Degradability of Polysaccharides Multilayer Films in the Oral Environment: an in Vitro and in Vivo Study

ARTICLE *in* BIOMACROMOLECULES · JANUARY 2005

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Degradability of Polysaccharides Multilayer Films in the Oral Environment: an in Vitro and in Vivo Study

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Received September 14, 2004; Revised Manuscript Received November 23, 2004

Biomedical devices and modified biomaterial surfaces constitute an expanding research domain in the dental field. However, such oral applications have to face a very particular environment containing specific physiological conditions and specific enzymes. To evaluate their suitability in the development of novel oral applications, the degradability of polyelectrolyte multilayer films made of the natural polysaccharides chitosan and hyaluronan (CHI/HA) was investigated in vitro and in vivo in a rat mouth model. The films were either native or cross-linked using a water-soluble carbodiimide (EDC) in combination with *N*-hydroxysulfosuccinimide. The in vitro degradation of the films by different enzymes present in the oral environment, such as lysozyme and amylase, was followed by quartz crystal microbalance measurements and confocal laser scanning microscopy observations after being film labeled with CHI^{FITC}. Whereas native films were subjected to degradation by all the enzymes, cross-linked films were more resistant to enzymatic degradation. Films were also put in contact with whole saliva, which induced a slow degradation of the native films over an 18 h period. The in vivo degradation of the films deposited on polymer disks and sutured in the rat mouth was followed over a 3 day period. Whereas film degradation is fast for native films, it is much slower for the cross-linked ones. More than 60% of these films remained on the disks after 3 days in the mouth. Taken together, these results suggest that the multilayer films made of natural polysaccharides are of high potential interest for oral applications, especially as drug release systems, offering various degradation rates and consequent release characteristics.

Introduction

Polyelectrolyte multilayer (PEM) coatings have become a new and general way to functionalize surfaces whose applications range from optical devices to biomaterial coatings.[1,2] The technique is based on the alternate deposition of polyanions and polycations.[3,4] Film functionalization can be achieved by incorporating particles[5] or bioactive molecules into the architecture.[6,7] Various types of films can be prepared using synthetic polyelectrolytes, such as poly(styrene sulfonate) (PSS) or poly(allylamine hydrochloride)[3,8] synthetic polypeptides, specifically poly(L-lysine) (PLL) and poly(L -glutamic) acid (PGA),[9,10] or even natural polyelectrolytes, such as dextran, alginate, heparin, hyaluronan, and chitosan.[11−13] On account of their biocompatibility and nontoxicity, these latter films constitute a rapidly expanding field with great potential applications: preparation of biomimetic films,[11,14] drug release vehicles,[13,15] bioactive coatings either by drug incorporation or by the use of the intrinsic properties of the polyelectrolytes,[16,17] or build-up of cell adhesive or nonadhesive films.[11,18]

Despite the incorporation of precise functionalities into PEM films, only a few examples of functional multilayer assemblies designed to release incorporated materials have been described.[19,20] This can be achieved by gradually decomposing the film. Most of these decomposable assemblies concern synthetic polyelectrolytes, and the release mechanisms rely on the physicochemical properties of the film. Usually, the dissolution of PEM films is a consequence of a change in environmental pH (for hydrogen bonded multilayers[20] or a change in ionic strength.[19,21] Dubas and Schlenoff[21] and Schüler and Caruso[19] have demonstrated that salt induced dissolution can occur in high salt containing solutions. However, the transitions from stability to dissolution for these films are very rapid, and it does not appear to be feasible to control either the degradation rate or such decomposition in physiological conditions. Recently, Vazquez et al.[22] used a hydrolytically degradable polyelectrolyte, poly(β-amino-ester), in combination with poly(styrene sulfonate) (PSS) to build a film that is degradable in an aqueous environment. In these experiments, the film was degraded within about a day.

An alternative way to create biodegradable films for biomedical applications is to make use of the intrinsic properties of natural polyelectrolytes and of the potential

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10.1021/bm049425u CCC: $30.25
Published on Web 01/27/2005

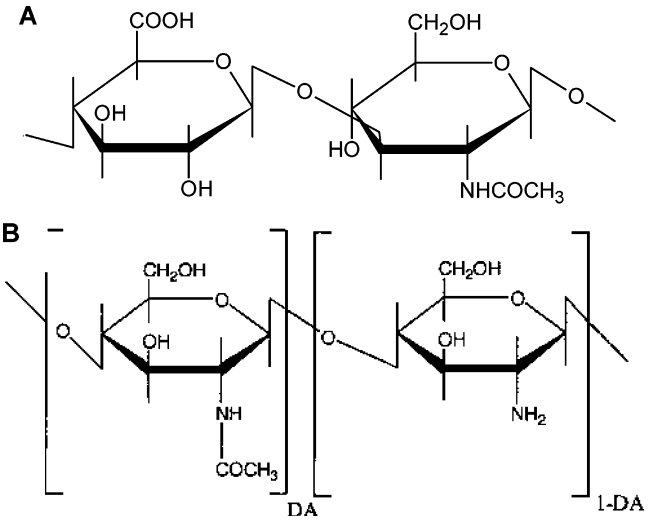

Figure 1. Molecular structures of the hyaluronan (A) and chitosan (B) repeating units. DA is the degree of acetylation of chitosan.

