Antibacterial paperboard packaging using microfibrillated cellulose

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Abstract The industry and consumers are focusing more and more on the development of biodegradable and lightweight food-packaging materials, which could better preserve the quality of the food and improve its shelf-life. In an attempt to meet these requirements, this study presents a novel bio-substrate able to contain active bio-molecules for future food-packaging applications. Based on a paperboard substrate, the development of an antibacterial bio-packaging material is, therein, achieved using a chlorhexidine digluconate (CHX) solution as a model of an antibacterial molecule, mixed with microfibrillated cellulose (MFC) and used as coating onto paperboard samples. AFM and FE-SEM analyses were performed to underline the nanoporous MFC network able to trap and to progressively release the CHX molecules. The release study of CHX was conducted in an aqueous medium and showed a lower proportion (20 %) of CHX released when using MFC. This led to the constant release of low amounts of CHX over 40 h. Antibacterial tests were carried out to assess the preservation of the antibacterial activity of the samples after the release studies. Samples remained active against *Bacillus subtilis*, with better results being obtained when MFC was used. The preservation of the quality of a model

food was finally evaluated paving the way for future promising applications in the food packaging industry.

Keywords Active paperboard packaging · Microfibrillated cellulose · Chlorhexidine digluconate · Antibacterial activity · Sustained release · Model food

Introduction

In response to the growing expectations of our society, the work conducted in the domain of active bio-packaging is increasing significantly (Pereira de Abreu et al. 2012). The development of more biodegradable and lightweight food-packaging materials (Johansson et al. 2012), which could offer a better protection of the product in terms of quality (freshness, taste) and shelf-life, is expected. Active packaging is one way of meeting these requirements, as it aims to extend the shelf-life and to maintain or improve the condition of the packaged product (European Council and Parliament 2004). Its market is already well developed in Japan and in the USA, and is now expanding in Europe.

Active packaging can be classified in two main categories: (i) scavengers and (ii) release packaging systems. Scavengers aim to absorb specific elements responsible for the degradation of the packaged food, such as oxygen or ethylene (Pereira de Abreu et al. 2012). Release systems aim to release substances into the environment surrounding the food, such as carbon dioxide or antibacterial molecules. Antibacterial packaging, in particular, aims to inhibit or reduce the growth of bacteria or fungi, which develop under specific temperature or humidity conditions on an appropriate medium that provides them with nutrients (Suppakul et al. 2003).

To our knowledge, this sub-family is still not commercialized in Europe (unlike some kinds of scavenger packaging systems), despite the increasing interest of researchers.

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Given the various kinds of antibacterial materials used today, such as microcapsules (Madene et al. 2006), antibacterial polymeric films (Cerisuelo et al. 2012) or coated foils (Dogan et al. 2009), the combination of active agent and cellulosic substrate creates a number of attractive opportunities. Indeed, cellulosic substrates such as paper or paperboard are usually used as food-packaging materials. Their price remains lower than that of biopolymers, and from a consumers' point of view, they are immediately linked to biodegradability and recyclability. Nevertheless, when taking into account the few studies published in the subject area, the development of active packaging combined with cellulosic substrates remains relatively low compared to their development as films (Guillard et al. 2009). This is mainly due to the fact that the industries focus on the incorporation of the active component directly into the polymeric matrix itself.

The major studies on active cellulosic substrates focused on the incorporation of active agents, such as essential oils, into wax coating for paper packaging (Rodríguez et al. 2007). Essential oils, such as cinnamaldehyde and carvacrol were also introduced into other paper coating slurries like soy protein isolates (SPI) and octenyl-succinate modified starch (Ben Arfa et al. 2007). In spite of the positive results obtained in each case, the main concern was the organoleptic modification of food due to the use of essential oils.

Organic acids, which are standard preservative agents, were also introduced into coating slurries for paper-based materials as they inhibit the growth of bacteria and fungi (Khwaldia et al. 2010). Some studies focused on the introduction of sorbic acid into wax-coated papers, which were then used as sausage or cheese packaging materials (Labuza and Breene 1989). Lactic, propionic and acetic acids were also tested as antibacterial compounds and introduced into chitosan coating for papers. However, the lactic acid/chitosan slurry combination was the only one showing an antibacterial activity against *Bacillus subtilis* (Vartiainen et al. 2004). Other antibacterial compounds were also directly mixed with coating formulations for paper packaging, such as nisin (Vartiainen et al. 2004) or silver nanoparticles (Nassar and Youssef 2012), but no viable solution has so far been developed for future commercialization.

