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# Synthesis and Characterization of Polyamide of Trans-4-hydroxy-L-proline used as Porogen Filler in Acrylic Bone Cement

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**ABSTRACT:** The aim of this study was to synthesize on a larger scale, an experimental polyamide based on an amino acid of trans-4-hydroxy-L-proline. The polyamide of trans-4-hydroxy-L-proline has been used as porogen filler (i.e., a hydrophilic pore generating material) in nondegradable acrylic bone cement. In *in vitro* studies, this hydrophilic filling component has been shown to form porosity within the acrylic bone cement in an aqueous environment. The formation of *in situ* porosity in the acrylic polymer matrix is believed to improve the fixation between the cement and the living bone. Namely, a porous structure can support bone ingrowth and strengthen the mechanical connection between the acrylic bone cement and the bone. The monomer, trans-4-hydroxy-L-proline methyl ester, was prepared from trans-4-hydroxy-L-proline by means of two steps, and the monomer was then polymerized to polyamide of trans-4-hydroxy-L-proline. The polymerization was carried out using a melt polycondensation method. The molecular weights ( $M_n$ ) of the produced polyamides were between

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1800 and 3600. The products were characterized by FTIR and  $^1\text{H-NMR}$  spectroscopy.

**KEY WORDS:** porogen filler, acrylic bone cement, biodegradable polymers, poly(amino acids), trans-4-hydroxy-L-proline, polyamide of trans-4-hydroxy-L-proline.

## INTRODUCTION

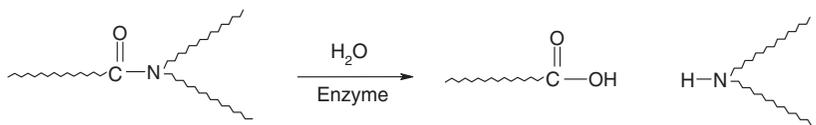
**B**one, as a living hard tissue, can heal itself when fractured. In the case of a serious defect, where a part of the bone is lost, a bone reconstruction is needed. In recent years, both degradable and non-degradable biomaterials have been studied for bone defects [1–7]. Biodegradable polymers have the ability to break down, biologically safely and relatively quickly, into the raw materials of nature and disappear from the body, because they contain hydrolyzable chemical bonds (e.g., esters, amides, or orthoesters). Nevertheless, many biodegradable polymers or their composites with bioactive substances are occasionally too weak in load-bearing applications for bone defects. Therefore, nondegradable polymers (e.g., polymethylmethacrylate (PMMA) or related polymers) with better mechanical strength have been commonly used. Moreover, degradation of the materials used as reconstructive parts in the bone or teeth is in some cases not desired (e.g., acrylic bone cements) [8–10].

Acrylic bone cements in total hip replacements were first performed in the 1960s [11]. In these replacements, the implant devices are held in place by nondegradable acrylic bone cement because it achieves a stable fixation between the implant and the bone. However, the plain unmodified acrylic bone cements prevent bone ingrowth into the cement layer, so new bone formation is limited to the interface between the bone cement and the bone. In fact, nondegradable orthopedic materials should also contain a pore-generating filling component that would produce porosity of a suitable degree in moist conditions. The formation of *in situ* porosity within the acrylic bone cement is supposed to improve the fixation between the orthopedic implant and the living bone, because a porous structure can support bone ingrowth and strengthen the mechanical connection between the bone cement and the bone. The pore size should be large enough (from 100 to 500  $\mu\text{m}$ ) for new bone ingrowth [12]. Basically, porosity should develop immediately after the material has been connected to living tissue (e.g., during the initial days).

Many studies have been done on the bioactive acrylic bone cements, where the acrylic matrix contains bioactive substances [13–17].

The bioactive substances seem to increase the bone ingrowth into the acrylic bone cement. In these composites, the porous phase is not formed very quickly, because in most cases the inert polymer matrix covers the outermost layer of well-embedded filler particles, thus hindering the porosity formation. Moreover, the inorganic bioactive filler particles do not degrade fast enough to form porosity during the first days after implantations. Espigares et al. have developed a partially degradable acrylic bone cement composite that also has hydroxyl apatite as the bioactivity inducer. The degradable component was based on corn starch and cellulose acetate blends [18]. In the present study, the aim is to synthesize an experimental polyamide based on an amino acid of trans-4-hydroxy-L-proline that has been used as a hydrophilic pore generating material in nondegradable acrylic bone cement [19,20]. By adding the hydrophilic porogen oligomer with low molecular weight to nondegradable matrixes, it is possible to create porosity during the first week in moist conditions [21,22]. This porogen oligomer is more precisely poly(amino acid).

