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IN VITRO DEGRADATION OF POLY (DL-LACTIDE-ε-CAPROLACTONE) NERVE GUIDES

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Abstract

The aim of this study was to evaluate the in vitro degradation of a poly (DL-lactide-ε-caprolactone) nerve guide with an initial molecular weight (Mw) of 1.1 x 10⁶ kg/kmol in phosphate buffer (pH = 7.4), after different time intervals, ranging from 7-98 days. We evaluated the changes in Mw, dry and wet volume, dry and wet mass and pH of the buffer. In addition, cryo scanning electron microscopical (cryo SEM) evaluation of the degrading nerve guides was carried out to evaluate the effect of the degradation on the surface of the nerve guide.

The degradation of the poly (DL-lactide-ε-caprolactone) nerve guides is characterized by a slow decrease in Mw and a change in physical properties after 10 weeks. Cryo-SEM analysis showed holes with a diameter of approximately 0.3 μm 5 weeks after degradation. After 8 weeks of degradation, cracks could also be observed.

From this study, it seems likely that the temperature at which the nerve guides are stored (in this study at room temperature), has a great impact on the evaluated parameters.

Key Words: Biodegradable, nerve regeneration, nerve guide, in vitro degradation, cryo-scanning electron microscopy.

Introduction

The use of degradable biomaterials in clinical applications is of increasing interest in medical practice (Ali, 1993), especially for temporary therapeutic applications (Vert et al., 1992). For example, degradable drug release systems, degradable dental membranes and degradable hydroxyapatite coatings are already used routinely. A new application for biodegradable materials is the use of a nerve guide for nerve reconstruction. After reconstruction of a nerve defect, the nerve guide directs the outgrowing nerve fibers towards the distal nerve stump, whilst preventing neuroma formation and the ingrowth of fibrous tissue into the nerve gap. After serving its function as a temporary scaffold, the nerve guide may degrade completely. The rate of degradation is of utmost importance for the ultimate quality of nerve regeneration. If, for example, the nerve guide would degrade too slowly, some slow-degrading fragments of biomaterial might negatively influence nerve function due to the formation of scar tissue. This may in turn lead to constriction of the nerve, or it might cause chronic irritation at the implantation site (Den Dunnen et al., 1995).

In previous studies a copolymer of 50% DL-lactide and 50% ε-caprolactone was used as a nerve guide (Den Dunnen et al., 1995, 1996, 1997a,b). The lactide component contained 85% L-lactide (LLA) and 15% D-lactide (DLA). Nerve regeneration through this nerve guide was already quite acceptable (Den Dunnen et al., 1996), and the amorphous poly (DL-lactide-ε-caprolactone) degraded completely within one year (Den Dunnen et al., 1997b). However, the degradation was characterized by swelling of the biomaterial, starting between 1 and 2 months after implantation (Den Dunnen et al., 1995, 1996). The swelling was caused by water uptake by the copolymer (Den Dunnen et al., 1995), due to the formation of low molecular weight degradation products, increasing...
the osmolarity in the biomaterial. The swelling of the nerve guide has a negative effect on the speed and quality of the nerve regeneration due to compression of the regenerating and maturing nerve fibers (Den Dunnen et al., 1995). The amount of swelling depends on the mass of polymer and the degradation rate of the biomaterial, which in turn, depends on the type of polymer, crystallinity, weight, average molecular weight ($M_w$) and porosity/effective surface per volume ratio of the biomaterial (Den Dunnen et al., 1997b).

To study degradation of subcutaneously implanted poly (DL-lactide-$\epsilon$-caprolactone), bars of materials ($M_w$ 1.06 x 10$^6$ kg/kmol) were evaluated (Den Dunnen et al., 1997b). After an implantation period of 3 months, the degradation was characterized by swelling of the degrading polymer up to 300% (i.e., 200% water uptake).

The aim of the present study was to evaluate the in vitro degradation of a poly (DL-lactide-$\epsilon$-caprolactone) nerve guide with a $M_w$ of 1.1 x 10$^6$ kg/kmol in phosphate buffer (PB) (pH = 7.4), after different time periods, ranging from 7-98 days. To do so, the changes in $M_w$, dry- and wet volume, dry- and wet mass and pH of the PB were evaluated. In addition, cryo-scanning electron microscopical (cryo SEM) evaluation of the degrading nerve guides was carried out to evaluate the effect of the degradation on the surface of the nerve guide.