presence in vivo of different enzymes in biological fluids. For instance, all the natural polyelectrolytes and proteins present in tissues and fluids can be cleaved by specific enzymes: this is the case for collagen by collagenase, for hyaluronan by hyaluronidase,[23] for chitosan by chitosanase, and other enzymes. Serizawa et al. started to explore such possibilities by investigating, using quartz crystal microbalance, the degradation of chitosan/dextran sulfate films in the presence of chitosanase.[24] They found a more rapid hydrolysis when dextran sulfate constitutes the outermost layer of the film. These authors also demonstrated that DNase can hydrolyze DNA/poly(diallyl dimethylammonium chloride) films in a controlled manner provided that the concentration of both Mg^{2+} and Ca^{2+} ions in the medium is adjusted.[25] If the films are to be used in a biomedical application, the nature of the enzymes present in vivo must be taken into consideration. This will largely depend on the nature of the fluid in contact with the coated material in its specific location. In the oral environment, biomaterials will be in contact with saliva, which contains many proteins and enzymes such as lysozymes and α-amylase.[26] Polyelectrolyte multilayer films can be used for many potential oral bioapplications such as antimicrobial protection[27,28] or antiinflammatory protection.[29] Most biomedical applications are dependent on two factors, the biocompatibility of the multilayer films and the stability of the films in vivo, especially for a controlled release of a peptide or other molecule. The biocompatibility of various polyelectrolyte multilayers made of polysaccharides and polypeptides has already been evaluated.[7,17]

In the present work, we have investigated the degradability properties of chitosan/hyaluronan (CHI/HA) films in vitro and in vivo in the oral environment over several days. Chitosan is obtained after N-deacetylation of chitin by alkaline treatment (Figure 1), chitin being the second most abundant naturally occurring polysaccharide.[30] Chitin is found in crustacean and insect exoskeletons and is also synthesized by some unicellular organisms. Hyaluronan (HA) (Figure 1) is a highly hydrated polysaccharide of great biological interest.[31] It possesses lubricating functions in the cartilage and participates in the control of tissue hydration, water transport, and in the post-traumatic inflammatory response. It is widely used in cosmetic formulation and shows promise in tissue engineering applications.[32,33] These two polysaccharides are biocompatible, nontoxic, and biodegradable by enzymatic hydrolysis with chitosanase,[24] α-amylase,[26] lysozyme, and hyaluronidase.[34] Both have already been widely used in biomedical applications and have interesting intrinsic properties.[35] Chitosan in solution or as a hydrogel is particularly used in pharmaceutical drug formulations, in sustained release of water-soluble drugs,[30,36] and also exhibits antibacterial and antimicrobial activity.[37,38] Tissue engineering based on chitosan and hyaluronan hydrogels is also promising.[39,40] Chitosan and hyaluronan can be easily chemically modified[41−43] and bonded to various molecules such as cell-targeted prodrugs[44] and carbohydrates,[45] which could be released upon film hydrolysis.

Chitosan and hyaluronan have already been used for oral applications as hydrogel or membranes.[37,46] Recently, we showed that these polysaccharides can also form PEM films in acidic conditions in the presence of 0.15 M NaCl. Because the film growth is exponential, film morphology and thickness can be estimated by confocal laser scanning microscopy using fluorescently labeled chitosan. The hydrolysis activity of whole saliva on these films, as well as, specifically, lysozyme and α-amylase enzymatic activity, will be more precisely investigated. Lysozyme (14 kDa) is able to hydrolyze chitin and chitosan.[47] α-Amylase (45−60 kDa) is an abundant salivary enzyme that catalyzes the hydrolysis of α(1,4) glycosidic bindings between glucose residues of polysaccharides. Amylase has already been found to hydrolyze chitosan in solution.[47] For our in vivo experiments, the films were deposited on polymer disks and sutured in the oral cavities of rats. Film cross-linking was performed using a water soluble carbodiimide[48] to investigate the possible relations between physicochemical properties of the film and modifications in its biodegradability.

Materials and Methods

Polyelectrolyte and Enzyme Solutions. HA (sodium hyaluronate, 4×10^5 g/mol) was purchased from Bioiberica (Spain). HA is a polyanionic macromolecule with a $pK_a \approx 2.9$[32] and with a negative charge at pH = 4.5. It has a low charge density since only one residue from two is charged.[49] CHI (chitosan, oligo-saccharide of low molecular weight, LMW = 5×10^3 g/mol) was purchased from Medipol (Switzerland). According to the manufacturer, the supplied degree of acetylation (DA) is below 20%. CHI is a weak base, a positively charged polyelectrolyte in acidic condition with a $pK_a \approx 6$.[49,50] Sodium dodecyl sulfate (SDS) was purchased from Sigma, and sodium chloride (purity 99.5%) was obtained from Fluka (St. Quentin Fallavier, France). All solutions were prepared using ultrapure water (Milli Q-plus system, Millipore) with a resistivity of 18.2 MΩ cm. Fresh polyelectrolyte solutions at 1 mg/mL were prepared by dissolution of polymer in filtered saline solutions. CHI and HA were dissolved at 1 mg/mL in 0.15 M NaCl in water and were gently stirred overnight. The pH of the polyelectrolyte solutions was adjusted to 4.5 with 0.1 M acetic acid. For both polyelectrolytes, taking into account their molecular

weight, the concentrations were below the critical concentration c^*.[51,52] $(CHI/HA)_i$ architectures were built with i number of deposited layer pairs. Fluorescently labeled chitosan (CHI^{FITC}) was prepared according to a published protocol.[11]

Lysozyme (L6876, Sigma) and α-amylase (A4551, Sigma) were prepared in the NaCl solution at pH 5 at 1 mg/mL and 500 U/mL, respectively. Natural saliva was obtained from human after stimulation by paraffin chewing and used immediately at 37 °C. For long-term observations, saliva was changed every 12 h.