Poly(amino acids) and pseudo-poly(amino acids) are two classes of biodegradable polymers. Poly(amino acids) have amide bonds and pseudo-poly(amino acids) have ester bonds in their backbone: both of these polymers are produced from amino acids (e.g., from tyrosine, lysine, or hydroxyproline) [23,24]. In the literature, synthesis of N-protected polyesters of trans-4-hydroxy-L-proline and trans-4-hydroxy-L-proline have been reported by Putnam and Langer [25]. Some years ago, we reported our synthesis to produce the polyesters of N-benzyl protected trans-4-hydroxy-L-proline and trans-4-hydroxy-L-proline [26]. A degradation study of the polyester of trans-4-hydroxy-L-proline has been reported by Lim et al. [27]. Their degradation study showed that the polyester degraded rapidly, namely, its molecular weight decreased to half in less than 2 h. Both the polyamide and the polyester of trans-4-hydroxy-L-proline contain a naturally occurring and nontoxic building block. More precisely, trans-4-hydroxy-L-proline is a constituent in collagens, gelatin, and other proteins [28]. Therefore, this degradation product can be digested by cells, because it is present in the normal metabolic components of living tissues. The polyamide of trans-4-hydroxy-L-proline like proteins has hydrolyzable amide bonds in its structure (Figure 1). This synthetic polymer-containing hydroxyl group, in every repeating unit, readily absorbs water, and with lower molecular weights, it is even quite easily hydrolytic-dissolvable. This study was limited to synthesizing on a larger scale (>50 g) an experimental polyamide based on an amino acid of trans-4-hydroxy-L-proline.

