Periodontal Tissue Regeneration Using Enzymatically Solidified Chitosan Hydrogels With or Without Cell Loading

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This study is aimed to evaluate the *in vivo* biocompatibility and periodontal regenerative potential of enzymatically solidified chitosan hydrogels with or without incorporated periodontal ligament cells (PDLs). To this end, chitosan hydrogels, with ($n=8$; CHIT + CELL) or without ($n=8$; CHIT) fluorescently labeled PDLs, were prepared and transplanted into rat intrabony periodontal defects; untreated defects were used as empty controls ($n=8$; EMPTY). After 4 weeks, maxillae were harvested, decalcified, and used for histological, histomorphometrical, and immunohistochemical assessments. The results showed that PDLs remained viable upon encapsulation within chitosan hydrogels before transplantation. Histological analysis demonstrated that the chitosan hydrogels were largely degraded after 4 weeks of implantation, without any adverse reaction in the surrounding tissue. In terms of periodontal regeneration, alveolar bone height, alveolar bone area, and epithelial downgrowth were comparable for CHIT, CHIT + CELL, as well as EMPTY groups. In contrast, both CHIT and CHIT + CELL showed a significant increase in functional ligament length compared with EMPTY. From a cellular perspective, the contribution of chitosan hydrogel-incorporated cells to the periodontal regeneration could not be ascertained, as no signal from transplanted PDLs could be detected at 4 weeks post-transplantation. The results demonstrated that enzymatically solidified chitosan hydrogels are highly biocompatible and biodegradable. Moreover, chitosan hydrogels without cell loading can improve periodontal regeneration in terms of functional ligament length, indicating the great potential of this hydrogel in clinical applications. Further work on the use of chitosan hydrogels as cell carriers is required.

Introduction

PERIODONTITIS, A COMMON inflammatory disease caused mainly by dental plaque microorganisms,¹ is characterized by irreversible loss of periodontal tissues, which include gingiva, alveolar bone, root cementum, and periodontal ligament (PDL). Conventional treatments for periodontitis, including scaling and root planing are usually successful in preventing the progression of the active disease, but the regeneration of lost tissues remains a clinical challenge.

Recently, periodontal tissue engineering, using supportive biomaterials and stem/progenitor cells, has been applied as a promising alternative to conventional periodontal treatments.^{2–5} Biomaterials offer tissue engineering and regenerative medicine a powerful tool in the form of cell delivery vehicles and scaffolds. A three-dimensional scaffold, serving as a space maintainer to temporarily support stem/progenitor cell attachment, proliferation, and differentiation, is an important element for tissue engineering.⁶ However, a diseased periodontal environment usually lacks robust stem/progenitor cells. Therefore, transplantation of *ex vivo* ex-

panded stem/progenitor cells is hypothesized to facilitate periodontal regeneration.⁷

Among various biomaterials, chitosan has received major attention in tissue engineering and regenerative medicine due to its biocompatibility,⁸ biodegradability,^{9,10} tissue regeneration capacity,¹¹ anti-inflammatory effects,¹² and antimicrobial activity.¹³ Chitosan can be applied as a biomaterial in different forms, of which hydrogel vehicles are of great interest, as they offer several advantages, including high cell seeding efficiency, easy handling, and good formability to fill irregular defects.¹⁴ Chitosan hydrogels can be prepared by either chemical or physical crosslinking of chitosan chains. The drawbacks of chemically crosslinked chitosan hydrogels include the potential cytotoxicity of applied chemical crosslinkers, such as glutaraldehyde, and the risk of altering the initial properties of chitosan through chemical modifications of the primary structure.^{15,16} A possible means to avoid the aforementioned disadvantages is to prepare physically crosslinked chitosan hydrogels, which can be obtained by increasing the pH of acidic chitosan solutions without the use of chemical crosslinking agents.^{9,17,18} Chenite *et al.*¹⁹ reported that pH-induced

chitosan hydrogels can be generated through homogeneous neutralization of chitosan solutions by ammonia generated *in situ* from enzymatic hydrolysis of urea. In our previous work, chitosan gelation kinetics of this system were addressed.²⁰ The results demonstrated that the gelation time of chitosan hydrogels can be precisely controlled by variation of the urea and urease concentrations, which provides the opportunity to set the desired gelation time for various applications.