Automatic Buildup of the Polyelectrolyte Multilayered Films. For confocal microscopy, fluorimetry, and in vivo experiments, $(CHI/HA)_i$ films were prepared with an automatic dipping machine (Dipping Robot DR3, Kirstein and Riegler GmbH, Germany) on 12 mm glass slides (VWR Scientific, France) cleaned with 10 mM SDS and 0.1 N HCl and extensively rinsed. The glass slides were introduced vertically in a custom holder which was dipped for 15 min into a first polyelectrolyte solution (CHI, 12 mL) and subsequently rinsed in three different beakers containing the 0.15 M NaCl solution at pH = 4.5. The slides were dipped in the first rinsing beaker (350 mL) and once for 150 s in the two other rinsing beakers (40 mL each). The slides were then dipped into the oppositely charged polyelectrolyte solution (HA, 12 mL) followed by the same rinsing procedure. The robot was programmed to move to fresh rinsing solutions after each deposition of six layers. Slides were then stored at 4 °C until use in 24-well culture plates.

Film Cross-Linking Procedure and Characterization by Fourier Transformed Infrared Spectroscopy. For the chemical cross-linking of the films, a previously published protocol that was applied to $(PLL/HA)_i$ films[48] was followed. It is based on the reaction of activated carboxylic sites with primary amine groups[53] in the presence of a water soluble carbodiimide, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) and of *N*-hydrosulfosuccinimide (sulfo-NHS) (both purchased from Sigma). Briefly, EDC and sulfo-NHS were prepared at 400 and 100 mM, respectively, in a 0.15 M NaCl solution at pH 4.5. One mL of the mixed EDC/Sulfo-NHS solution (v/v) was deposited in the wells containing the $(CHI/HA)_{24}$-CHI^{FITC} coated glass slides or in 1.5 mL tubes containing polymer disks and left for 12 h at 4 °C.

For film cross-linking characterization, $(CHI/HA)_9$ films deposited on a ZnSe crystal were investigated by in situ Fourier transform infrared (FTIR) spectroscopy in attenuated total reflection (ATR) mode with an Equinox 55 spectrophotometer (Bruker, Wissembourg, France). All the experimental details have been given previously.[54] The experiments were performed in deuterated 0.15 M NaCl solution at pH ≈ 4.5. The parameters and configuration used for the acquisition during the cross-linking reaction have already been detailed.[48] The films were cross-linked with the EDC/NHS solution, and spectra were acquired before and after cross-linking.

Film Degradation Analysis by Quartz Crystal Microbalance (QCM). The $(CHI/HA)_i$ film build-up process and its subsequent degradation by enzymes and by saliva were followed by in situ quartz crystal microbalance (QCM-Dissipation, Qsense, Sweden).[55,56] The quartz crystal is excited at its fundamental frequency (about 5 MHz) as well as at the third, fifth, and seventh overtones (corresponding respectively to 15, 25, and 35 MHz). Changes in the resonance frequencies δf and in the relaxation of the vibration once the excitation is stopped are measured at the four frequencies. The apparatus also gives access to the dissipation D of the vibrational energy stored in the resonator. From these parameters, the film thickness can be determined using the model developed by Voinova et al.[57] $(CHI/HA)_6$ films were built at 25 °C by successive injections of 500 μL of the polyelectrolyte solutions in the measuring cell (12 min adsorption for each layer) and a subsequent rinsing with 500 μL of the NaCl solution (injected in about 10 s). This number of layer pairs was chosen to ensure that at least two frequencies/dissipations (5 and 15 MHz) could be acquired during the whole experiment. After film build-up, the temperature was raised to 37 °C in 2 °C steps over about 2 h, and 1 mL of the enzyme or saliva solutions was injected and left at rest overnight at 37 °C.

Film Degradation Observations by Confocal Laser Scanning Microscopy (CLSM). For the CLSM experiments, film coated glass slides were prepared with the dipping robot. CHI^{FITC} was adsorbed as the ending layer. As an example, $(CHI/HA)_{24}$-CHI^{FITC} corresponds to a film composed of 24 pairs of layers on top of which a final CHI^{FITC} layer has been deposited. The configuration of the microscope and the parameters used for the CLSM observations on a Zeiss LSM510 microscope have been given elsewhere.[58] One mL of the enzyme solution or of saliva was deposited on the film coated glass slides (introduced in a 24-well culture plate) and kept in an incubator at 37 °C for a given period of time (from 3 to 24 h). For the observations, the 12 mm glass slides were introduced in a custom chamber and observed by imaging series of consecutive overlapping optical sections ($x-y$ images at different depth z). Orthogonal vertical sections were computed to image an ($x-z$) section of the film.