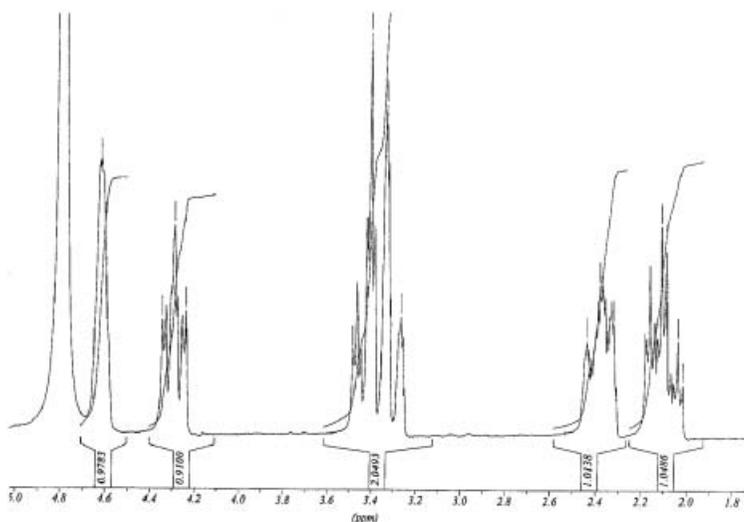


**Figure 1.** A hydrolyzable amide bond that breaks down to form a carboxyl acid and an amino group.

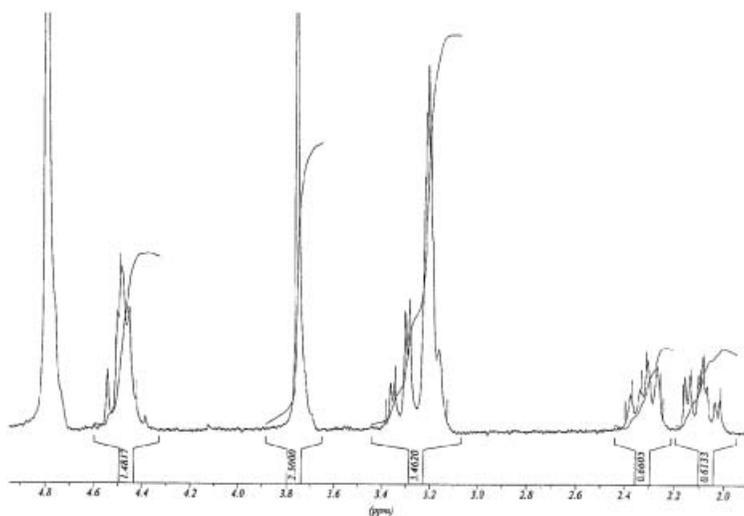
## EXPERIMENTAL

### Synthesis of Polyamide of Trans-4-hydroxy-L-proline

Trans-4-hydroxy-L-proline methyl ester hydrochloride salt was synthesized from trans-4-hydroxy-L-proline (50 g, 381.3 mmol, 100 mol%, Applichem, Darmstadt, Germany) in a mixture of anhydrous methanol (200 mL) and acetyl chloride (32.7 mL, 457.6 mmol, 120 mol%, Merck-Schuchardt, Hohenbrunn, Germany). First, the dried methanol (200 mL) was precooled and stored in an ice/salt bath at  $-10^{\circ}\text{C}$ , after which acetyl chloride was added to the methanol extremely slowly (30 min). Trans-4-hydroxy-L-proline was mixed with 200 mL of dried methanol, to which the HCl-methanol mixture was added. The mixture obtained was then stirred at a refluxing temperature for 48 h in an argon atmosphere. Trans-4-hydroxy-L-proline methyl ester hydrochloride salt was isolated, and the crude material was purified by flash column chromatography. Column chromatography was carried out on silica gel 60 (70–230 mesh, Merck, Darmstadt, Germany). Trans-4-hydroxy-L-proline methyl ester hydrochloride salt (Figures 3 and 5B) was obtained as a white, crystalline solid (52.2 g, 287.5 mmol, 75 mol%), Rf. 0.28 (methanol : ethyl acetate (1 : 1), ninhydrin), m.p.  $164.5^{\circ}\text{C}$ . Trans-4-hydroxy-L-proline methyl ester was prepared from trans-4-hydroxy-L-proline methyl ester hydrochloride salt using an ion exchange resin. Strongly basic gel-type ion exchange resin (400 g, Amberlite IRA-400, Fluka Chemie GmbH, Buchs, Switzerland) was stirred with 500 mL of 2 M NaOH (Natriumhydroxide, Akzo Nobel, Eka Nobel AB, Bohus, Sweden) at room temperature for 5 min. The resin was then washed with distilled water until the pH value was ca 7. After this, the resin was filtered, dried, and rinsed with anhydrous methanol. An aliquot of synthesized trans-4-hydroxy-L-proline methyl ester hydrochloride salt (20.8 g) was dissolved in dried methanol (400 mL), and the solution was then eluted several times through a gel-type ion exchange resin column (Amberlite IRA-400 in OH-form). The solvent was evaporated and the trans-4-hydroxy-L-proline methyl ester (Figures 4 and 5c) obtained was



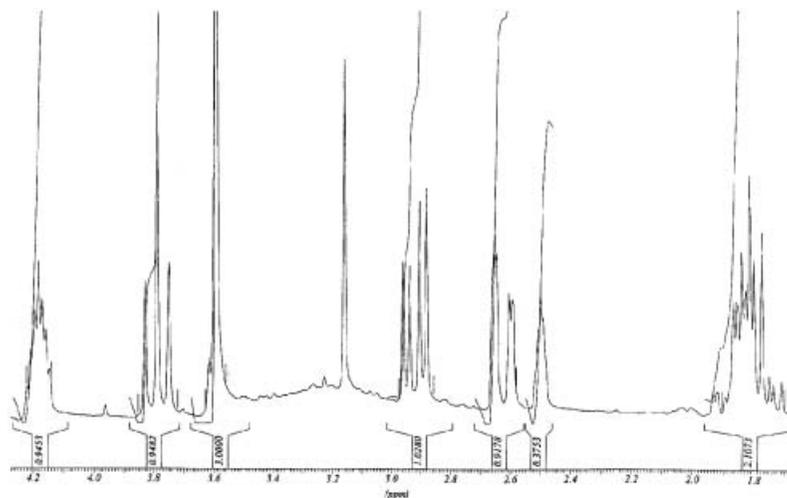
**Figure 2.**  $^1\text{H-NMR}$  spectra of trans-4-hydroxy-L-proline.



**Figure 3.**  $^1\text{H-NMR}$  spectra of trans-4-hydroxy-L-proline methyl ester hydrochloride salt.

a colorless, viscous liquid (12.0 g, 82.39 mmol, 72 mol%), Rf. 0.63 (methanol : ethyl acetate (1 : 1), ninhydrin).