Further research is thus needed to overcome some unresolved issues still prevailing like: (i) the impact of the active agents on the organoleptic properties of the packaged food, (ii) the controlled release of the active agents for a long-term antibacterial activity, and (iii) the biodegradability, recyclability and inertness of the active packaging materials.

Considering all the previous studies and challenges, we suggest, in the present study, a new kind of biodegradable active packaging material able to release the antibacterial compound over a longer period of time in order to improve the shelf-life of the packaged products.

This new material was developed using a solution containing chlorhexidine digluconate (CHX) as an antibacterial model agent. Chlorhexidine digluconate is the most efficient chemical agent in plaque control and is mainly used for oral hygiene (Houston et al. 2002). Although CHX is not mentioned in the list of food additives, it was used as model since it offers many advantages. Indeed, due to its cationic nature (Giménez-Martín et al. 2009), CHX is efficient against a wide range of bacteria. In addition to this, owing to the presence of its two Cl-atoms, CHX is easily detected by elemental analysis so that its presence in the substrate can be evaluated.

Based on our previous study dealing with the release of CHX introduced into the paper coating (Lavoine et al. 2014c), CHX was coated onto the surface of paperboard using a microfibrillated cellulose (MFC)-based coat.

MFC is produced from cellulosic fibers by applying a strong mechanical treatment. In 1985, the first MFC manufacturing process was patented by Turbak et al. 1985. It consisted in the defibrillation of wood pulp under high pressure using a homogenizer. Today, improved mechanical treatments have been developed, and pre-treatments (Isogai et al. 2011; Pääkkö et al. 2007) are also applied to reduce the high energy cost resulting from the use of mechanical treatments (Spence et al. 2011). The appeal for using MFC has increased considerably in the last few years, owing to its nanometric dimensions (diameters in the range of 10–50 nm and lengths higher than 1 μm (Siró and Plackett 2010)), to the natural entanglement of the microfibers, and to its ability to form strong films. MFC is indeed extensively used in nanocomposites (Siró and Plackett 2010) due to its mechanical reinforcing properties, as a film essentially for its barrier properties (Syverud and Stenius 2009), as an aerogel for its nanoporous network and lightweight (Korhonen et al. 2011), as a coating slurry for its barrier properties and its ability to improve the printing quality (Ridgway and Gane 2012), and more recently as a drug delivery system (DDS) (Kolakovic et al. 2012b; Valo et al. 2013).

Research focusing on the development of novel drug delivery systems was conducted by taking into account the different kinds of materials obtained with MFC. For example, Valo et al. 2013 developed MFC aerogels containing drugs and studied the influence of the sources of MFC on the release of the drug. Kolakovic et al. 2011 worked with spray-dried MFC as a tablet excipient, and as a sustained drug release system (Kolakovic et al. 2012a), and also investigated drug loaded MFC films (Kolakovic et al. 2012b). These films displayed promising results, since the release of drugs was sustained for 90 days. It was also confirmed by a more recent study focusing on food-packaging applications, which proposed the addition of lysozymes (antibacterial proteins) into MFC films (Cozzolino et al. 2013). Following these previous studies, we demonstrated that MFC coated onto paper

substrates can also be used as a delivery system for different kinds of molecules (Lavoine et al. 2014a, c). These innovative studies were the first to investigate an active packaging application using a MFC coating process. Thanks to its nanoporous network preserved during the process, the MFC substrate ensured the slow and gradual release of molecules into an aqueous medium (like water or agar). However, no tests were conducted using food and the elaboration of a 3-D packaging has so far never been considered.

In the present study, we therefore applied this MFC/active molecule coating strategy to a paperboard packaging material for 3D applications. The homogeneity of the coating was observed by SEM and AFM analyses. X-ray microanalyses were carried out to characterize the CHX distribution on the paperboard surface and throughout its thickness. This structural characterization was related to the release study of CHX, which was performed in an aqueous medium and analyzed by UV-Vis spectrophotometry. Antibacterial tests were then performed to evaluate the activity of the coated paperboard against *Bacillus subtilis*. This new active material was finally tested with a food model to assess its impact on the quality of the food, paving the way for future food packaging applications.

