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A dural substitute based on oxidized quaternized guar gum/porcine peritoneal acellular matrix with improved stability, antibacterial and anti-adhesive properties

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ABSTRACT

Trauma and neurosurgery often result in dural defects and are followed by serious complications or even death, finding suitable dural replacement materials to repair the defective dura has important clinical significance. Porcine peritoneal acellular matrix (PPAM) is a promising alternative material, but its poor stability makes it difficult to meet the various needs of dural reconstruction. In this work, we developed a novel antibacterial cross-linking agent oxidized quaternized guar gum (OQGG) and used it for the first time to stabilize PPAM to construct a dural mater substitute (OQGG-PPAM). The results showed that 1.5% OQGG-PPAM presented suitable mechanical property as well as good thermal stability and resistance to enzymatic degradation. It also exhibited good antibacterial activity and good anti-leakage ability. Furthermore, 1.5% OQGG-PPAM not only exhibited excellent cell compatibility but also significantly stimulated the secretion of bFGF and VEGF from seeded cells which was convenient for dural remodeling. *In vivo* experiment, it also exhibited the excellent histocompatibility and good anti-adhesion property. This study showed that OQGG can be used as a novel antibacterial cross-linking reagent for crosslinking natural tissues and 1.5% OQGG-PPAM was a potential candidate material for dura mater substitute.

The dura mater is an important anatomical structure of the central nervous system [1]. As a natural barrier, its integrity is of great significance for protecting the brain tissue and maintaining neuroelectrophysiological activities [2]. Once the dura mater is defective, it could lead to complications such as cerebrospinal fluid leakage, intracranial infection, meningoencephalopathy, epilepsy [3–5]. If the dura mater can be properly repaired during the operation, the above complications can be significantly reduced [6]. At present, dura mater substitutes mainly include homogenous materials, natural materials, synthetic materials and acellular materials. The applications of the first three materials are limited by sources [7,8], insufficient mechanical strength [5,9,10] and chronic inflammation [11–13].

Various acellular materials, which mainly include the pericardium [14], dermis [15], and small intestinal submucosa (SIS) [16], have recently attracted increasing attention as dural substitutes owing to their good biocompatibility and bioactivity. They can provide the specific 3D architecture and bioactive substances that similar to the extracellular matrix (ECM), which facilitate cell adhesion, proliferation and migration, thus promoting tissue repair and regeneration [17,18]. Among them, porcine peritoneum (PP) with a wide range of sources and excellent biological and physical properties has become one of the preferred materials. In addition, it rarely adheres to the brain tissues of the recipient after surgery due to its extreme smooth surface [19–21]. Despite the many advantages of porcine peritoneal acellular matrix (PPAM) in the repair of dura mater, its widespread use is limited by its poor mechanical strength and high degradability. Finding suitable crosslinking agents for chemical modification of PPAM can address these problems [22]. Up to now, some crosslinking agents such as glutaraldehyde (GA) [23], genipin [24], 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) [25] have been developed. However, they have presented various problems in the applications. The GA-fixed materials show high cytotoxicity, while the genipin-fixed materials present the dark blue appearance, and the EDC fixed-linked materials exhibit poor stability.

Guar gum (GG), with galactomannan as the main ingredient, is chemically composed of linear chains of (1→4)-linked β-D-

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mannopyranosyl residues partially substituted with (l→6)-linked α-D-galactopyranosyl side-groups in a ratio of 1.5 to 2 [26]. GG not only exhibit excellent solubility in both hot and cold water, but possess rich hydroxyl groups, which provides opportunities for its modification such as quaternization or oxidation [27,28]. Based on this, we first proposed a new method of preparing materials that the quaternary ammonium grafting reaction of GG with *N*-(3-chloro-2-hydrohypropyl)trimethylammonium chloride (CHPTAC) and then undergoing oxidation reaction with sodium periodate ($NaIO_4$) can obtain a new type of oxidized quaternized guar gum (OQGG) as a crosslinking agent with antibacterial function. Compared with other biological crosslinking agents, the application of OQGG in crosslinking acellular matrix can not only improve the mechanical properties of the acellular matrix through the cross-linking reaction between its aldehyde groups and the amino groups of the acellular matrix and then maintain structural stability of the acellular matrix for a longer time, but also introduce its quaternary ammonium groups into the acellular matrix to endow it with antibacterial effect which is conducive to reducing the postoperative infections and adhesions.

In this study, we designed a novel OQGG-PPAM composite as a dure mater substitute using a biologically derived porcine peritoneal acellular matrix (PPAM) as a matrix and bio-functionally chemically modified by a novel antimicrobial cross-linking agent (OQGG). OQGG was first prepared and characterized. Subsequently, we adopted OQGG to crosslink PPAM, investigated the cross-linking characteristics, biomechanical properties, ultrastructure, enzymatic hydrolytic resistant ability, thermodynamics, antibacterial properties, water absorption and anti-leakage properties, and cytocompatibility of OQGG-PPAM. Finally, the inflammation and adhesion reactions of OQGG-PPAM *in vivo* were also studied in this research. Related experimental procedures were provided in Section A in Supporting information.