The monomer, trans-4-hydroxy-L-proline methyl ester, was used in the polymerization step and some polymerization experiments were



**Figure 4.**  $^1\text{H}$  NMR spectra of *trans*-4-hydroxy-L-proline methyl ester.

*Table 1. Some characteristic bands to interpret the products using FTIR.*

Position of band/ $\nu(\text{cm}^{-1})$	Assignment of signal
2500–3000	Carboxylic acid
2400–2700	Ammonium salt
1740	Methylester
1640	Amide

carried out to upscale the production of polyamide of *trans*-4-hydroxy-L-proline (Table 2). In one polymerization experiment, the isolated monomer ester had been combined from a few synthesis pots of *trans*-4-hydroxy-L-proline methyl ester. The polymerization reaction flask was charged with the isolated monomer ester ( $m = 50\text{--}100\text{ g}$ ) and subjected to melt-condensation at elevated temperatures in a vacuum. The reaction system had also been equipped with an acetone/liquid nitrogen trap. Before the reaction, the monomer was agitated during the first 5 min by flushing the system with argon. Then, the monomer was heated to between 100 and 120°C, and the catalyst, calcium acetate hydrate (from 0.5 to 2.5 mol%, Fluka Chemie GmbH, Buchs, Switzerland), was added. Thereafter, the reaction was further heated to between 150 and 200°C in a vacuum for 30 min–3 h, depending on

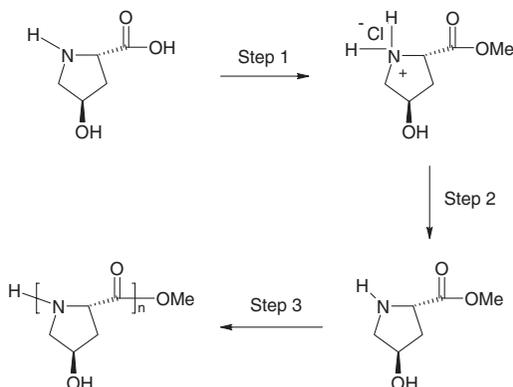
the reaction conditions being tested. The reaction system was stirred using a powerful electronic stirrer (Heidolph, RZR 2102 control, Schwabach, Germany).

### Characterization

The trans-4-hydroxy-L-proline methyl ester hydrochloride salt was analyzed on thin-layer chromatography (TLC) plates (i.e., precoated aluminum plates, Merck Kieselgel 60 F<sub>254</sub>, Merck, Darmstadt, Germany) and visualized with UV light ( $\lambda = 254$ ) or dipped in ninhydrin solution. The Fourier transform infrared spectrometer (FTIR, Spectrum One, Perkin Elmer, Beaconsfield Bucks, UK) was used, and the spectra were measured using the attenuated total reflection (ATR) sampling accessory. In addition, <sup>1</sup>H nuclear magnetic resonance spectroscopy (NMR) spectra were recorded on a Bruker AM200 or AM500 NMR spectrometer (Bruker Fällanden, Switzerland). For the determination of the molecular weight of oligomers, the viscosity measurements were carried out using an Ubbelohde viscometer (Schott-Geräte GmbH, Mainz, Germany). An Ubbelohde-type viscometer was used to determine the molecular weights in 2,2,2-trifluoroethanol when using five standards of poly(ethylene glycol) with  $M_w$  between 400 and 10,000. The poly(ethylene glycol) standards (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were used as calibration agents with molecular weights ( $M_w$ ) of 400, 2000, 4600 and 10,000. All the standards and samples of synthesized oligomers were dissolved in 2,2,2-trifluoroethanol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to prepare solutions at concentrations of 10 mg/mL. The flow rates of each standard and sample were measured five times at 50°C, and the average flow rates were used in the final calculations. The average molecular weights of oligomers were determined from the calibration line ( $R^2 > 0.95$ ) of poly(ethylene glycol).