In Vivo Studies in Rat Mouth. In vivo studies were conducted on eight male Wistar rats following the principles of animal welfare. Sixteen heat-cured poly(methyl-methacrylate) (PMMA) disks, 1 mm thick × 4 mm diameter (Probase, Ivoclar, Lichtenstein), were prepared as buttons with two holes at their center to facilitate the suture. The disks were divided into two groups: eight disks were covered with a native $(CHI/HA)_{24}$ film, and eight disks were covered with a cross-linked $(CHI/HA)_{24}$ film using the automatic dipping machine (DR3, Kirstein GmbH, Germany). To obtain a well-coated surface, a precursor film composed of poly(ethylene imine) (PEI), poly(styrene sulfonate) (PSS), and poly(allylamine hydrochloride) (PAH) (i.e., PEI-(PSS/PAH)-PSS) was first deposited on the polymer disks. For each animal, one disk was fixed with a polyglactine 910 suture (3/0 VICRYL, ETHICON, Johnson and Johnson Intl., Belgium) to a zone in the cheek between the molars and the incisors to prevent any risk of extraction by teeth. Six out of the 16 disks were not recovered, suggesting that there was a strong mechanical tongue activity around them. Ten disks were recovered: six disks after 6 h (three of each group), two disks after 48 h (one of each group), and two

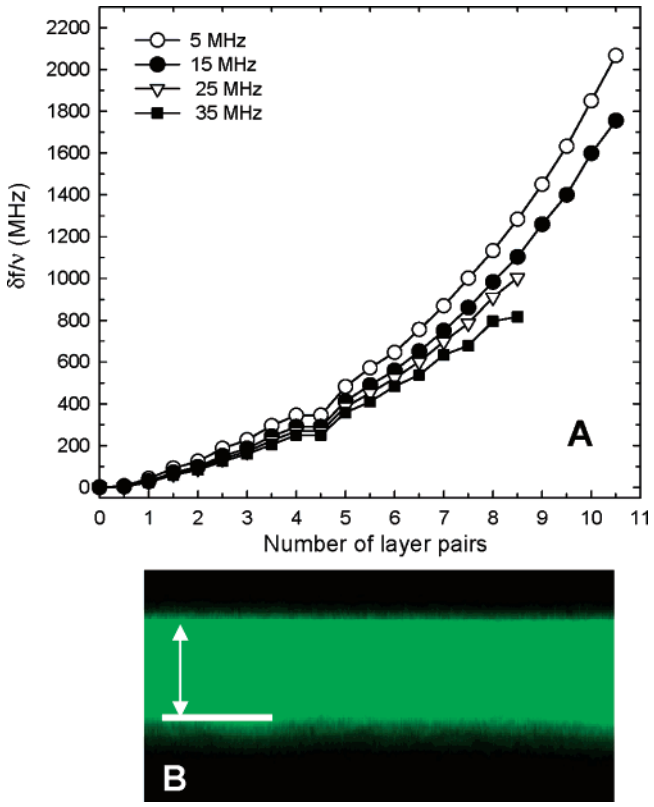

Figure 2. Film growth followed by QCM-D for a $(CHI/HA)_{10}$-CHI film built in 0.15 M NaCl at pH = 4.5 (CHI, MW = 5×10^3 g/mol; HA = 4×10^5 g/mol). Confocal laser scanning microscopy observation of a $(CHI/HA)_{24}$-CHI^{FITC} film built in the same conditions. Film thickness is about 6.5 μm (line).

disks after 72 h (one of each group). All disks were stored in a 0.15M NaCl solution and viewed under epifluorescent illumination (excitation filter, 480/40 nm; dichroic filter, 505 nm; and emission filter, 510 nm) with a Leica MZFLIII stereomicroscope and also by confocal laser scanning microscopy. The stereomicroscope images were analyzed by Image J software (NIH, Bethesda, MD) to determine the film area remaining on the disk, which was divided by the total disk area (the result being explained in percentage).

Results and Discussion

Film Characterization and Cross-Linking. Because (CHI/HA) film growth is exponential (Figure 2A), film thickness rapidly reaches several micrometers for film containing a few tens of layers. The mean thickness (±SE) of a $(CHI/HA)_6$ film was estimated at 175 ± 18 nm by QCM (mean of three independent experiments). For high numbers of deposited layers and much thicker films (of the order of at least 1 μm), confocal microscopy is used to determine the film thickness. This is done after the film is labeled with CHI^{FITC} as ending layer, and on *z*-sections obtained by CLSM, the green band corresponding to CHI diffusion being measured.[11] For a $(CHI/HA)_{24}$-CHI^{FITC} film with CHI of MW = 5kDa, the film thickness is of about 6 μm (Figure 2B).

To investigate the possibility of cross-linking the CHI/HA films, a protocol based on the reaction of activated carboxylic sites with primary amine groups[53] in the presence of a water soluble carbodiimide, EDC, in combination with sulfo-NHS, was used. The cross-linking reaction in the

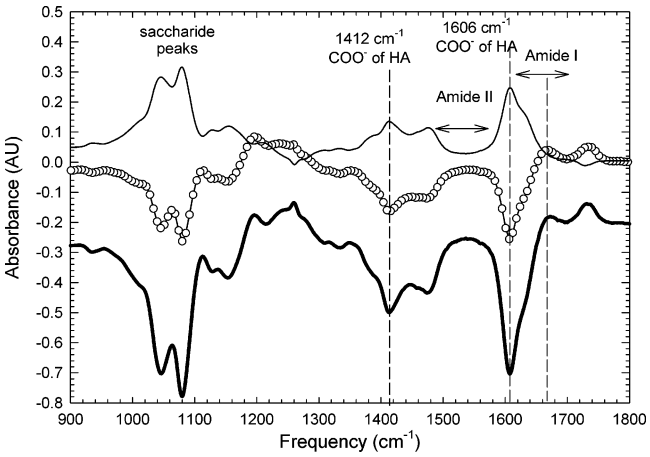

Figure 3. Film cross-linking followed by FTIR-ATR: spectra of a native $(CHI/HA)_8$ film (thin line) and of the same film after the cross-linking procedure and the final rinsing step (-○-). The difference between the two spectra (before and after cross-linking) is also represented (thick black line shifted downward for a better visualization).