Regarding cell population for periodontal regeneration, various cell types have been evaluated, including bone marrow stromal cells (BMSCs), periodontal ligament cells (PDLs), alveolar periosteal cells (APCs), dental pulp cells (DPCs), and dental follicle cells (DFCs).^{2,4,5,21} Tsumanuma *et al.* transplanted BMSCs, PDLs, and APCs in canine one-wall intrabony defects to compare the regenerative potential between cell sources.⁴ After 8 weeks, significantly more well-oriented periodontal PDL fibers and newly formed cementum were observed upon transplantation of PDLs. In addition, an organ culture study performed on tooth root surfaces showed that new alveolar bone and PDL-like tissues were formed only by PDLs, but not by MSCs, DPCs, or DFCs.⁵ These results suggest that PDLs are the most suitable cell population for periodontal regeneration.

In our previous study, the suitability of enzymatically solidified chitosan hydrogel was evaluated for delivery of PDLs *in vitro*.²⁰ The results showed that this hydrogel can encapsulate PDLs and support their survival *in vitro* for up to 30 days. In addition, PDLs released from the hydrogel upon degradation were able to form colonies and differentiate into the osteogenic lineage. However, the *in vivo* biocompatibility of this chitosan hydrogel is not clear and the regenerative capacity of this system in an animal model still needs to be confirmed. Therefore, the aim of the present study was to evaluate the biocompatibility and regenerative potential of the enzymatically solidified chitosan hydrogel with or without incorporated PDLs for periodontal regeneration *in vivo*. Based on our previous experiments,^{2,22,23} a validated rat maxillary periodontal defect model was chosen and PDLs from green fluorescent protein (GFP) transgenic rat were applied to allow cell tracing after transplantation.

Materials and Methods

Materials

Chitosan (average molecular weight 500 kDa, degree of deacetylation 85%; Hepe Medical Chitosan GmbH, Halle, Germany), urea (Invitrogen, Carlsbad, CA), and urease (type III from Jackbeans, U1500-20kU; Sigma, St. Louis, MO) were used as received. Chitosan was dissolved in 0.125 M HCl at a concentration of 2.5 w/v%. Urea (50 U/mL) was dissolved in MilliQ at a concentration of 10 M and sterilized by filtration over a 0.2 μ m filter. Urease was dissolved in cell culture medium, which contained Alpha-Minimum Essential Medium (α -MEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma), 100 U/mL penicillin, and 50 U/mL streptomycin (Gibco).

Isolation of PDLs

Primary PDLs were retrieved from one GFP transgenic Sprague Dawley rat (Japan SLC, Inc., Shizuoka, Japan) as described previously.² Briefly, PDLs were derived from

extracted incisors of the GFP rat. The primary cell extraction procedure was performed according to the method described by Brunette *et al.*²⁴ Cells were expanded in a cell culture medium. Upon 70–80% confluency, cells were subcultured for three passages and afterward cryopreserved in α -MEM containing 20% fetal calf serum and 10% dimethyl sulfoxide until use. After defrosting, cells from the fifth passage were used.

Cell encapsulation

Cell encapsulation was performed 1 day before *in vivo* implantation. Chitosan hydrogels were prepared as described previously.²⁰ In brief, 1 mL chitosan solution was mixed with 0.67 mL urease solution (50 U/mL). Subsequently, 6.25 μ L of the urea solution was added and mixed, which resulted in urea hydrolysis and pH increase. Before gelation, 200 μ L chitosan solution containing 37.5 mM urea and 30 U/mL urease were mixed with 50 μ L proliferation medium containing 3×10^6 PDLs. After gentle mixing, 20 μ L of the mixture (1.2×10^7 cells/mL) was quickly injected in presterilized Teflon molds ($\varnothing 2.5$ mm), followed by incubation at 37°C for 30 min. Afterward, the newly formed gels were transferred to nonadherent tissue culture 24-well plates. Proliferation medium was added and refreshed 30 and 60 min after encapsulation.