OQGG with a QGG/$NaIO_4$ feeding ratio of 1:1.5 was selected as a cross-linking reagent for subsequent experiments and its aldehyde content was 4.8 mmol/g. In addition, the decellularization method in this study could not only effectively reduce immune antigenicity in PP, but also maintain its structure complete. More details could be found in Section B of the Supporting information. The mechanism and main reactions of PPAM fixation with OQGG were shown in Fig. S3 (Supporting information). OQGG can react with the free amino groups of lysine, hydroxylysine or arginine residues within PPAMs through their aldehyde groups to form imine structure units. The FTIR spectra were used to clarify the changes of specific groups during the OQGG-fixation (Fig. 1a). For OQGG-PPAM, the peak of the bending vibration in the N-H bond plane of the amino group and the superimposition of the imine bond at 1630 cm^{-1} was observed to be sharper than that of PPAM. In addition, the C-N stretching vibration peak of the amino group at 1080 cm^{-1} and the in-plane bending vibration peak of NH at 878 cm^{-1} were weakened in OQGG-PPAM compared with PPAM. These were the results of the fixation of PPAM with OQGG and the consumption of amino groups within PPAM by fixation. Figs. 1b-d showed SEM images of PPAM, 1.5% OQGG-PPAM and GA-PPAM. The larger magnification images were shown in Fig. S4 (Supporting information). As shown in Figs. 1b-d, as compared to the messy and loose fiber of PPAM, OQGG-PPAM presented thicker and stronger fiber bundles in which the fibers tended to be regular and dense; moreover, the space between the fibers became narrow. This was due to that the crosslinking OQGG acted as a "bridge" linking the fibers. Similarly, regular dense fibers of GA-PPAM were also resulted from the cross-linking modification of GA.

The degree of cross-linking reaction can be evaluated by estimating the number of free amino groups in the sample tissues, called crosslink degree. The crosslink degree of OQGG-PPAM increased with the increase of the OQGG's concentration (from 0.5% to 2%) as shown in Fig. 2a. However, the crosslink degree of 2.5% OQGG fixed samples did not increase significantly, which might be due to that OQGG has reached a saturated state in these solutions, that was, the steric hindrance of OQGG made it difficult to react with some amino groups within PPAM even if the content of aldehyde groups increased. Compared with OQGG-PPAM, which had the largest crosslink degree of 88%, GA-PPAM had a crosslink degree of 97% because GA was a small molecule resulted in smaller steric hindrance. The thermal stability of the samples was also increased after cross-linking as shown in Fig. S5 (Supporting information).

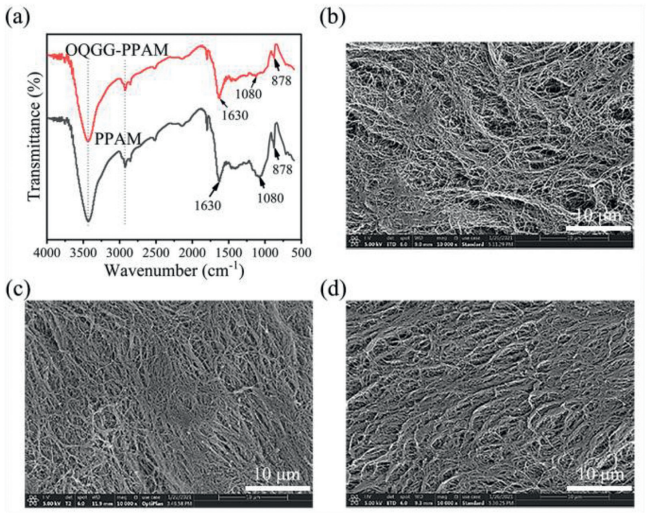

Fig. 1. (a) FTIR spectra of PPAM and OQGG-PPAM; SEM images of PPAM (b), 1.5% OQGG-PPAM (c) and GA-PPAM (d).

The suitable biomechanical strength is essential for dura mater substitutes to withstand squeezing and stretching, maintain intracranial pressure, and prevent cerebrospinal fluid leakage. The stress-strain curves, tensile strength, elongation at break, and tearing strength of PPAM, 0.5%-2.5% OQGG-PPAMs and GA-PPAM were showed in Figs. 2b and c and Fig. S6 (Supporting information). It could be seen from Fig. 2b that the tensile strength of OQGG-PPAM (1.65 ± 0.23 ~ 7.59 ± 0.58 MPa) was significantly higher than that of PPAM (1.15 ± 0.10 MPa) and increased with the OQGG's concentration (from 0.5% to 2%). This increase in tensile strength benefited from the cross-linking between the aldehyde group of OQGG and the free amino group of PPAM, which made the fiber network more intertwined and complex, so that it was not easy to be broken under the action of external force. Fig. 2b showed that the elongation at break of OQGG-PPAM began to decrease when the OQGG's concentration exceeded 1%, indicating the increase of brittleness of the materials. This was due to the fact that the oriented aggregation of the fibers at a certain degree of cross-linking will easily cause stress concentration, which lead to an increase in brittleness and thus reduce the elongation at break of materials. In addition, GA-PPAM had more excessive cross-linking due to the small steric hindrance of GA, resulting in that the tensile strength was increased while the elongation at break was greatly reduced. Compared with PPAM and GA-PPAM, OQGG-PPAM had a tensile strength similar to that of human dura mater (3.28-7.86 MPa) [29] while showing suitable flexibility, which was convenient for bending and repairing in practical applications. The evaluation of the tear strength of the dura mater substitute is necessary for its practical application. Fig. 2c showed that the tear strength of PPAM was about 8 N, while the tear strengths of cross-linked samples were 12–17 N, which was significantly improved after fixation and both were higher than the tear strength value of dura mater in