## RESULTS

In the first step, the trans-4-hydroxy-L-proline was first converted into methylester by an acid-catalyzed esterification (Scheme 1), which is one of the fundamental processes in organic chemistry [29]. The catalyst was prepared *in situ* from acetyl chloride and methanol. The acetyl chloride reacted with methanol extremely exothermically. According to these reaction experiments, esterification with methanol could easily be carried out on a larger scale. The upscaling was carried out by reducing the intermediary concentration. The solvent was evaporated,



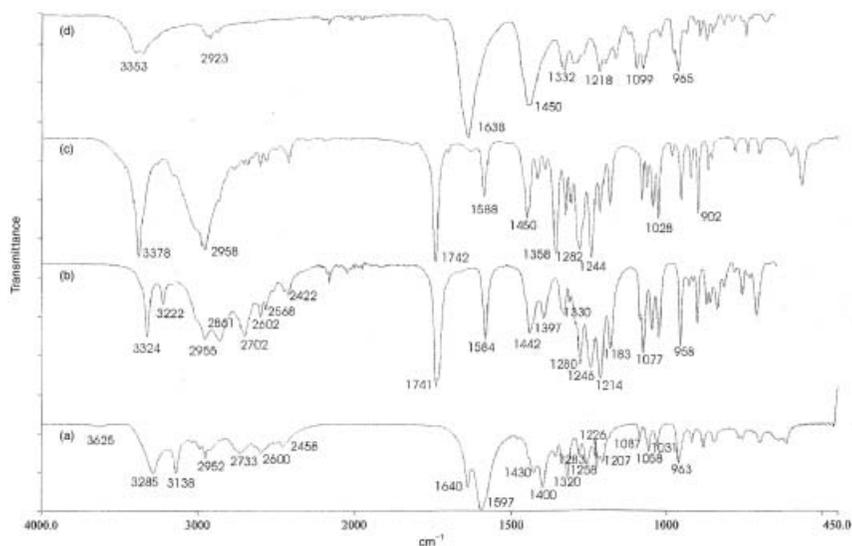
**Scheme 1.** The synthesis route of polyamide of trans-4-hydroxy-L-proline by the melt polycondensation method.

*Table 2. The test conditions for polymerization experiments.*

Test	$T_{MAX}$ /°C	Catalyst/mol%	Time/h	$M_v$
1	200	1	1.5	1800
2	200	0.5	0.5	2000
3	150	2.5	2	2800
4	200	0.1	1	3200
5	150	1.2	3	3600

and the crude product was purified using flash chromatography. The yield from this synthesis step was ca 75 mol%. The formed HCl of trans-4-hydroxy-L-proline methyl ester hydrochloride was released using an excess of gel-type anion exchange resin. Before the releasing reaction, the chloride ions occupying the exchange sites in the resin had been replaced by hydroxide ions. The yield from this synthesis step was ca 72 mol%. The synthesis of the polyamide of trans-4-hydroxy-L-proline was done by melt-condensation polymerization. The polymerization experiments are shown in Table 2. The studied factors affecting the molecular weight were the temperature, the amount of the catalyst, and the reaction time. According to the results of these experiments, the polymerization is relatively easy to upscale to the extent that 50–100 g of polyamide can easily be prepared. During the polymerization, the reaction mixture became increasingly viscous. The formed polyamide was fibrous, and the achieved molecular weight ( $M_v$ ) was between 1800

and 3600. The molecular weight ( $M_v$ ) of the polyamide was 3600, when the reaction conditions were as follows: the reaction temperature was 150°C, the amount of catalyst 1.2 mol%, and the reaction time 3 h. The FTIR and the NMR spectroscopy methods were used to study the structural properties of the products. According to the FTIR spectra, the most important signal for analyzing the structure of trans-4-hydroxy-L-proline methyl ester hydrochloride salt was the formed methylester signal at wave number 1741  $\text{cm}^{-1}$ . Moreover, it was found that there were five bands between wave numbers 2568 and 2956  $\text{cm}^{-1}$  that could be assigned to the ammonium salt structure. When the ammonium salt structure had been released from trans-4-hydroxy-L-proline methyl ester hydrochloride salt, the ammonium salt bands disappeared (Figures 5B and C). In the  $^1\text{H-NMR}$  spectrum, the methylester peak was a singlet at  $\delta 3.75$  ppm, when analyzing the structure of trans-4-hydroxy-L-proline methyl ester hydrochloride salt (Figure 3). After the ammonium salt was released from its structure, the chemical shift of methylester was found at  $\delta 3.56$  ppm as a singlet (Figure 4). In the FTIR spectrum of polyamide, the amide signal existed at wave number 1628  $\text{cm}^{-1}$  (Figure 5).