presence of EDC/sulfo-NHS was more precisely followed by FTIR-ATR. This technique was a powerful tool for following the cross-linking reaction kinetics for PLL/hyaluronan films[48] and for poly(L-lysine)/poly(L -glutamic) acid (PLL/PGA) films.[59] The detailed mechanism of the cross-linking reaction with EDC combined to sulfo-NHS has previously been given.[48] By FTIR, carboxylate peaks, saccharide peaks, and amide bands can be unambiguously identified. Figure 3 shows a typical spectrum of a $(CHI/HA)_8$ film deposited on a ZnSe crystal before contact with the EDC/NHS solution. The peaks attributed to asymmetric and symmetric -COO- stretches (1606 and 1412 cm^{-1}, respectively) from HA can be clearly identified.[60] The amide I band from HA in D_2O appears in the 1630−1700 cm^{-1} region,[60] and the C═O band for chitosan appears at around 1620 and 1660 cm^{-1}.[61] Characteristic bands of saccharide peaks representative of skeletal vibrations include the C−O stretching at 1082 and 1032 cm^{-1} and that at 1159 cm^{-1}.[61,62]

After the film has been brought in contact with the EDC/NHS solution for 10 h, the spectrum has changed. In particular, the intensity of the peaks attributed to the carboxylic groups (1606 and 1412 cm^{-1}) has decreased, and at the same time, the intensity of the amide band around 1660 cm^{-1} has increased. This change identifies the reaction between the ammonium groups of CHI and the carboxylic groups of HA. The characteristic bands of the saccharide peaks also decrease during the cross-linking reaction. This suggests the formation of other bonds such as ester bonds, which involve hydroxyl groups of polysaccharide and carboxylic groups or acid anhydride formed by the reaction between two carboxylic groups.[63] Such a reaction was proposed by Tomihada et al. to explain the cross-linking of pure HA gels by EDC.[63] Conversely, the intensity of the peak attributed to the ester bond at 1740 cm^{-1}[64] increases. Here, the reaction most probably occurs between chitosan and hyaluronan molecules since comparable observations were not made during the cross-linking of (PLL/HA) films.[48]

The difference between the final spectrum (after rinsing the film that has been in contact for 10 h with the EDC/NHS solution) and the spectrum of the film before contact

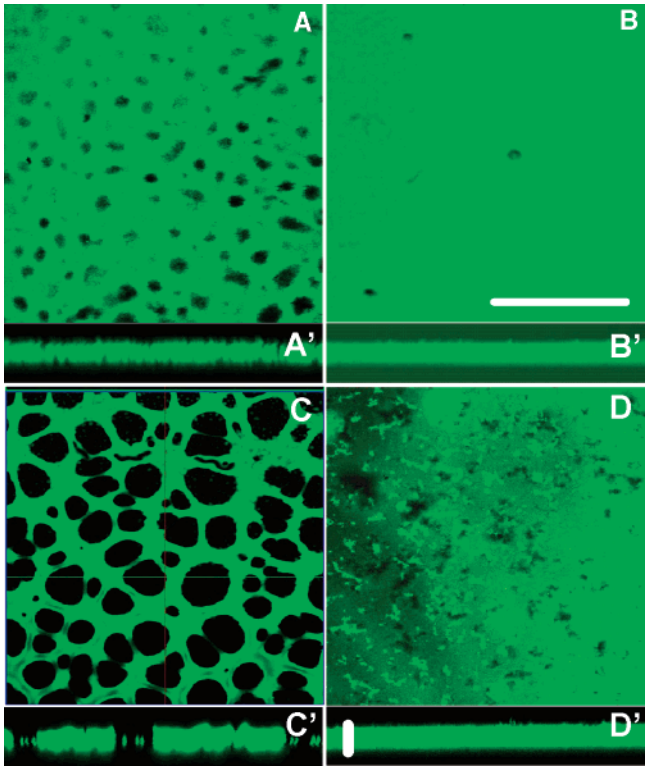

Figure 4. Enzymatic degradation of native (left-hand side) and cross-linked (right-hand side) $(CHI/HA)_{24}$-CHI^{FITC} films observed by CLSM: lysozyme at 1 mg/mL after 17 h on a native film (A, top view and A′, vertical section through the film) and on a cross-linked film (B and B′); α-amylase at 500 U/mL after 15 h on a native film (C, top view and C′ side view) and on cross-linked film (D and D′). Image sizes are 230 × 230 μm for (A−D, scale bar is 100 μm) and 230 × 16 μm for (A′−D′, vertical scale bar is 6 μm).

with the EDC/NHS solution is strong evidence for structural modification occurring in the film upon cross-linking. Unfortunately, FTIR is only a semiquantitative method, and no quantification of the cross-linking density can be achieved.