Materials and methods

MFC suspension preparation and characterization

The preparation steps and the characterization methods of the MFC suspension are described in details in our previous study (Lavoine et al. 2014c). More information can be found in the supporting material S1.

Sample preparation and characterization

A4 paperboard sheets were supplied by Cascades La Rochette (France). With a basis weight of 300 g/m², this paperboard was made of virgin pulp and coated on one side to facilitate printing. The other side was left uncoated in order to follow the coating process we developed, as described below.

Three different slurries were coated onto the paperboard surface:

- (1) The chlorhexidine digluconate (CHX) solution was purchased from Sigma-Aldrich (France). Its concentration was 20 wt % in water. This solution was diluted to 2 wt % in deionized water and coated as such onto the surface of the paperboard.
- (2) The MFC suspension was coated as such: at 2 wt %, following homogenization using an Ultra-Turax (France) for 5 min.

- (3) A slurry containing CHX/MFC was prepared by mixing 10/90 % of CHX at 20 wt % and MFC at 2 wt %, respectively. As a result, 100 mL of this slurry contained about 2 g of dried CHX and 1.8 g of dried MFC. The slurry was stirred and homogenized using an Ultra-Turax (France) for 5 min, and then coated onto the surface of the paperboard.

The coating of these solutions and suspensions was performed using a bar coating process (Endupap, France) as described in our previous study (Lavoine et al. 2014b).

For each slurry the coat weights were obtained by measuring the basis weight of each sample previously cut into 10×10 cm² strips and dried at 105 °C, for 1 h, using the previously described contact drying system and a Lhomargy scale (± 0.001 g).

SEM images of the surfaces of the coated paperboard were obtained using an Environmental Scanning Electron Microscope (E-SEM, Quanta200®, The Netherlands). The samples were mounted onto a metal stub with a double sided carbon tape, and observed at a working distance of 10.0 mm, under high pressure at 15 kV. The Back-Scattered Electron Detector (BSE) was also used to visualize the chemical contrast of our samples, before obtaining high-resolution compositional maps of the surfaces of the samples using an Energy Dispersive X-Ray Spectroscopy Detector (Bruker, USA).

The nanoporous MFC network formed during the coating was identified and studied by Atomic Force Microscopy (AFM). Small pieces of 5×5 cm² of MFC-coated paperboard were glued onto aluminium sample holders and scanned by tapping-mode using a Dimension Icon with a Scan Analyst (Bruker, USA). The images were recorded at room temperature with a standard silica cantilever (Bruker, USA).

Several AFM and E-SEM analyses were performed and the most representative pictures were selected.

The distribution and quantification of CHX

The distribution of the CHX molecules in the paperboard samples was observed by X-Ray microanalysis using an elemental mapping mode of the distribution of the chlorine atoms as recorded at the surface and in the cross-section of each sample.

The exact amount of CHX introduced into the paperboard samples was measured according to two different methods: (1) calculation of the difference in weight and (2) an elemental analysis. The differences in weight between the dried coated

samples and dried paperboard reference samples were calculated to evaluate the dried coat weight, and thus, to determine the quantity of CHX introduced into each slurry. Measurements were performed after drying each $10 \times 10 \text{ cm}^2$ sample for 1 h, at 105°C using a contact drying system and a Lhomargy high resolution scale ($\pm 0.001 \text{ g}$). These measurements were repeated up to three times for each sample.

The weighed values were then compared with those calculated from the percentage of Cl-atoms obtained after an elemental analysis. The elemental analysis was performed by the Service Central d'Analyse of the CNRS (France). Two measurements per sample were carried out.

Table 1 summarizes the different quantities of CHX and MFC, respectively, coated onto the surface of the paperboard samples, and measured either using the difference in weight or after an elemental analysis.

Release study of CHX in an aqueous medium

The release of CHX was studied following two protocols: (i) continuous and (ii) intermittent diffusions, as previously described in Lavoine et al. 2014c. The complete protocols and conditions of experiment are described in the supporting material S1.

Antibacterial activity of coated paperboard samples

The antibacterial efficiency of the coated-paperboard samples was evaluated against *Bacillus subtilis* according to the standard AFNOR EN 1104 (disc-diffusion assays). The protocol is detailed in the supporting material S1.