In vitro assays

Cell survival was determined using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) after 1 day of culture. Hydrogel samples were washed in phosphate buffered saline (PBS) and incubated for 30 min in PBS solution with 2 mM calcein-AM and 4 mM ethidium homodimer at 37°C. After incubation, samples were rinsed again in PBS, and then photographed using a Zeiss Imager Z1 equipped with an AxioCam MRc5 camera and operated using the AxioVision 4.6.3 software (Carl Zeiss Microimaging GmbH, Göttingen, Germany). Percentage of live cells was quantified using the ImageJ software (National Institute of Health, Bethesda, MD) from three samples.

For histological analysis, samples were fixed in 10% phosphate buffered formalin for 2 h. Thereafter, samples were dehydrated in graded ethanol and embedded in paraffin. After deparaffinization in xylene and rehydration through graded series of ethanol, sections of 6 μ m were cut with a microtome (Leica RM2165, Nussloch, Germany).

Animals

Twelve healthy male athymic nude rats (Hsd:RH-Foxn1^{nu}; Harlan Laboratories, Horst, the Netherlands) were used as the recipient animals. The Animal Ethics Committee of the Radboud University, Nijmegen approved the study protocol (RU-DEC-2013-143). All procedures were in accordance with the national guidelines for the care and use of laboratory animals. The recipient rats were 6-week old at the start of the experiment and had a known specific pathogen-free status.

Surgical procedures

To minimize peri- and postoperative pain, rats received subcutaneous injection of carprofen (5 mg kg⁻¹, Rimadyl; Pfizer Animal Health BV, Capelle aan de IJssel, the Netherlands) preoperatively and on the first 2 days postoperatively.

After intubation, general anesthesia was maintained with a mixture of nitrous oxide, 2.5% isoflurane, and oxygen through a constant volume ventilator. Bilateral intrabony three-wall periodontal defects were created mesially to both maxillary first molars as described previously^{2,25} (Fig. 1A–F). With the aid of 2.5× magnifying loupes and strong light, a 3-mm-long full thickness incision was made along the alveolar ridge. A local adrenaline-containing dental anesthetic (Ultracain D-S®; Aventis Pharma BV, Gouda, the Netherlands) was applied to reduce bleeding. Flaps were raised to expose the root surface and alveolar bone. Subsequently, a three-wall intrabony defect was created along the root surface using a piezoelectric device (OT5 B-tip, Ø1.7 mm, Piezosurgery®; Mectron, Carasco, Italy). Thereafter, the remaining alveolar bone, PDL, and cementum were carefully removed from the root surface using a less abrasive OP5-tip. This tip was also used to create the final defect ($W \times L \times D$; $2 \times 2 \times 1.7 \text{ mm}^3$), with monitoring the dimensions by a periodontal probe. Thereafter, defects were rinsed with sterile saline and dried with sterile gauze. Subsequently, the defects were randomly left empty ($n=8$; EMP-TY), treated with either chitosan hydrogel only ($n=8$; CHIT) or chitosan hydrogel encapsulated with 0.24×10^6 rat GFP⁺ PDLCs ($n=8$; CHIT + CELL). Chitosan hydrogels as prepared in Cell Encapsulation section were gently picked up from nonadherent tissue culture 24-well plates and placed in defects by a tweezer. Finally, flaps were repositioned and sutured with resorbable sutures (Vicryl® 5-0; Ethicon Products, Amersfoort, The Netherlands).

Histological preparation

After 4 weeks, rats were deeply anesthetized before 10% formalin cardiovascular perfusion. Complete maxillae were harvested and excess tissues were removed. Subsequently, the maxillae were split into two parts through the palatal median line and fixed in buffered 10% formaldehyde for 24 h. Afterward, specimens were decalcified in 10% EDTA at room temperature. Decalcification end point was confirmed using X-ray. Thereafter, specimens were dehydrated in a graded series of ethanol and embedded in paraffin. Mesio-distal sections (6 µm thickness) were cut with a microtome (Leica RM2165). Sections were stained with Hematoxylin and Eosin (HE) for general tissue survey. For ligament and epithelial tissue observation, adjacent sections were stained with Azan staining and Elastica-van Gieson (EVG) staining, respectively.