**Materials and Methods**

**Preparation of the nerve guides**

The biodegradable nerve guides in this study were composed of a copolymer of 50% DL-lactide and 50% $\epsilon$-caprolactone. The lactide component contained 85% L-lactide (LLA) and 15% D-lactide (DLA). For the preparation of this copolymer, stannous 2-ethylhexanoate was used as a catalyst. The reaction temperature was 130°C and the duration of the reaction was 15 days.

A solution of 3 wt% of the amorphous copolymer in chloroform was prepared. This solution was dip-coated on a glass mandrel with a diameter of 1.1 mm. Finally, the polymer was manually removed from the mandrel. The preparation procedure is described in detail by Den Dunnen et al. (1996). This technique resulted in a nerve guide with an internal diameter of approximately 0.9 mm, a wall thickness of 0.20 mm and an external diameter of 1.1 mm.

After preparation, each nerve guide ($M_w$ of 1.1 x 10$^6$ kg/kmol), with a length of 12 mm, was stored in 150 $\mu$l 0.1 M KH$_2$PO$_4$ and 0.1 M Na$_2$HPO$_4$.2OH (PB, pH 7.4) at room temperature (RT).

**pH, mass and volume measurements**

Twenty-eight nerve guides were evaluated for changes in mass and volume. Every week, during the 14 weeks of storage of the nerve guides in 150 $\mu$l PB at RT, 2 nerve guides were evaluated for dry- and wet masses using a balance with a 10$^4$ gram accuracy (Sartorius 1207 MP2; Sartorius, Göttingen, Germany). To evaluate the changes in dry- and wet volume of the degrading nerve guides, a new technique was developed (Fig. 1). The wet volumes were measured by placing each nerve guide in a certain volume of demineralized water. The rise of this volume corresponds with the wet-volume of the nerve guide.

Before measuring the dry masses and volumes, the nerve guides were dried for 24 h at 37°C using silicagel. From these measurements, information is obtained about the decrease in mass and volume of the degrading biomaterial and the amount of water uptake. The changes in dry mass during degradation at $t$, is calculated by dividing the dry mass at $t$, by the mass measured at $t_0$, multiplied by 100%. The water uptake per volume of biomaterial at $t$, is calculated by dividing the change in volume of the nerve guide at $t$, by the wet volume of the nerve guide at $t$, multiplied by 100%.

In addition, the pH of the PB of 2 samples was measured at all time intervals, using a "pH-meter" (Sentron®2001; Sentron, Roden, The Netherlands). Before measuring the pH, the "pH-meter" was calibrated, according to standard operating procedures.

**Macroscopy**

The nerve guides were also evaluated for changes in macroscopy at all time intervals. The nerve guides were evaluated for swelling and changes in color of the degrading polymer.

**M$_w$ measurements**

The degrading nerve guides were evaluated for changes in the $M_w$. Every week, the $M_w$ of 2 samples was measured, using Gel Permeation Chromatography (GPC) in combination with Light Scattering (as a detector), using chloroform as a solvent. Polystyrene was used as a standard in our calibration curve.

**Preparation of the nerve guides for cryo-scanning microscopy**

Cryo-scanning microscopy evaluation of the degradation of the nerve guide was carried out after 0, 5, 8, 10, and 12 weeks. Thin (100-200 $\mu$m) slices of the nerve guides were mounted on copper specimen grids (75 mesh). Subsequently, cryo-fixation was carried out using a Reichert-Jung (Vienna, Austria) MM 80 slammer at -190°C (in liquid N$_2$). The frozen nerve guide was inserted into the Oxford Instruments
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Figure 1. Schematic representation showing the set-up designed to measure the changing volumes of the nerve guides. After storage, a nerve guide was placed in a constant volume of demi-water (a). The rise of this volume is removed via a capillary tube and subsequently weighed (b). This weighed volume corresponds with the volume of the nerve guide.