Qualitative evaluation of the quality of the food

Using the ArtiosCad® software, the final template of a folding box ($10 \times 10 \times 5 \text{ cm}^3$) was designed on A4 paperboard sheets. The dimensions of the box were chosen so that a majority of the paperboard could be used. The folding boxes were cut, creased and folded into 3-D paperboard packaging using a cutting and creasing laboratory converting pilot (Kongsberg, Esko, France). Three boxes of each coated paperboard sample were made.

Pork liver was chosen as a model food since its putrefaction is achieved within 3 days and is clearly noticeable to any

consumer by observing the change in color. Pieces of pork liver measuring about $5 \times 5 \text{ cm}^2$ were cut using a sterilized blade. One piece was placed inside each paperboard box and brought into contact with the active coated paperboard surface. The boxes were closed, wrapped and sealed in polyethylene (PE) plastic bags using a heated seal press, and were then stored in a fridge at $4\text{--}5^\circ \text{C}$ for 5 days, i.e., more than 2 days after the use-by date.

After the storage period, each sample was photographed using a camera under normal light. The images were submitted to a panel of consumers (20 witnesses) responsible for judging the degradation of the liver. Each consumer classified the liver samples from the least fresh to the freshest by giving them a grade ranging from 1 to 5; with five corresponding to the freshest liver. The highest grade which could be reached was thus 100 (i.e., 20×5), corresponding to the pork liver analyzed just after its purchase.

Results and discussion

Observation and characterization of the MFC suspension

The MFC suspension is a white aqueous gel (Supporting material S2, Fig. S2.1a) made of an entanglement of microfibrillated cellulose. Even after a consequent dilution of the suspension, it was quite difficult to isolate only one microfiber. It is well-known that an accurate measurement of their length is difficult despite recent scientific studies on that subject (Shinoda et al. 2012).

An estimation of their diameter was nevertheless achievable from a FE-SEM image (Supporting material S2, Fig. S2.1c). A mean value of $33 \pm 8 \text{ nm}$ was obtained. This value corroborates with other measurements found in the literature (Siró and Plackett 2010). Generally, the diameter of MFC ranges between 10 and 50 nm, no matter what treatment is used to produce it. Images obtained by light microscopy (Supporting material S2, Fig. S2.1b), and the high viscosity of the gel proved the homogeneity of the fibrillation process. It is worth verifying the homogeneity because to date no standard about MFC exists in spite of the development of ISO working groups (Marrapese 2013). As a result, some researchers call MFC microfibrils with smaller diameters, or

Table 1 Amount of CHX and MFC (g/g of dried sample) coated onto the paperboard samples

Paperboard samples	Weighed compounds (g/g of dried sample)	Methods	
		Difference in weight	Elemental analysis
CHX-coated	CHX	0.010 ± 0.003	0.0055 ± 0.0002
CHX/MFC coated	CHX	0.056 ± 0.009	0.021 ± 0.002
	MFC	0.036 ± 0.013	not measurable

fibers with larger diameters. In our case, we confirmed that a good quality and a homogeneous MFC suspension was produced and further used as a coating.

MFC-coated paperboard

Five MFC layers were successively deposited onto the surface of the paperboard to ensure a homogeneous coat. A complete characterization of these samples was already published in our previous works (Lavoine et al. 2014b). With five MFC layers, a coat weight of about 7 g/m^2 was achieved. Our previous study proved that the MFC layers counterbalanced the loss of mechanical properties induced by the successive wetting/drying cycles of the aqueous coating process. The MFC even improved some mechanical properties, such as the bending stiffness. It was, thus, possible to produce a paperboard box coated with a thin MFC layer (7 g/m^2) having similar mechanical properties to a box coated with 17 g/m^2 of polyethylene. The use of this MFC layer as a sustained release system could then be investigated in the present study.

The observation of the nanoporous MFC network is a key aspect for this application. This network plays a major role in the controlled release of CHX. The sample surfaces were, therefore, analyzed by SEM and AFM. Figure 1 shows SEM images of the surface of the paperboard sample taken with SE and BSE detectors.