Immunohistochemistry

GFP-positive cells from *in vitro* and *in vivo* samples were detected by immunohistochemical staining using an anti-GFP antibody (rabbit IgG, fraction, 1:500; Molecular Probes). Specimens from previous work² were used as positive control. After sections were deparaffinized, rehydrated, and rinsed in PBS, antigens were retrieved with sodium citrate buffer at 70°C for 10 min. Then, sections were preincubated in 10% normal donkey serum to inhibit non-specific staining for 60 min at room temperature. Next, sections were incubated with primary antibody (GFP, 1:500, rabbit anti IgG fraction; Molecular Probes) or PBS (negative control) overnight at 4°C in a humid atmosphere. Subsequently, sections were rinsed with PBS and incubated with a

biotin-conjugated donkey anti-rabbit IgG (Jackson Immuno-Research, West Grove, PA) for 1 h at room temperature. Then, sections were immersed in ABC complex solution in the dark for 45 min at room temperature. The final visualization of reaction products was performed by treatment of 3,3' diaminobenzidine substrate (Envision kit; Dako-Cytomation, Glostrup, Denmark) for 10 min at room temperature. The nuclei were slightly counter stained with Hematoxylin.

Histomorphometry

For the quantitative analysis of each specimen, up to four sections from the middle third of the defect area were evaluated. Sections were photographed using a Zeiss Imager Z1 equipped with an AxioCam MRc5 camera operated with the AxioVision 4.6.3 software (Carl Zeiss Microimaging GmbH). Area and linear measurements were performed on images of histological sections by a blinded assessor (X.Y.) using the ImageJ image analysis software (National Institute of Health, Bethesda, MD), as previous described² (Fig. 1G). The following quantitative results were calculated based on the formulations:

$$\text{Relative alveolar bone height} = \frac{L_{ac}}{L_{bg}} \quad (1)$$

where L_{ac} is the length of the mesial molar from apex (apical end of mesial root of the first molar) to the cusp (Fig. 1G ②), and L_{bg} is the length of bone gap, which was assessed as the distance between the alveolar bone level of the intact bone (dashed line IB) and the top of the new bone (Fig. 1G ①). A higher score indicates superior regenerated bone height.

$$\text{Relative new bone area} = \frac{A_{nb}}{A_{td}} \quad (2)$$

where A_{nb} is the area of new bone formation (Fig. 1G, area within green frames), and A_{td} is the area of the total defect (Fig. 1G, area within red frame).

$$\text{Relative epithelial downgrowth} = \frac{L_j}{L_{ac}} \quad (3)$$

where L_j is the length of the junctional epithelium from the cemento–enamel junction (dashed line CEJ) to the most apical extent (Fig. 1G ③), and L_{ac} is the length of the mesial molar from apex to cusp (Fig. 1G ②). A higher score indicates more epithelial downgrowth.

$$\text{Relative functional ligament length} = \frac{L_{ff}}{L_{ca}} \quad (4)$$

where L_{ff} is the length of functional ligament (Fig. 1G ④), and L_{ca} is the distance from the cemento–enamel junction (dashed line CEJ) to the apical end of the defect (Fig. 1G ⑤).

Collagen fiber bundles were defined to be functional if the angle between the long axis of the main fibers and the root surface was larger than 60°. ^{3,26}

Statistical analysis

Statistical analysis was carried out using the GraphPad InStat (GraphPad Software, San Diego, CA). Results were

FIG. 1. Surgical procedure and schematic drawing of histomorphometrical analysis. (A) Preoperative general observation; (B) Flaps were raised to expose the root surface and alveolar bone; (C) The created defect; (D) Hydrogel placement in the defect; (E) Flaps were repositioned; (F) Flaps were closed with resorbable sutures; (G) Schematic drawing illustrating the histometric analysis of periodontal tissue regeneration. The numbers from one to five indicate the length of bone gap, the length of the mesial molar from apex to the cusp, the length of the junctional epithelium from the cemento–enamel junction to the most apical extent, the length of functional ligament, and the distance from the cemento–enamel junction to the apical end of the defect, respectively. Dashed lines CEJ and IB indicate the cemento–enamel junction and the alveolar bone level of the intact bone, respectively. Area within the green frames indicate the area of new bone formation.

statistically evaluated by one-way ANOVA, followed by a Tukey–Kramer Multiple Comparisons Test. Results were considered statistically significant at $p < 0.05$.