Film Degradation by Enzymes and Saliva in Vitro: CLSM Observations and QCM Measurements. To investigate the biodegradability of the films in vitro, films were put in contact with saliva enzymes such as lysozyme and α-amylase and with natural saliva. Film degradation was followed by QCM and by confocal microscopy. For QCM, thin films made of six layer pairs were built and then put in contact with the solutions, to follow the signal over a long time period. For CLSM observations, thick $(CHI/HA)_{24}$-CHI^{FITC} films were used since they allow clear film morphology and thickness visualization. Native and cross-linked films were compared. The native films were degraded by both lysozyme and α-amylase, with however, some differences in the resulting film morphologies. In fact, lysozyme is already known to cleave chitin and chitosan gels.[65−67] In its presence, pores appear in the film after several hours contact (Figure 4 A) and are visible on vertical sections (Figure 4A′). The action of α-amylase is also noticeable: a regular netlike pattern associated with a spider's web structure (Figure 4C,C′). This enzyme is able to cleave the α(1,4) glycosidic binding between glucose residues of polysaccharides and to degrade CHI.[47,68]

This result also suggests that the contact with certain enzymes enables three-dimensional regulation of the surface

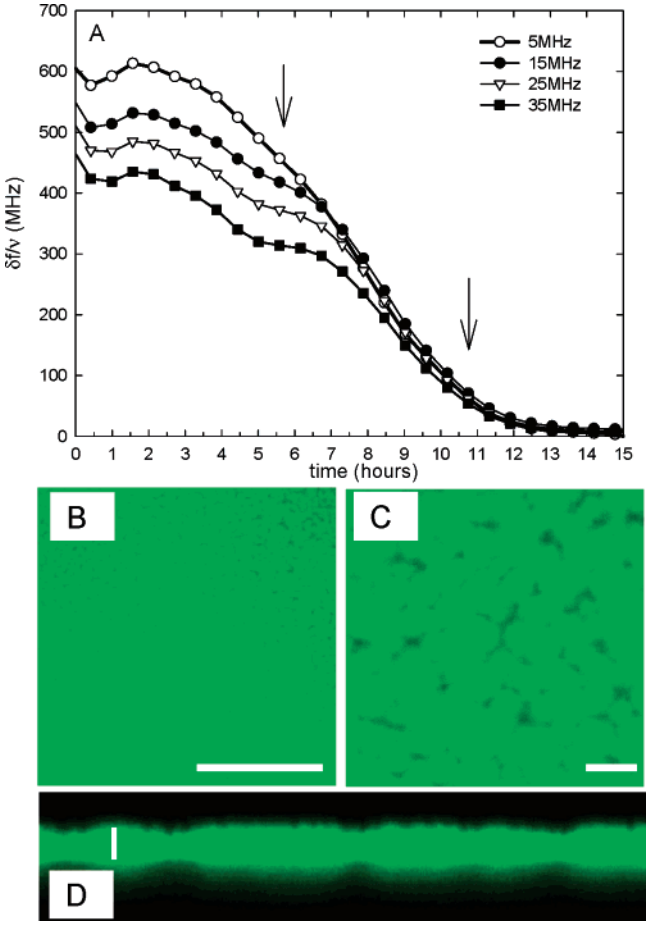

Figure 5. Differences in the QCM frequency shifts $-\delta f/\nu$ (A) as a function of time for a $(CHI/HA)_6$ film after contact with saliva: (○) 5 MHz, (●) 15 MHz, (▽) 25 MHz, and (■) 35 MHz. CLSM observations of a native $(CHI/HA)_{24}$-CHI^{FITC} films that has been in contact with saliva for 24 h: (B) scan size is 230 × 230 μm^2 (scale bar 100 μm) and (C) 57.6 × 57.6 μm^2 (scale bar 10 μm). Corresponding *Z*-section image (side view) of the same film (D) at 46.1 × 15.5 μm^2. Film thickness is about 4 μm (white line).

structure of the multilayer film and could be used, for example, to prepare films or membranes of various porosities. In contrast, the cross-linked films are much more resistant to all enzymatic degradation. No degradation was observed after contact with lysozyme (Figure 4B,B′). For α-amylase, a small superficial degradation was observable on the top view (Figure 4D), which was not visible on the film vertical section (Figure 4D′).

The effect of natural saliva on the native and cross-linked films was also investigated. QCM degradation of a native $(CHI/HA)_6$ film in contact with saliva at 37 °C was followed overnight (Figure 5A). The degradation is slow but progressive in the five initial hours, then a more rapid degradation is observable from 5 to 10 h, after which the film was totally degraded. On the CLSM observations of a $(CHI/HA)_{24}$-CHI^{FITC} film that was in contact with saliva for 24 h, a uniform degradation with the appearance of preferred degradation sites can be visualized (Figure 5B,C). On the film top views, a regular pattern of small nodules is visible, appearing as a grainy material. The side view image also presents a smooth and incomplete degradation with preferred dissolution areas on this thick film. It is thus clear that the native films are slowly and uniformly degraded in saliva.