No difference could be observed between the images of the reference paperboard and the CHX-coated paperboard, which was as expected. On the contrary, MFC-coated paperboard samples had a surface entirely covered by the MFC: no more pores were perceived. However, this homogeneity was not maintained overall following the coating process with the CHX/MFC mixture: some areas were not entirely covered. Charge interactions between CHX and MFC might induce the formation of aggregates, and result in a less homogeneous surface coverage. BSE images clearly revealed the presence of non-coated zones at the surface of CHX/MFC-coated samples, contrary to MFC-coated samples. Nevertheless, these heterogeneities did not drastically influence the release pattern of CHX as will be demonstrated further.

Figure 2 shows the AFM images of the MFC-coated paperboard samples.

The Atomic Force Microscopy is the only one, which accurately underlined the nanoporous MFC network at the surface of the samples. These pictures, of the surface of the MFC-coated paperboard, are quite rare and rather complicated to obtain, however they clearly prove that even after the coating, microfibrils were tightly entangled, as they were in the original suspension.

The effect of this network on the release of CHX will therefore be studied more extensively herein.

Release study of CHX into an aqueous medium

Two kinds of release protocols were implemented to study the diffusion of CHX into an aqueous medium. CHX-coated paperboard samples were compared with CHX/MFC-coated paperboard samples. In our previous study (Lavoine et al. 2014c), we proved that the most efficient release of CHX was achieved from papers coated with a mixture of CHX/

Fig. 1 SEM images ($\times 100$) of each sample surface using the classic Secondary Electron (SE – (a), (c), (e), (g)) and Back-Scattered Electron (BSE – (b), (d), (f), (h)) detectors. Images (a) and (b) show the paperboard reference, (c) and (d) the MFC-coated paperboard, (e) and (f) the CHX-coated paperboard, and (g) and (h) the CHX/MFC-coated paperboard samples

Fig. 2 AFM images of the surface of the MFC-coated paperboard sample: (a) Topography and (b) Phase

MFC, instead of papers impregnated in CHX and then coated with MFC. Consequently, in the present study, we limited our analysis to paperboard samples directly coated with the CHX/MFC mixture. To highlight the relevance of MFC, CHX-coated paperboard samples were prepared as a control. Nevertheless the amount of CHX introduced into both CHX- and CHX/MFC-coated paperboard differed (Table 1). Indeed, it was quite difficult to achieve a similar quantity using the bar coating process, especially with the CHX solution. Even after applying five successive layers, the total quantity of CHX coated onto the paperboard was very low compared to the quantity introduced into the MFC suspension.

The results of the continuous diffusion assays are presented in Fig. 3.

Results of both methods used to measure the amount of CHX released are plotted in Fig. 3a (difference in weight) and Fig. 3b (elemental analysis). Similar trends were observed in both cases: CHX-coated paperboard samples released a higher proportion of CHX than CHX/MFC-coated paperboard samples (e.g. 80 % vs. 10 % in 1 h; Fig. 3a). Furthermore, the plateau values were reached at the same time for both kinds of samples, i.e. in 1 h. Nevertheless, these plateau values inform us only about the highest proportion of CHX released for each kind of sample independently of the initial amount of CHX. When considering the curves representing the quantity of

CHX released (g) as a function of the time (h) (results not shown), the slopes of both curves were almost equal (about 0.2 g/h), proving that the release kinetic was similar for CHX and CHX/MFC-coated paperboard samples.

As a result, the nanoporous MFC network did not release the CHX molecules more slowly, but released them more gradually, i.e., in smaller proportions over time. The use of MFC as a delivery system thus tends to be promising, owing to the achievement of a controlled molecule release system. This will be further confirmed with the intermittent diffusion experiments.

A major concern in the comparison of both samples was the determination of the exact amount of CHX introduced into both samples. Considering the Fig. 3a and b, the total quantities of CHX reached were completely different depending on the quantification method applied. CHX-coated paperboard samples released 80 % of CHX in 1 h according to the quantification based on a difference in weight, whereas only 40 % of CHX was released in 1 h when considering the results obtained by elemental analysis. The same conclusion was drawn with the CHX/MFC-coated paperboard samples. Only 8 % of CHX was released according to the difference in weight method, against 20 % using the elemental analysis.