(Oxford, UK) Cryo preparation system CT 1500 HF, whereafter the temperature was set to -95°C. Subsequently, the sample was broken by a cryo-knife in order to obtain a clear fracture, and the surface was etched by sublimation of H₂O from the surface for 1 min and coated with a 1.5 x 10⁻⁹ m layer of Pt or Au/Pd (Denton Vacuum 1 inch sputter source; Denton, Cherryhill, NJ) at -120°C.

After this preparation of the nerve guide, the sample was placed into a Jeol (Tokyo, Japan) Field Emission Scanning Electron Microscope (6301 F). From the fracture surface, micrographs at -125°C were made with magnifications ranging from 4500x to 35000x, operating at 2 to 10 kV.

Results

Evaluation of pH, mass and volume

The pH of the PB in which the nerve guides were stored, was almost constant. A significant trend concerning the water uptake by the biomaterial over the course of the study, was not observed (Table 1). Changes in dry mass of the nerve guides during degradation was not observed either (Table 1).

The change in volume of the nerve guides in time, as well as the regression plot of these values are outlined (Fig. 2). A decrease of the dry volume of biomaterial during degradation can be observed. The maximum decrease observed after 11 weeks was approximately 40%. Linear regression analysis and ANOVA (analysis of variance) were performed, and showed a F-value of 7.039 and a p-value of 0.0199.

Macroscopical analysis

Changes in color of the degrading polymer were not observed during the study period. Swelling of the nerve guides was not observed either.

Molecular weight evaluation

After 4 weeks of degradation, the copolymer still has approximately the same weight average molecular weight (Mw) as the original copolymer, i.e., 9.8 x 10⁵ kg/kmol (Fig. 4). Five weeks after onset of the degradation study, the Mw starts to decrease slowly to 4.2 x 10⁵ kg/kmol after 13 weeks of degradation (Fig. 3).

Cryo-scanning analysis

Until 8 weeks, the cryo-breaking of the nerve guides for cryo-SEM analysis could easily be performed. At 10 and 12 weeks, it was difficult to obtain a clear fracture, because of a change in the physical

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Changes in dry mass</th>
<th>Water uptake (% of wet volume)</th>
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<tbody>
<tr>
<td>1</td>
<td>5%</td>
<td>13%</td>
</tr>
<tr>
<td>2</td>
<td>1%</td>
<td>21%</td>
</tr>
<tr>
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<td>4%</td>
<td>33%</td>
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<tr>
<td>4</td>
<td>2%</td>
<td>42%</td>
</tr>
<tr>
<td>5</td>
<td>2%</td>
<td>48%</td>
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<td>10</td>
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<td>14</td>
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Figure 2. Change in volume of the nerve guides as a function of time and the regression plot of the values measured (straight line).

Figure 3. Change in $M_w$ of the nerve guides as a function of time. ______ represents the molecular weights of the nerve guides measured in the present study. ----- represents the molecular weights of the rods measured by Den Dunnen et al. (1997b) .... represents the corrected values of the molecular weights.

Cryo-SEM observations of the fracture surface of the nerve guides (Fig. 4) showed a hilly aspect of the fracture surface of the nerve guide in the first week of degradation (Fig. 4a). During the first 5 weeks of degradation, some holes with a diameter of approximately 0.3 μm were formed (Fig. 4b). Eight weeks after onset of the degradation study, an increase in number of holes was observed (Fig. 4c), although the diameter of the holes remained equal. Furthermore, (surface) cracks could be observed after this degradation period (Fig. 4d). Ten weeks after onset of the degradation study, an increase in the number, as well as the size of the cracks, was observed (Figs. 5a,b).

Discussion

The aim of this in vitro study was to evaluate the short term degradation of an amorphous copolymer constructed of DL-lactide and ε-caprolactone, with an initial $M_w$ of $1.1 \times 10^6$ kg/kmol. This study is of interest since this amorphous copolymer is used for the construction of a biodegradable nerve guide (Den Dunnen et al., 1993b).