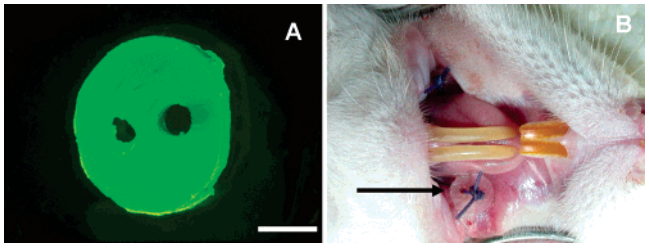

Figure 6. In vivo experiments in rat oral cavity. PMMA disks are coated on both sides with a $(CHI/HA)_{24}$-CHI^{FITC} film using the automatic dipping machine. A fluorescence stereomicroscope image of the film coated polymer disks (4 mm in diameter, scale bar 1.5 mm) was taken before implantation in the rat mouth. The disk was subsequently sutured to the rat's cheek (B).

The degradation seems to progress from top to bottom and could thus present advantages for the release of incorporated material, allowing a precise control of the sequences by which one or more components are progressively released. The cross-linked films totally resist saliva degradation over the same time period (data not shown).

Film Degradation in Vivo in the Oral Environment. In oral applications, the films were exposed to saliva in a rather harsh environment (including tongue mechanical action and chewing) as compared to the sole action of enzymes as tested in vitro. The films were thus placed in in vivo conditions to mimic a real environment. For these in vivo experiments, the $(CHI/HA)_{24}^{FITC}$ films were deposited on top of 4 mm diameter PMMA disks (Figure 6A), which were subsequently sutured to the rat's cheek (Figure 6B). This location was chosen to avoid direct contact with the teeth and to ensure a strong tongue mechanical action. Thus, the outer side of the disk was in contact with the tongue and the inner side with the cheek, which means that the latter side was protected more against the mechanical action and was mostly exposed to saliva.

After different time periods varying from 6 h to 3 days in the mouth, disks were recovered, and both sides were observed using a fluorescence stereomicroscope (Figure 7) and CLSM (Figure 8). The area of the film remaining on the disk surface was measured using Image J software and expressed as percentage of the total surface area (Table 1).

The native films are rapidly degraded in the mouth on both cheek and tongue side. On the cheek side, only 12% of the native film remains after 6 h (Figure 7A) and 8% after 2 days, and no film traces are visible after 3 days (Figure 7B,C). Only 7% of the native film remains on the tongue side after 6 h, and the film is totally degraded after 3 days (images not shown).

The cross-linked films are more resistant to degradation. Considering the cheek sides, after 6 h, more than 80% of the film remains (Figure 7D). Observations of disks implanted in different rats after 2 or 3 days show approximately 65 and 75% of film remaining, respectively (Figure 7E,F). These data indicate that a large fraction of the cross-linked film is still on the polymer disks after 3 days of implantation in vivo. Results for the tongue side show the same trend (images not shown), except that the percentage of cross-linked film remaining on the polymer disk is systematically lower. After 6 h, 75% of the film remains on the tongue side, whereas only 55 and 40% remain after 2 and 3 days (Table 1). Confocal images obtained by projection of a whole *z*-series acquisition confirm these observations (Figure 6). Only small amounts of the native (uncross-linked) films remain after 6 h and 2 and 3 days (Figure 8A−C). In contrary, the disks coated with the cross-linked films are still almost entirely covered after 6 h (Figure 8D). After 2 days, degradation of the film is clearly visible, and the film has a granular aspect (Figure 8E), which is even more pronounced at 3 days (Figure 8F). This suggests potential applications to protect implantable materials over a week in the oral environment.

It is obvious that the film coated polymer disks are not only subjected in vivo to saliva degradation but also to a mechanical degradation due to the rat's chewing. This explains why the tongue side is always more degraded than the cheek side. The mechanical action of the rat's teeth may be responsible for the extremely rapid degradation observed for the native films, which were not so rapidly degraded in vitro by enzymes and by saliva. Cross-linked films, which were barely degraded in vitro, are also partially degraded in the harsh in vivo conditions. One way to reduce the mechanical degradation would be to attach the film to the

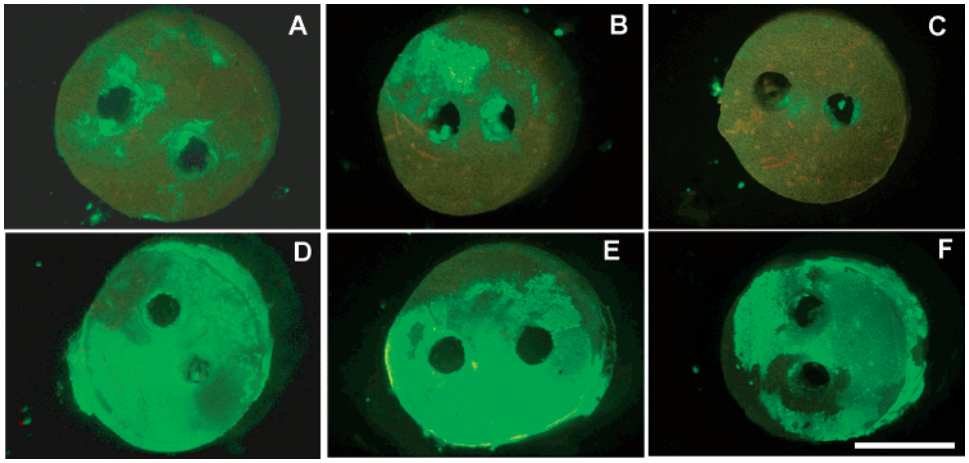

Figure 7. Observation of the native and cross-linked $(CHI/HA)_{24}$-CHI^{FITC} coated polymer disks after implantation in rat oral cavity (each image is a different disk). Fluorescence stereo microscope images of the polymer disks coated with native (A−C) and cross-linked (D−F) $(CHI/HA)_{24}$-CHI^{FITC} films that have been placed in vivo for different time periods in rat oral cavity: after 6 h (A and D), after 2 days (B and E), and after 3 days (C and F). Image sizes are 6.4 × 4.8 mm^2 (scale bar 2 mm).