Differences between both methods are quite significant. This proves that the results obtained using the difference in

Fig. 3 Continuous diffusion of CHX into an aqueous medium. CHX-coated paperboard samples (diamond) are compared with CHX/MFC-coated paperboard samples (square). The amount of CHX released (%) was measured using the (i) difference in weight (graph a) and the (ii) elemental analysis (EA) (graph b)

weight, method generally used, cannot be used in the case of paperboard samples. The quantity of CHX was, indeed, too low to be accurately quantified with a scale: for paperboard samples of about 3 g, a mean value of only 0.015 g of CHX was quantified by elemental analysis just after coating. The results determined by elemental analysis only will thus be further considered.

As a result, around 50 % less of CHX was released in presence of the MFC coating. The ability of the MFC to enable the release of smaller proportions might be due to its high surface area, which increases the number of interactions with CHX, compared to a paperboard.

For a similar reason, the whole quantity of CHX was not released after 15 h of release in water. The adsorption and covalent bonding of CHX onto cellulosic fibers were already studied and confirmed in a previous study (Giménez-Martín et al. 2009), which could thus explain the results observed. The mapping of the Cl-atom (from CHX) of our samples before and after the releasing assays was then conducted to confirm these interactions between cellulose and CHX as well (Supporting material S2, Fig. S2.2). The distribution of the Cl-atoms throughout the thickness of the samples was simultaneously performed, and demonstrated the presence of CHX in samples even after the release tests were conducted (Fig. 4).

It is worth noting that before the release assays were conducted, the whole quantity of CHX was mainly located on the surface of the paperboard. After the release assays, CHX mainly migrated throughout the thickness of the paperboard. This can possibly explain why a large quantity of CHX was still not released at the end of our assays. When a thick and porous material (like paperboard) is used, CHX is able to diffuse in two directions: into the release medium and into the thickness of the material. With thinner materials, such as paper, the diffusion may also happen in both “directions”. Since paper is rapidly saturated by the molecules, CHX mainly diffuses into the release medium (Lavoine et al. 2014c). According to the distribution profiles (Fig. 4), very few CHX molecules were released during the continuous diffusion

experiments. On the contrary, during intermittent diffusion experiments, a larger quantity of CHX was released, as the medium was renewed every 30 min after each sampling. Profiles of the distribution of Cl-atoms throughout the paperboard thickness showed that CHX was still present in samples, but only in very low amounts (Fig. 4).

Figure 5 presents the results of the intermittent diffusion experiments. In this case, the abscissa does not represent the time, but the number of washing steps and the cumulative amount of CHX released into the aqueous medium is plotted as a function of the number of washing steps, i.e. the number of times the medium was renewed every 30 min. CHX-coated samples continuously released significant amounts of CHX after each medium renewal until a maximum value of about 100 % of released CHX was reached. On the contrary, very small quantities of CHX were released from paperboard samples coated with CHX/MFC after the first medium renewal. During the first 30 min, excessive amounts of CHX were entirely released (about 20 %). This corroborates the concept of burst effect proposed by Raso et al. 2010. After this first washing step, the proportion of CHX released was very similar between all of the medium renewals and did not exceed 5 %.

After 84 washing steps, the entire quantity of CHX initially introduced in CHX-coated samples was released (according to the elemental analysis). CHX/MFC-coated samples, however, released CHX during 51 washing steps. The quantity released after these 51 steps was only 25 %. This suggests that CHX was still released from these samples, but only in very small amounts. The experiments were stopped when three consecutive samples displayed a zero absorbance, although some samples continued to release CHX. It was, therefore, difficult to complete the CHX release experiments, due possibly to the fact that CHX was trapped in the MFC network.

Surprisingly, CHX was completely released from the CHX-coated paperboard samples after 84 washing steps, whereas only 18 washing steps were necessary, when coated onto paper samples (Lavoine et al. 2014c). The reason for this

Fig. 4 Distribution profiles of the Cl-atoms throughout the thickness of the CHX- (a) and CHX/MFC-coated (b) paperboard

Fig. 5 Cumulative amount of CHX released (%) from the CHX- (black diamond) and CHX/MFC-coated paperboard (grey square) as a function of the number of washing steps

discrepancy could mainly be due to the multilayer structure of paperboard since the quantity of CHX introduced into the paperboard samples was ten-times lower than the quantity introduced into the paper samples. As CHX was diluted in an aqueous solution, it had the opportunity to penetrate into the whole structure of the paperboard. CHX was, thus, able to interact chemically with each layer of paperboard, which drastically slowed down its release. The structure of paperboard, therefore, allows for a slower release of CHX compared to paper.