Results

Cell viability in the chitosan hydrogel

After 1-day culture, chitosan hydrogels encapsulated with PDLCs were processed with LIVE/DEAD viability assay. The results showed that PDLCs were viable (Fig. 2), as evidenced by the appearance of large numbers of live cells (appearing in green) and only few dead cells (appearing in red). Quantitative analysis showed that $96.8\% \pm 1.7\%$ of cells were viable in chitosan hydrogels.

General observations animal experiment

One sample from EMPTY and one sample from CHIT were lost after one rat died during surgery. The remainder of the rats showed uneventful healing and gained weight

FIG. 2. Viability of PDLCs incorporated into chitosan hydrogels after 1-day culture, visualized by LIVE/DEAD staining (live cells appear in green, dead cells in red). PDLCs, periodontal ligament cells.

TABLE 1. NUMBER OF DEFECTS, HARVESTED AND EVALUATED SPECIMENS FOR DIFFERENT GROUPS

	<i>Number of defects</i>	<i>Number of harvested specimens</i>	<i>Number of evaluated specimens</i>
EMPTY	8	7 (1 lost during surgery)	6 (1 lost during histological processing)
CHIT	8	7 (1 lost during surgery)	7
CHIT + CELL	8	8	6 (2 lost during histological processing)

during the 4-week study in a similar fashion. No clinical signs of inflammation were observed in any of the experimental groups, neither throughout the postoperative period, nor at tissue harvest.

Descriptive histology

In the HE-stained overview sections, two specimens from CHIT+CELL and one specimen from EMPTY were lost

due to the difficulty in trimming and embedding small-size specimens in the right direction. Consequently, data were obtained from 19 out of 22 retrieved surgically created periodontal defect specimens (Table 1).

In general, the apical end of cementum/ligament removal was observed in all experimental groups (Figs. 3 and 4). The healed defects were occupied by epithelium, connective tissue, and newly formed alveolar bone, marked as e, c, and b in Figure 3, respectively. In some specimens, newly

FIG. 3. Histological observations of the different treatment groups (Hematoxylin and Eosin staining): (A) EMPTY; (C) CHIT; (E) CHIT+CELL. Higher magnifications of panels (A), (C), and (E) are shown in panels (B), (D), and (F), respectively. In some specimens, newly formed cellular cementum (blue arrow), PDL (green arrow), and new bone formation in the defect margin areas (yellow arrow) and in the middle of the defect area (black arrow) was observed. The locations indicated by b, c, and e represent newly formed alveolar bone, connective tissue, and epithelium, respectively.

FIG. 4. Histological observations of the different treatment groups (Elastica-van Gieson staining): (A) EMPTY; (C) CHIT; (E) CHIT+CELL. Higher magnifications of panels (A), (C), and (E) are shown in panels (B), (D), and (F), respectively. Blue arrow indicates ankylosis; yellow arrow indicates resorption lacunae; green arrow indicates the penetration of connective tissue and black arrows indicate gel remnants.

formed cellular cementum (bone-like substance covering the root, blue arrow in Fig. 3B), PDL (the specialized connective tissue that attaches the cementum to the alveolar bone, green arrow in Fig. 3B), and ankylosis (fusion of tooth surface and alveolar bone with obliteration of the PDL, blue arrow in Fig. 4B) was observed. The newly formed cellular cementum was limited to the apical part of the defect. Occasionally, the presence of resorption lacunae (yellow arrow in Fig. 4B) was detected on the surface of root dentin. None of the specimens showed complete periodontal regeneration. For all CHIT and CHIT+CELL specimens, chitosan hydrogel remnants (yellowish islands as indicated by the black arrows in Fig. 4D, F) were observed. None of the specimens showed signs of inflammation or adverse tissue responses.

For EMPTY, all specimens exhibited new bone formation. On the denuded dentin surface, resorption lacunae were detected in two, and ankylosis was observed in five out of six specimens. Some specimens (two out of six) exhibited new cellular cementum formation, which was limited to the apical part of the defect. As shown in Azan staining, the cellular matrix had organized into bundle-like structures

(blue color) running parallel to the dentin surface (black arrows in Fig. 5A, B).