An ideal nerve guide should provide optimal conditions for the outgrowing and maturation of nerve fibers, and degrade immediately after serving this function, in order to prevent chronic nerve com-
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Figure 4. Cryo scanning electron micrograph (cryo SEM) of a poly (DL-lactide-ε-caprolactone) nerve guide. (a) A hilly aspect of the fracture surface of the nerve guide can be seen in the first week of degradation. (b) Five weeks after the onset of the degradation study, holes of approximately 0.3 μm are formed. (c) After 8 weeks, the biomaterial becomes more porous, an increase in number of holes can be seen. (d) Detail from the hole shown in (c). Notice the beginning of crack formation (arrows).

Pression (Ducker and Hayes, 1968). On the other hand, a nerve guide should not degrade too fast, because fibrous tissue can then grow into the nerve gap and hamper the regeneration and maturation of the nerve fibers. Therefore, this degradation study model will provide relevant information for finetuning the degradation rate of the nerve guide. The evaluation periods of this study ranged from 1-14 weeks, because the regeneration of nerve fibers and maturation of a 1-cm gap in the sciatic nerve of the rat occurs in the same time span (Robinson et al., 1991; Den Dunnen et al., 1993a; Dellon and Mackinnon, 1988).

This study showed that the degradation of poly (DL-lactide-ε-caprolactone) nerve guides did not result in major or minor fluctuations in pH. This means that
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also in the in vivo environment the degradation of the
guide will have no effect on the local pH, especially
since in vivo buffer effects are much more effective.

From the Mw measurements in this study it can be
observed that the Mw is constant until 4 weeks. There-
after, the Mw decreases (i.e., $1.1 \times 10^6$ kg/kmol)
slowly to $4.2 \times 10^5$ kg/kmol.

The degradation of subcutaneously implanted DL-
lactide and ε-caprolactone polymeric bars were
evaluated in a separate study (Den Dunnen et al.,
1997b). In that in vivo study, three months after
degradation, the copolymer still had the same Mw as
the original copolymer, i.e., $1.0 \times 10^6$ kg/kmol (Fig.
3). The degradation was characterized by swelling of
the degrading polymer up to 300%. Four months after
implantation the Mw had suddenly decreased to
approximately $8 \times 10^5$ kg/kmol (Fig. 3). This cor-
responded with the sharp decrease in volume of the
copolymer. The Mw values in that study however,
were obtained by measuring the bulk of the degrading
copolymer and some rat tissue. It is likely that in the
fibrous tissue, surrounding the bulk of biomaterial,
molecules with a lower Mw are present, which was not
measured. So the Mw values measured in that study
needed to be corrected (Den Dunnen, 1996). This was
done by measuring the Mw spectrum from the rat
tissue, these values were subtracted from the original
Mw values. The corrected values are outlined in Fig.
3. From that Figure it can be concluded that the rate
of polymer degradation in this study is much slower
than the corrected Mw values.

This difference in rate of polymer degradation
was probably caused by the fact that in this degra-
dation study the nerve guides were stored at RT,
whereas in the in vivo study, the degradation took
place at approximately 40°C (body temperature of the
rat). Although other parameters (as for example
enzymatic activity) may be of influence too.

In the present study, an appreciable loss of the
original mass of the nerve guides during the entire
evaluation period was not observed (Table 1). It must
be noted that the evaluation period was only 14 weeks.
However, Den Dunnen et al. (1995) found a 7% loss
of the initial weight after 10 weeks in his study. This
implies that in this study, the degradation of poly (DL-
lactide-ε-caprolactone) was relatively slow, which
could also be explained by the fact that in this
degradation study the nerve guides were stored at RT.

The decrease in Mw (i.e., from $1.1 \times 10^6$ kg/kmol
to $4.2 \times 10^5$ kg/kmol), does not correspond with a
sharp decrease in volume of the biomaterial, which
was observed by de Dunnen et al. (1997b). However,
the dry volume of the nerve guides decreased 30% 
during a 14 week period.