Although the CHX release was uninterrupted over a longer period of time by the CHX-coated samples, the addition of MFC showed yet its relevance since only 25 % of CHX was released after 50 washing steps against 65 % for the CHX-coated samples (Fig. 5).

These results corroborate thus those obtained by continuous diffusion. The nanoporous MFC network allowed for a more gradual release of the CHX molecules into water, but after reaching a certain level, this network seemed to prevent the CHX left from being released.

Antibacterial tests were, thus, carried out in order to verify whether an antibacterial activity could still be observed in spite of this interrupted diffusion, and in spite of the low amounts of CHX released over time.

Antibacterial activity against *Bacillus subtilis*

The antibacterial activity of coated-paperboard samples was evaluated before and after CHX release assays. The bacterium targeted in our tests is a non-pathogenic organism, named *Bacillus subtilis*. These tests were qualitative in nature and only indicated whether the samples were antibacterial or not,

prior to and following the intermittent diffusion experiments. In our case, the antibacterial activity of CHX/MFC-coated samples after release assays was expected, since the whole quantity of CHX was not released after 51 washing steps, whereas in the case of the CHX-coated samples, no activity was expected, since 100 % of the CHX introduced was released after 84 washing steps.

The results of the antibacterial tests are presented in Fig. 6.

Before the release study, the samples were quite active against *Bacillus subtilis*: inhibition areas of 712 and 905 mm² for CHX and CHX/MFC-coated samples, respectively, were measured. Compared to the Penicillin control samples, which displayed inhibition zones of approximately 1560 mm², CHX was relatively efficient against these bacteria, and diffused perfectly into the agar.

When considering the results obtained after the intermittent diffusion experiments, both samples still exhibited an antibacterial activity (Fig. 6).

The CHX-coated paperboard samples, the whole quantity of CHX of which was released during the intermittent diffusion experiments according to Fig. 5, still had an inhibition area of approximately 330 mm², i.e., a decrease of 54 % compared to the samples before the release study. The low quantity of CHX remaining (not measurable with the UV-Vis spectrophotometer but detectable with the profile curves Fig. 4) did not prevent the samples from being active against *Bacillus subtilis*.

The same pattern was observed with CHX/MFC samples: after the diffusion experiments, the inhibition areas were reduced by approximately 60 %. Thus, even after a prolonged diffusion into an aqueous medium, renewed several times, the samples were still highly active. This was expected since only

Fig. 6 Antibacterial tests of the samples before and after the intermittent releasing assays. The areas of inhibition (mm²) were calculated from the diameter of the inhibition zones formed by the active samples, without considering the sample surface (samples of 16 mm in diameter)

25 % of the CHX was released (Fig. 4). Nevertheless, the MFC did not prevent the remaining CHX from being released into the agar as it was previously assumed (no signal was detected by UV-Vis spectrophotometer). According to the results, small amounts of CHX were still released into the agar, which maintained the antibacterial activity of the samples throughout the release assays.

It is worth noting, however, that no clear difference in the antibacterial activity was observed between both samples (without and with MFC). Following the release tests, each paperboard sample remained active against *Bacillus subtilis*, and displayed a similar inhibition zone. This could be expected since CHX is a strong antibacterial agent with a very low minimum inhibitory concentration (MIC) against the bacteria studied. Other kinds of bacteria with a stronger metabolism should consequently be tested, since the low amount of CHX remaining in the samples after the release assays may not be efficient against more virulent bacteria.

Evaluation of the quality of food

A possible future application for the antibacterial paperboard designed could be in food packaging. To verify such an application, pork liver was chosen as a model food to carry out preliminary experiments on food quality. Pork liver is a sensitive meat with a short shelf-life, even under standard storage conditions (fridge at 4–6 °C). Its degradation is very rapid and clearly noticeable without any complicated measurements for the consumers. It also has a high water content preventing it from drying during usual storage periods, which makes it an ideal food model.