For CHIT, light microscopic analysis showed that the chitosan hydrogels were largely degraded (Fig. 4C) without any adverse reaction in the surrounding tissue. Penetration of connective tissue (red color, green arrow in Fig. 4D) into the remaining fragments (yellowish color) was observed. Newly formed bone was observed in all specimens, mainly originating from the apical part of defects. Besides new bone formation being present in the defect margin areas (yellow arrow in Fig. 3C), new bone tissue was observed in the middle of the defect area as islets (black arrow in Fig. 3D). Along the dentin surface, resorption lacunae were detected in one, ankylosis in three, and new cellular cementum formation in three out of seven specimens. In addition, the supracrestal connective tissues exhibited functional orientation of the fibers (blue color), which obliquely inserted into the dentin surface (black arrows in Fig. 5D).

Specimens from CHIT+CELL showed comparable histological results to CHIT. Newly formed bone was mainly observed at the mesial/apical defect edges. Immature bone

FIG. 5. Representative images for ligament formation from three treatment groups (Azan staining): (A) EMPTY; (C) CHIT; (E) CHIT + CELL. Higher magnifications of panels (A), (C), and (E) are shown in panels (B), (D), and (F), respectively. Black arrows indicate the orientation of newly formed fibers.

FIG. 6. Anti-GFP immunohistochemical staining: (A) GFP⁺ cells were visualized by brown staining in the positive control; (B) The negative control showed no specific immunoreactivity; (C) At 4 weeks post-transplantation, no GFP signal could be detected in the *in vivo* specimens; (D) PDLCs were GFP positive before implantation. GFP, green fluorescent protein.

islets were scattered amongst the remnants of scaffold material. Along the dentin surface, resorption lacunae were detected in two, ankylosis in three, and new cellular cementum formation in two out of six specimens. Moreover, functional orientation of fibers (blue color) was observed in the supracrestal connective tissues (black arrows in Fig. 5F).

GFP immunohistochemistry

The immunohistochemical staining succeeded in demonstrating GFP signal in the positive control (Fig. 6A). The negative control showed no specific immunoreactivity in the related tissues (Fig. 6B). At 4 weeks posttransplantation, no GFP signal was detected in any of the *in vivo* specimens (Fig. 6C). Examination of the immunohistochemically stained sections revealed that PDLCs embedded in chitosan hydrogel were GFP-positive before implantation (Fig. 6D).

Histomorphometrical analysis of periodontal regeneration

Histomorphometrical measurements of periodontal regeneration are presented in Figure 7. The relative alveolar bone height was 6.1 ± 1.4 , 5.9 ± 4.0 , and 4.6 ± 1.4 , for EMPTY, CHIT, and CHIT+CELL, respectively, with no statistical difference between the experimental groups ($p > 0.05$). Comparable values ($p > 0.05$) of relative alveolar bone area were measured for all groups, ranging from 0.3 ± 0.1 for EMPTY, 0.2 ± 0.1 for CHIT, to 0.2 ± 0.1 for CHIT+CELL. The relative epithelial downgrowth in EMPTY, CHIT, and CHIT+CELL was 0.2 ± 0.1 , 0.2 ± 0.1 , and 0.2 ± 0.1 , respectively, without significant differences between the experi-

mental groups ($p > 0.05$). For relative functional ligament length, the Azan-stained sections showed that around 9% of denuded root surface in the EMPTY (0.09 ± 0.04) was covered by functional collagen fibers. In contrast, sections from CHIT (0.35 ± 0.09) and CHIT+CELL (0.38 ± 0.19) showed a significantly ($p < 0.01$) higher relative functional ligament length than EMPTY. In addition, CHIT demonstrated a comparable score for relative functional ligament length to the CHIT+CELL, with more than 35% of the denuded root surface covered by functional collagen fibers.