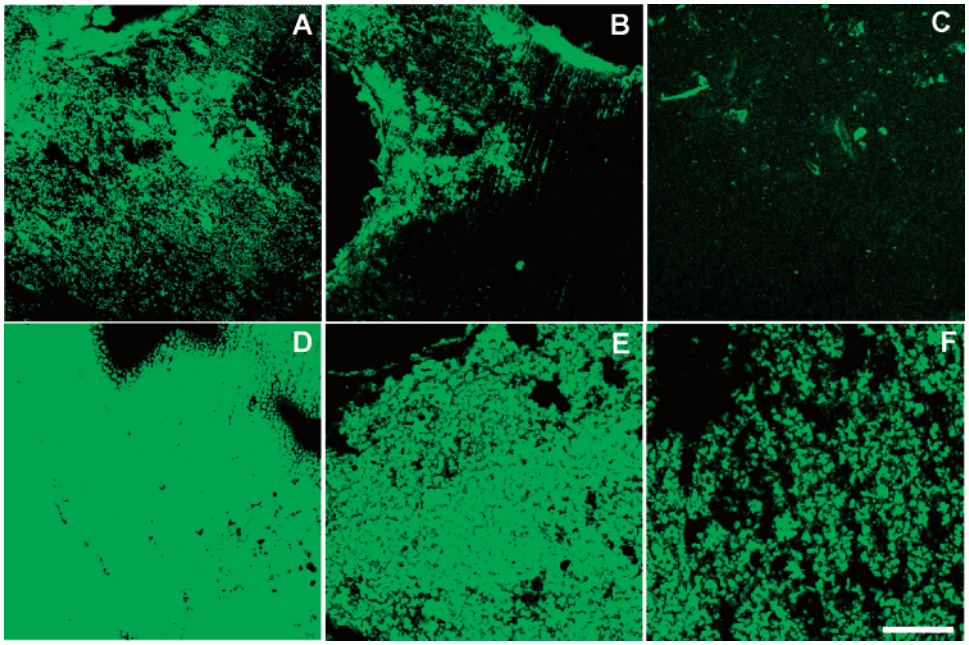

Figure 8. CLSM observations of native (A−C) and cross-linked (D−F) $(CHI/HA)_{24}$-CHI^{FITC} coated slides that have been placed in vivo in rat oral cavity for different time periods: after 6 h (A and D), after 2 days (B and E), and after 3 days (C and F). Image sizes are 921 × 921 μm^2 (scale bar 200 μm). Each image is the projected image of a whole *z*-series acquisition.

Table 1. Percentage of $(CHI/HA)_{24}$-CHI^{FITC} Film Remaining on the Polymer Disks after Various Times in Vivo in the Rat Mouth

type of film	6 h (%)	day 2 (%)	day 3 (%)
	tongue side		
native	≈7	≈10	<2
cross-linked	≈75	≈55	≈40
	cheek side		
native	≈12	≈8	<2
cross-linked	≈ 81	≈65	≈77

polymer disks by creating covalent bonds between PMMA and the film. Such a mechanism has already been used to graft hydroxyapatite to PMMA.[69] Recent work in our laboratory suggests it is possible to modulate the degree of cross-linking of the film (Rudolphe Obeid, personal communication). This indicates that the degradation rate of cross-linked films may be more finely tuned.

It may also be possible to combine fully degradable polyelectrolytes, such as the polysaccharides, with less degradable polyelectrolytes, including synthetic ones, such as poly(styrene sulfonate) or polyamino acids such as poly(L-glutamic acid). It would now be of interest to embed drugs into the multilayered assembly for further controlled release of the drug.

Conclusion

We successfully demonstrated that polyelectrolyte multilayers made of chitosan and hyaluronan can be degraded by specific enzymes present in saliva and by saliva in vitro. Film degradability was followed using quartz crystal microbalance and observed by confocal laser scanning microscopy after film labeling with CHI^{FITC}. The native films were rapidly degraded, whereas the cross-linked films, which contain amide and ester bonds, are resistant to degradation in vitro. The in vivo degradation of the films deposited on polymer disks and grafted into the rat oral cavity was also very rapid for the native films. However, dissolution was much slower for cross-linked films; more than 60% of the films remained on the disks after 3 days in the mouth. These results suggest that multilayer films made of polysaccharides are of high potential interest for oral applications, especially as drug release systems.

Acknowledgment. We thank L. Le Bars and C. Betscha for their technical help and B. Senger for the quantitative QCM data analysis. We are grateful to Anne Braun, Ian Robbins, and Dale Hunter for careful reading of this manuscript and for their critical comments. This work was supported by the Institut Français pour la Recherche Odontologique (IFRO) through a fellowship (2004) (C.P.). We also thank J. Mutterer for access to the CLSM platform used in this study, which was cofinanced by the Région Alsace, the CNRS, the Université Louis Pasteur, and the Association pour la Recherche sur le Cancer.

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BM049425U