Meat is not generally preserved directly in contact with the paperboard packaging. But a novel application could be investigated with this new bio-material. We recently proved that the MFC coating significantly improved the water absorption properties of the paperboard (Lavoine et al. 2014b). Thus, within the context of a meat-packaging application, the antibacterial paperboard samples could be added into plastic

Fig. 7 Evaluation of the quality of the pork liver samples after storage. The pie chart (b) shows the total number of points assigned to each food sample (a) by the consumer (20 witnesses)

baskets, instead of the current tissues used to absorb the excessive blood. Furthermore, CHX is only active by direct contact between the packaging material and the food, contrary to volatile molecules, and represents a perfect model of antibacterial food additives such as sorbic acid for example. The food, therefore, needs to be directly in contact with the antibacterial paperboard to be protected.

3-D boxes were built with A4-paperboard sheets (Supporting material S2, Fig. S2.3a) coated or not with antibacterial molecules. CHX/MFC-coated samples were tested and compared with the reference paperboard (uncoated) and the polyethylene-coated (PE) paperboard.

Similar pieces of pork liver were placed in contact with the coated side of the paperboard box (Supporting material S2, Fig. S2.3b). Each box was then wrapped into a plastic bag and placed in the fridge at 4 °C. Various control samples were also added: pork liver directly after purchase (called reference in Fig. 7), pork liver wrapped in a plastic bag alone and pork liver in contact with Penicillin discs wrapped in a plastic bag.

The degradation of the liver samples was judged by a panel of 20 various consumers (different ages, genders, and eating habits). They had to classify each picture and give it a grade between 1 and 5. The highest grade was assigned to the freshest pork liver. Thus, according to this evaluation, the pork liver analyzed directly after its purchase got a maximum of 100 points (20*5). Fig. 7 presents the results.

The PE-coated paperboard got the lowest grade. The pork liver packaged in the active paperboard samples was better graded than those packaged in the reference paperboard and the plastic bag, but appeared less fresh than the pork liver with the Penicillin. Penicillin preserved the quality of the pork liver by reducing its oxidation and the development of aerobic bacteria.

According to these results, the CHX/MFC-paperboard samples were able to preserve the quality of the pork liver much better than the other paperboard samples devoid of any antibacterial agent. CHX has quite a strong antibacterial activity, though, according to the antibacterial test results, this activity was less efficient than Penicillin.

These results, although encouraging for a future food packaging application, remain qualitative.

Further tests should be conducted following this study and the perspectives it has unveiled for the food-packaging industry. Food tests are usually quite sensitive since many parameters come into play (nutrient, water content, salt, etc.), and this preliminary study was a promising foretaste of such deeper analyses.

Consequently, others tests have to be performed to analyze the migration and the impact of the antibacterial agent on the organoleptic properties of food. Another kind of food could be tested such as bread, which spoils more slowly and could allow the measurement of a long-term antibacterial activity. After inoculation of a well-chosen food, the evolution of the

bacteria growth over time could also be studied to prove the antibacterial effectiveness of such new packaging material.

Toxicity is also an additional parameter to be considered since CHX can be ingested by humans, without affecting their health until a threshold is reached. Other antibacterial molecules should be tested and compared to the results obtained with CHX, which already ensures an effective inhibition of bacteria.

Conclusion

CHX, a model antibacterial agent, was introduced into the nanoporous MFC network, preserved during its coating onto a paperboard substrate. This network allowed a sustained release of the antibacterial agent into the aqueous medium: the amount of CHX released was reduced by 20 % owing to MFC. These results confirmed our team findings on the coating of antibacterial agents in combination with MFC.

The MFC suspension is a biodegradable, lightweight, and porous layer able to contain a sufficient quantity of active compounds to enable proper antibacterial properties. The antibacterial activity of the paperboard samples against *Bacillus subtilis* was, indeed, preserved, even after the release study was completed. Furthermore, owing to the paperboard ability to be creased and folded, active boxes were made and their ability to preserve the food quality was tested with pork liver as a model food. The CHX/MFC coated paperboard samples were able to better preserve the freshness of the pork liver compared to the other samples, in particular the polyethylene-coated paperboard samples.

This study thus, revealed the relevance of using MFC in the elaboration of a lightweight antibacterial bio-material. Further food tests and other antibacterial compounds should nevertheless be tested following this study and the promising perspective it has unveiled for the food-packaging industry.

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Conflict of interest All the authors declare that there are no conflicts of interest.

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