Discussion

The objective of the current study was to evaluate the *in vivo* biocompatibility and periodontal regenerative potential of enzymatically solidified chitosan hydrogels with or without incorporated PDLCs. To this end, GFP-positive PDLCs were encapsulated in chitosan hydrogels and transplanted into a rat periodontal defect model. The results from this study demonstrate that enzymatically solidified chitosan hydrogels are biodegradable. In addition, this hydrogel was well-tolerated in the periodontal defect, as there were no undesirable local responses observed in the recipient rats. Moreover, the hydrogel was favorable to induce functional orientation of fibers compared with empty controls in the current rat model. PDLCs remained viable upon encapsulation within the chitosan hydrogels before transplantation, although no GFP signal was detected at 4 weeks posttransplantation in the defect region.

To explore the efficacy of chitosan hydrogel as a potential implantation material in periodontal regeneration, an intrabony defect with limited spontaneous healing was used.²⁵ This

FIG. 7. Histomorphometrical measurements. There was no statistical difference between all groups in terms of alveolar bone height (A), alveolar bone area (B), and epithelial downgrowth (C). Samples from EMPTY showed a significantly negative effect with respect to the functional ligament measurements than the CHIT and CHIT+CELL groups (D). ** $p < 0.01$.

defect was surgically created, which is dissimilar to naturally occurring periodontitis caused by plaque. However, compared with plaque-induced periodontal breakdown, surgically prepared defects help keep the initial conditions of different experimental groups almost identical, which increases the reproducibility of the model system.

Many reports have shown that chitosan-derived materials have high biocompatibility.^{27–30} Chitosan hydrogels used in the current study were fabricated using urea and urease. According to our previous work,²⁰ chitosan hydrogel containing 37.5 mM urea and 30 U/mL urease was used since its gelation time (11.7 ± 0.6 min) is suitable for clinical handling of cell encapsulation. Our previous work has demonstrated that the urea can be totally converted by hydrolysis within 15 min.²⁰ However, the *in vivo* influence of the remaining urease in this hydrogel are not clear. This study proved that enzymatically solidified chitosan hydrogel also showed good biocompatibility, as there were no undesirable local responses observed in the recipient rats. Light microscopy analysis demonstrated that this hydrogel was largely degraded *in vivo* after 4 weeks and there was no inflammatory cell infiltration around the grafted material, indicating that there was no adverse effect from the limited amount of remaining urease.

Our results further confirmed that enzymatically solidified chitosan hydrogel is a promising biomaterial for periodontal tissue regeneration based on several findings. First, CHIT displayed superior development of the ligament structure compared with EMPTY. For measurements on the ligament structure, the quantity as well as the orientation of newly formed collagen bundles were taken into account as reported previously.^{3,51} The insertion of functionally oriented collagen bundles into hard tissues may lead to improved sensing and protection toward mechanical load from mastication.³² In this study, the enhancement of PDL regeneration in CHIT probably because chitosan hydrogel functioned as a space maintainer to preserve a space for the formation of new PDL. It has been demonstrated that a scaffold suitable for periodontal tissue regeneration should be able to maintain a space within the wound site for cells to grow into.^{33,34} Second, CHIT exhibited relatively less ankylosis (three out of seven samples) compared with EMPTY (five out of six samples). Ankylosis is a pathological fusion of the dentin or cementum of a tooth root to the alveolar bone.³⁵ Ankylosis may occur when primary proliferation of cells from alveolar bone takes place, for which the speed of PDLCs in repopulating the root surface determines whether or not ankylosis occurs.³⁰ Therefore, our results indicated that this chitosan hydrogel might enhance PDL regeneration by allowing/promoting repopulation of the relatively slow growing PDLCs on the root surface. Third, the chitosan hydrogel used in the present study supports new bone formation, as new bone tissue were formed as islets, which were scattered amongst the remnants of scaffold material. A previous study demonstrated that chitosan collagen composite, but not collagen alone was able to stimulate ectopic bone formation in a subcutaneous location.³⁶ Although the results of this previous study appear to support the possible osteoinductive property of chitosan, the mechanism of this phenomenon is not clear. It has been reported that chitosan upregulated the expression of type I collagen mRNA and the alkaline phosphatase activity of PDLCs.³⁷ In addition, previous studies have demonstrated that chitosan can enhance platelet adhesion, aggregation, activation, and the subsequent

growth factor release, including epidermal growth factor, platelet-derived growth factor-AB, and transforming growth factor,^{38,39} which are valuable for new bone formation.⁴⁰ However, to clarify the mechanism of the effect of chitosan on osteogenesis, additional studies will be necessary. Taken together, these above findings suggest that this chitosan hydrogel without cell loading is able to support periodontal regeneration as superior development of the ligament structure and less ankylosis were observed in CHIT compared with EMPTY, indicating the great potential of this hydrogel in clinical applications of periodontal regeneration.