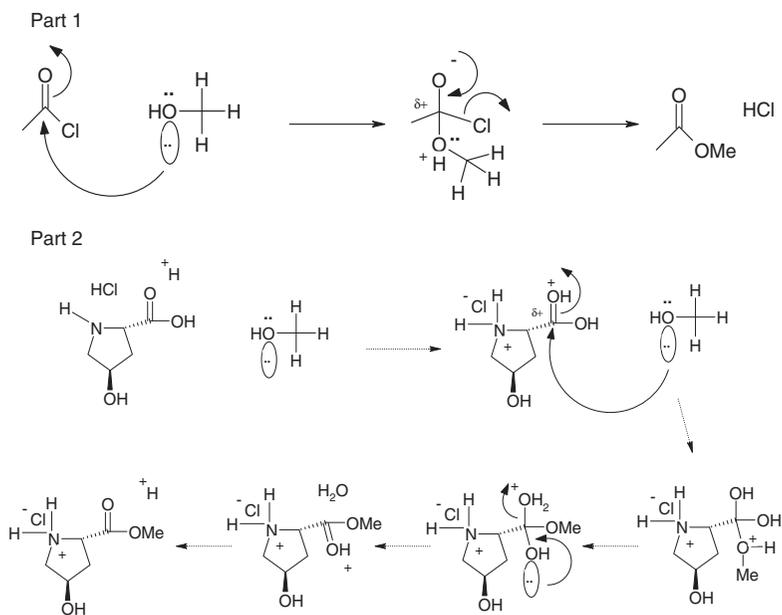


**Figure 5.** FTIR spectra: (a) trans-4-hydroxy-L-proline, (b) trans-4-hydroxy-L-proline methyl ester hydrochloride salt, (c) trans-4-hydroxy-L-proline methyl ester, and (d) polyamide of trans-4-hydroxy-L-proline.

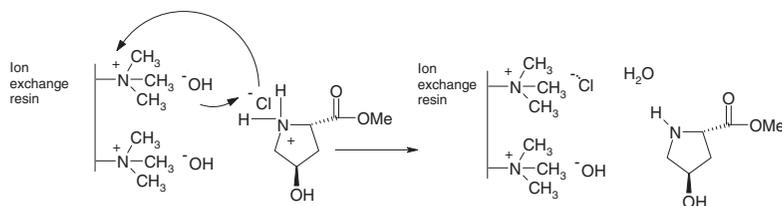
## DISCUSSION

The synthesis of the polyamide of *trans*-4-hydroxy-L-proline was conducted in three steps: (1) methyl esterification, (2) releasing of ammonium salt, and (3) polycondensation. The reaction mechanism of the esterification used in synthesis step 1 is illustrated in Scheme 2. Rapid proton transfer started the esterification reaction after hydrochloride acid had formed in the reaction with acetylchloride and methanol. After HCl-catalyzed esterification, the formed amino acid ester has ammonium salt in its structure that has to be released. In ion exchange chromatography, a charged support separates molecules according to the amount of charge they carry. In the ion exchange reversible chemical reaction, chloride ions from *trans*-4-hydroxy-L-proline methyl ester hydrochloride salt were exchanged for similarly charged hydroxide ions (Scheme 3). In this synthesis step, an excess of ion exchange resin was used for preparing *trans*-4-hydroxy-L-proline methyl ester.

The *trans*-4-hydroxy-L-proline methyl ester monomer was subjected to melt-polycondensation at elevated temperatures ( $>150^{\circ}\text{C}$ ) in a vacuum. The monomer unit had two functional groups (i.e., methylester



**Scheme 2.** The reaction mechanism in the esterification step.



**Scheme 3.** The reaction mechanism in the treatment with the ion exchanger resin.

and amine). The polyamide results from a reaction of monomer units, accompanied by the loss of methanol (i.e., leaving group) that was distilled from the reactor. This polycondensation reaction has a nucleophilic substitution character. One necessary component for nucleophilic substitution is the leaving group. This polymerization reaction must be carried out in a vacuum at a high temperature, because methanol is a poor leaving group. Therefore, the obtained molecular weight was relatively low. However, according to the test polymerizations, the polyamide of *trans*-4-hydroxy-L-proline can be prepared relatively easily on a larger scale. The molecular weight of the polymer varied from 1800 to 3600, depending on the selected conditions of the reaction, such as the amount of catalyst, the temperature, and the reaction time. The molecular weight was 3600, when the amount of catalyst was 1.2 mol%, and the polymerization was allowed to occur at a lower temperature (150°C) during 3 h. To improve this polymerization step by trying to obtain higher molecular weights, other leaving groups and catalyst types should be studied.