Figure 5. (a) Cryo scanning electron micrograph
(cryo SEM) of a poly (DL-lactide-ε-caprolactone)
nerve guide after 10 weeks. Cracks are clearly visible
and an increase of the diameter of the holes was
observed. (b) Cryo scanning electron micrograph
(cryo SEM) of a poly (DL-lactide-ε-caprolactone)
nerve guide after 12 weeks.
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In the first place, these differences could be explained by temperature differences, as stated before. Secondly, the M_w values in the study of Den Dunnen et al. (1997b) were obtained from an in vivo model. In vivo, several proteins and enzymes are present, which can be responsible for a cleavage in ester bonds, which in turn will result in an increase in the hydrolytic degradation (Kulkarni et al., 1971; Pitt et al., 1981).

In the present study, swelling of the biomaterial during degradation was not observed. A change in color of the degrading polymer from transparent to opaque in this study was not observed either. In previous studies, it was found that the degradation of the amorphous poly (DL-lactide-ε-caprolactone) nerve guide was characterized by swelling of the biomaterial, starting between 1 and 2 months after implantation (Den Dunnen et al., 1995, 1996). It was stated that the swelling of the biomaterial was caused by the absorption of water in the degrading copolymer, whereafter the biomaterial became opaque (after drying, the copolymer became transparent again).

Due to the relatively slow degradation of the biomaterial in this short term study, swelling of the biomaterial as well as a change in color was not observed. The difference in temperature of the storage media lowers the degradation rate, although no swelling or decrease in dry mass of the nerve guide could be observed. At the same time, an increase of the diameter of the holes in the biomaterial was observed, immediately followed by a decrease in M_w thereafter. After 12 weeks of the onset of the degradation study, an increase in the number of cracks was observed, again immediately followed by a decrease in M_w at week 13.

In conclusion, the degradation of the poly (DL-lactide-ε-caprolactone) nerve guides in this study was characterized by a slow but steady decrease in M_w with time. Furthermore, cryo-SEM analysis showed holes with a diameter of approximately 0.3 µm 5 weeks after degradation, and cracks after 8 weeks. A decrease of the dry volume of biomaterial during degradation was observed. Loss of dry mass of the nerve guides, as well as swelling of the biomaterial during the 14 week period was not observed. The discrepancy in the results with the study of Den Dunnen et al. (1997b) is probably caused by the fact that in this degradation study the nerve guides were stored at RT, whereas in the other studies the samples were stored at approximately 40°C, resulting in a slower degradation rate. Although other parameters (as for example enzymatic activity) may be of influence too.

In the near future, an in vitro biodegradation study of the poly (DL-lactide-ε-caprolactone) nerve guide stored in different media at different temperatures, in which several proteins and enzymes are present, will be performed. This will provide essential information with regard to the influence of proteins and enzymes on the degradation of poly (DL-lactide-ε-caprolactone) nerve guides.

Acknowledgements

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Discussion with Reviewers

S.W. Shalaby: Is it possible that the early formation of holes is due to evaporation of trace amounts of chloroform?

Authors: No, the early formation of holes is not due to evaporation of trace amounts of chloroform. In the past, cytotoxicity tests [MEM-extract test (72 h)] were carried out for toxic leachables and proved that the nerve guides were non-toxic and contained no chloroform (since chloroform is cytotoxic).

M.S. Shoichet: How were the in vivo nerve guides assessed for changes in molecular weight? Did the rat tissue really dissolve in chloroform? And did you really inject this into the GPC?

Authors: Poly (DL-lactide-ε-caprolactone) dissolves in chloroform. Cell membranes mainly consist of phospholipids, which also dissolve in chloroform. This solution was injected in the GPC.

M.S. Shoichet: You discuss fine-tuning the degradation rate of the nerve guide: what is the desired degradation rate?

Authors: Indeed, fine-tuning of the degradation rate of the nerve guide is of great importance. The desired degradation rate of a nerve guide must be in accordance with the length of the nerve gap and the speed of nerve regeneration. A longer nerve gap desires a slower degradation rate (and the other way around). In a 1 cm gap in the rat, the nerve guide may lose its strength after 2 months. In humans however, first there is a latency period of 3 weeks. Thereafter nerves regenerate with a rate of approximately 1 mm per day. After bridging the nerve gap, the nerve guide should also stay intact during the first phase of maturation. In a 1 cm gap in humans, the nerve guide should therefore stay intact somewhat longer than in a 1 cm gap in the rat.