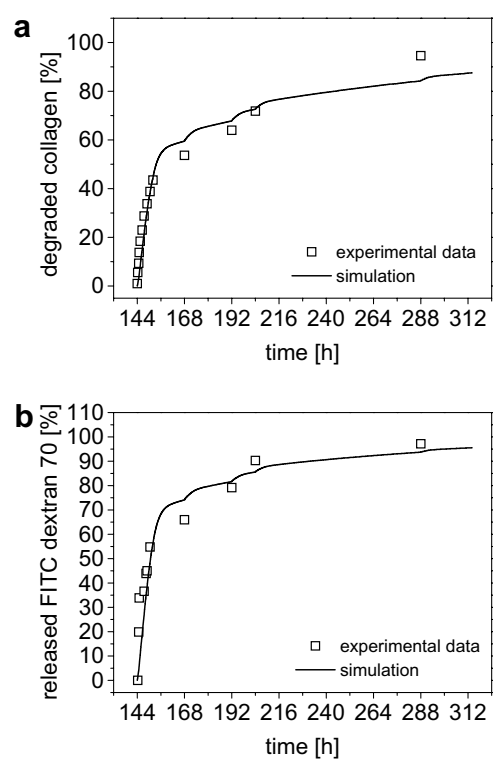

Fig. 9. Simulation of collagen degradation (a) and FITC dextran 70 release (b) of 10 mm equine non-cross-linked minirods (0.1 μg/ml enzyme was added after 6 d; dots represent experimental data).

very good agreement. In the next step, the drug release due to the matrix degradation was investigated. The same matrix as before, now loaded with uniformly distributed FITC dextran 70, was considered. An initial concentration of FITC dextran 70 of $C^0_{Ai} = 1.36\ \mu mol/cm^3$ was assumed, which is supposed to be physically immobilized. The parameter $\vartheta = 1.5$, which describes the dependence of C_{Ai} on C_C, was mathematically fitted. The constant σ in $C_{Ai} = \sigma C^{\Theta}_C$ is completely determined by knowing ϑ, C^0_{Ai} and C^0_C. Finally, as in the case of enzyme, the diffusion coefficient of drug in water was determined to be $D^0_A = 1.7 \times 10^{-3}\ cm^2/h$ and β_A was set as 1.0. The results of the numerical simulation and the experimental data are plotted in Fig. 9b. Again a very good agreement between simulated and experimental results was noticed. As an intrinsic result of the numerical simulation, concentration profiles of collagen, enzyme, enzyme–collagen complex, product and drug can be visualized at interesting time points (Fig. 10). These concentration profiles can be used for a better understanding of the complex processes which occur simultaneously. The collagen concentration decreased steadily from the boundaries to the interior of the minirod (Fig. 10a). Simulations demonstrated that the minirods were degraded throughout the complete device without a dramatic change in dimensions. This is consistent with the assumption that dense collagen implants undergo bulk erosion. The concentration profile of FITC dextran 70 inside the collagen minirods showed that drug delivery started already before collagen degradation occurred (Fig. 10b). This observation confirmed the assumption that the incorporated FITC dextran 70 could be divided into two drug pools, a mobile drug fraction, predominantly released by diffusion processes in the beginning, and immobilized drug molecules which could only be released in the course of matrix erosion. For further details on the mathematical model and the numerical techniques, in particular, a sensitivity study, refer to [7]. Basically, sensitivity of the calculated profiles was observed only with respect to parameters describing the non-linearities in the model, namely parameters n and γ of Eqs. (3)–(12). Simpler models of the literature, without such non-linearity (especially assuming a linear cleavage process, i.e. $\gamma = 1$), would not describe the processes adequately because of a much more slower degradation as compared to the experimental results. The mathematical fit of one of the fitted parameters (see Table 1) does not modify the degradation and release profiles completely in their shapes. Hence, the good agreement between experimental and numerical data is not achieved by an appropriate fit of parameters but is due to the capability of the model to describe enzymatic matrix degradation and simultaneous drug release properly.