The obtained product, polyamide of *trans*-4-hydroxy-L-proline, was brittle and easy to crush manually in a mortar. Therefore, it has been used as a hydrophilic pore generating material in nondegradable acrylic bone cement. The mean particle size of powders varied between 10 and 500  $\mu\text{m}$ . Hydrophilic porogen filler particles that are embedded inside the nonresorbable polymer matrix degrade in contact with body fluid. The remaining porous polymer matrix is supposed to provide a framework for new tissue ingrowth. Normally, the porous phase in acrylic bone cement is not formed easily onto the outermost surface, because in most cases the inert polymer matrix covers the filler particles, thus hindering porosity formation (e.g., when using inorganic bioactive filler). An optimal resorbable filler material should swell and dissolve very easily in contact with water or body fluid, and it should be a biocompatible and nontoxic material. Generally, good filler materials are believed to be swellable hydrogels or hydrophilic polymers [30]. The phenomenon of porogen filler swelling might also compensate for

the shrinkage of the bone cement matrix in the polymerization process, and it would also hold the implant device tightly in place by means of more stable fixation.

Polyamide of trans-4-hydroxy-L-proline was used as porogen filler in acrylic bone cement on the following basis: (a) it breaks the outermost surface of the composite, and forms a porous structure in moist conditions, (b) it has a low molecular weight, hence dissolves quite easily in water, (c) it is relatively easy to prepare by a three-step synthesis procedure, and (d) its degradation products should be nontoxic for mammals. In fact, the use of polyamide of trans-4-hydroxy-L-proline in biomedical devices is a natural choice, because its building block is only trans-4-hydroxy-L-proline that occurs widely both in the body and in nature [28]. According to ongoing *in vivo* tests, acrylic bone cement containing the polyamide of trans-4-hydroxy-L-proline used as porogen filler seems to have no foreign body reaction. Moreover, according to some preliminary studies, the degradation product of the polyamide of trans-4-hydroxy-L-proline is hydroxyproline. In addition, *in vitro* studies have shown that water can diffuse through acrylic bone cement containing porogen filler, resulting in a porous structure during the first week [21,22]. Because of the current demand of polyamide for trans-4-hydroxy-L-proline in ongoing experiments, the synthesis for producing polyamide of trans-4-hydroxy-L-proline on a larger scale was created, although polyamide of trans-4-hydroxy-L-proline is also commercially available.

In acrylic bone cement, porosity formation has an obvious effect on the mechanical properties [21]. Thus, the stresses might concentrate at the weaker interfaces of porous structures. According to our experiments, the weakening effect of the porous structure has been offset by reinforcing the matrix with glass fibers and a semi-interpenetrating network (semiIPN) structure [22,31]. Moreover, for plain acrylic bone cement, Schmitt et al. have shown *in vitro* that fatigue crack propagation resistance improves in a fluid environment [32]. Our investigations on producing porous bone cement have been limited to flexural and compressive properties, so more mechanical properties such as fatigue strength, fracture toughness, and fatigue crack propagation should be studied in more detail for composites containing porogen filler. In addition, some poly(amino acids) have been reported to have relatively slow hydrolytic degradation [33,34]. Thus, the biodegradation nature (hydrolysis) of the polyamide of trans-4-hydroxy-L-proline catalyzed by enzymes is under more detailed investigation. The polyamide of trans-4-hydroxy-L-proline is supposed to degrade rapidly, because it is hydrolytic-dissolvable with a low molecular weight (>5000).

The antigenicity of poly(amino acids) with more than three repeating units in a chain may be one problem [35]. Therefore, the antigenicity and cytotoxicity of the polyamide of trans-4-hydroxy-L-proline will be investigated, as well as the biocompatibility of both the polyamide and its degradation product.

## CONCLUSION

The polyamide of trans-4-hydroxy-L-proline can be prepared from commercially available trans-4-hydroxy-L-proline using a three-step synthesis method. According to this method, the polymerization is relatively easy to upscale to the extent that 50–100 g of polyamide is prepared. The polyamide of trans-4-hydroxy-L-proline can be used as porogen filler in nondegradable acrylic bone cement.

## ACKNOWLEDGMENTS

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