Our previous *in vitro* study²⁰ demonstrated that enzymatically solidified chitosan hydrogel can support PDLC survival *in vitro* for up to 30 days, which justifies the evaluation of this chitosan-based hydrogel as a cell delivery system for periodontal regeneration in an animal model. However, at the end of the 4-week transplantation period, no GFP signal was detected in any of the *in vivo* specimens, indicating that there are no exogenous healing cells in the defect area anymore. This result is different from our previous study, in which GFP-positive staining of implanted PDLCs was detected after 6-week implantation using a gelatin sponge as a cell carrier material.² As the same PDLCs and defect model were used in this study, this discrepancy is most likely caused by the difference in cell carriers. As GFP-positive cells were only detected in four out of eight specimens in our previous study,² the cells encapsulated in chitosan hydrogels most likely migrated out of the defect after implantation *in vivo*. Similarly, Tour *et al.* reported that implanted PDLCs decreased in number with time in a defect after transplantation in a calvarial defect model, from around 240 GFP⁺ cells/mm² defect area after 1 week to single or no positive cells detected after 10 weeks.⁴¹ Taken together, these findings suggest that transplanted cells tend to vanish with time after implantation.

In our study, no differences in periodontal regeneration were observed between CHIT+CELL and CHIT. Considering that comparable concentration of cells were encapsulated in the chitosan gels compared with previous studies using cell-laden biomaterials,^{2,42–44} the discrepancy between our results and the previous work is most likely not caused by different cell loading concentrations. Although additional work on the localization and contribution of transplanted cells to tissue regeneration are required, the transplanted cells probably vanished faster than the tissue regeneration process. This phenomenon of cell vanishing after implantation may be partially explained by the migration of the cells out of the defect without contributing to the regeneration process, as chitosan hydrogels were largely degraded after 4-week implantation *in vivo*, or the immediate cell death after implantation due to poor vascularization inside the entire cell-based construct.^{45,46} For the former reason, in future studies, it is of importance to enhance cell adhesion and proliferation, for example, by incorporating cell adhesive biomolecules. For the latter reason, future studies should be directed to improving cell survival after implantation. One possible approach is to simulate vascularization in cell-based constructs by coculture approaches of PDLCs with angiogenic cells (e.g., endothelial cells).⁴⁷ Another way to maintain cell survival after implantation is to generate bone tissue through the endochondral route, which resembles endochondral ossification by first forming a cartilage template *in vitro* and

subsequently allowing the cartilage to be replaced by bone *in vivo*.^{48–52} The rationale behind this approach is that chondrocytes are able to survive with limited nutrition and oxygen. Second, chondrocytes can secrete vascular endothelial growth factor in the hypertrophic stage, which is beneficial for blood vessel ingrowth.⁵³

The limitations of this study include the small model size and only one time point of observation. Due to the difficulty in handling specimens with small defect size, 3 out of 22 samples were lost during the histological processing. Although this model is suitable for initial studies, the biocompatibility and regenerative capacity of this chitosan hydrogel should be corroborated in a larger animal model in the future. A larger animal model would provide higher comparability with the human situation and thus would facilitate the translation from bench to bedside more optimally. To clarify the exact fate of transplanted PDLCs delivered by chitosan hydrogels, additional experiments with different observation periods are also necessary.

Conclusion

Enzymatically solidified chitosan hydrogel was demonstrated to display high biocompatibility and biodegradability. In addition, the implantation of hydrogel alone promotes functional ligament formation and reduces the incidence of ankylosis in the current rat model, indicating the application potential of this hydrogel for periodontal tissue regeneration. In contrast, no signal from transplanted PDLCs could be detected at the end of 4-week transplantation period, indicating that there are no exogenous healing cells in the defect area anymore.

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Disclosure Statement

No competing financial interests exist.

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