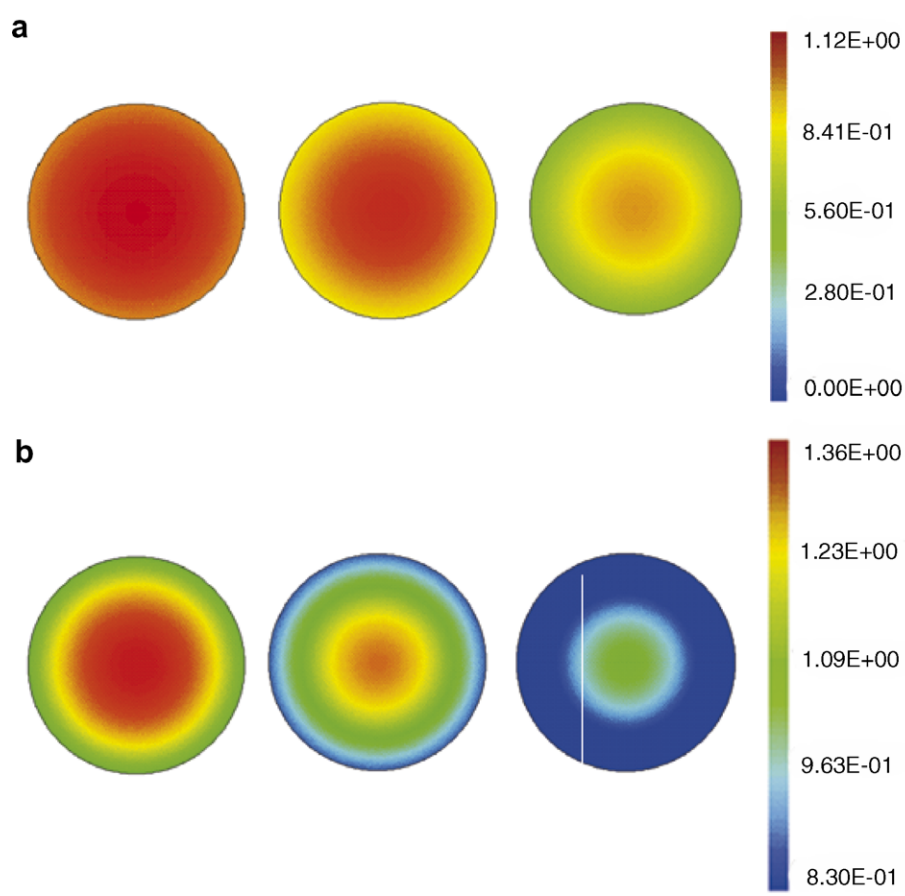

Fig. 10. Simulated concentration profiles of collagen (a) and FITC dextran 70 (b) in the matrix after 30, 60 and 120 min.

5. Conclusion and further prospective

In summary, it was demonstrated in this study that the release of FITC dextran from dense collagen devices depends on diffusion, swelling and erosion processes. It could be confirmed that the incorporated drug was composed of two pools. One part which was released due to diffusion and swelling processes and another population which was immobilized inside the collagen matrix and could only be delivered after device erosion. Studying the matrix erosion in more detail showed that collagen was degraded in vitro by bacterial collagenase following a Michaelis–Menten kinetic which is dependent on a Freundlich sorption. Furthermore, the dependency of the release profile on the molecular weight of the incorporated model compound and the matrix dimensions was investigated. Increasing either the molecular weight of FITC dextran or the length of the minirods led to a decrease in drug delivery. Using experimental and a comparatively small number of fitted data, a mathematical model was established, implemented and tested that describes the drug release of model drug compounds from collagen minirods during matrix degradation. Good correlation between numerical simulation and experimental data was observed for fully swollen matrices. The next step in developing a model which covers the complete drug release of a biodegradable collagen implant properly is to combine the model for drug release by diffusion [2] and the model presented in this paper.

Acknowledgements

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