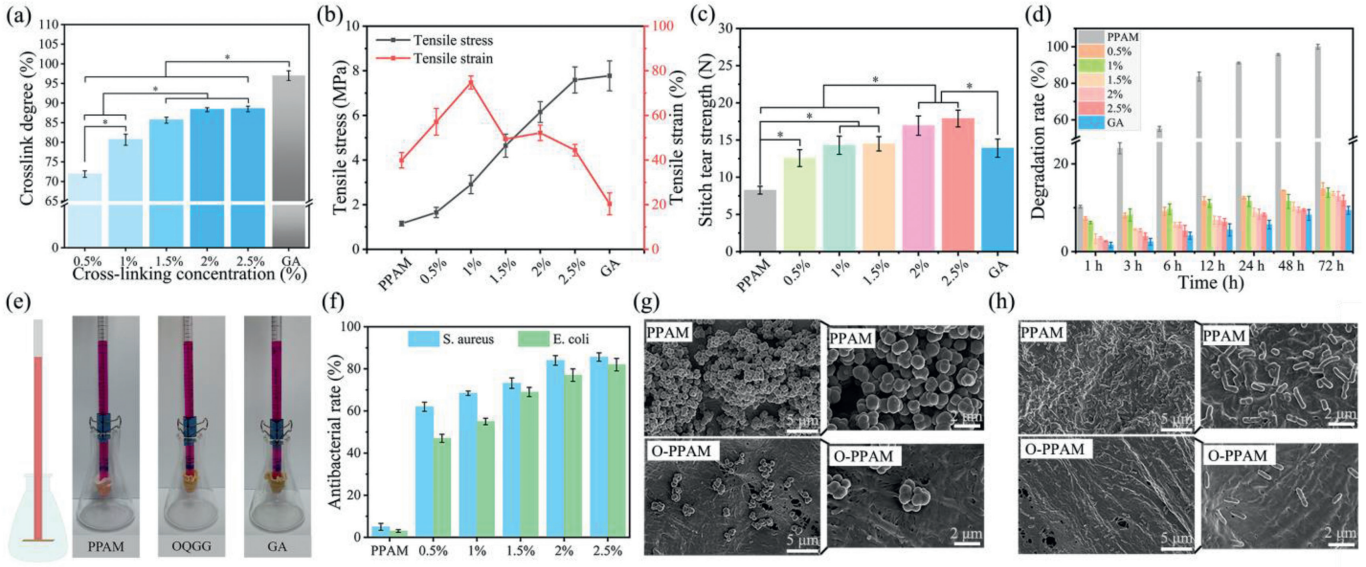

Fig. 2. (a) The crosslink degree of 0.5%-2.5% OQGG-PPAMs and GA-PPAM; The tensile strength and elongation at break (b), tearing strength (c), enzymatic hydrolysis weight loss rate (d) of PPAM, 0.5%-2.5% OQGG-PPAMs and GA-PPAM; (e) The anti-leakage performance of PPAM, OQGG-PPAM, GA-PPAM; (f) The antibacterial results for both *S. aureus* and *E. coli* of PPAM and OQGG-PPAM; SEM images of the morphologies of *S. aureus* (g) and *E. coli* (h) on the surface of PPAM and OQGG-PPAM.

the literature [5,6,30]. Excellent tear strength was conducive to the water-tight closure of sutures and the reduction of fluid leakage risk after operation.

The enzymatic hydrolysis weight loss rate of PPAM, 0.5%-2.5% OQGG-PPAMs and GA-PPAM at different time points was showed in Fig. 2d. All specimens degraded to varying degrees after treating with type I collagenase for a prescribed period time. Compared with the complete degradation of PPAM during 72 h hydrolysis process, the degradation rate of OQGG-PPAM and GA-PPAM was much lower, indicating that the cross-linking could present an effective resistance against enzymatic degradation. The reason why PPAM is less resistant against enzymatic degradation might be that the decellularization make the fibers of PPAM loose and expose more enzyme-attacked sites on collagen, while the cross-linking treatment led to dense fiber network which shield a large number of enzyme-attacked sites, and thereby the resistance of cross-linking simples to enzymatic hydrolysis are improved. The improvement of resistance against enzymatic degradation could not only reduce the cytotoxicity resulted from the small molecule-productions from degradation, but also provide implant materials with enough time to be gradually replaced by new tissue.

Anti-leakage simulation experiment (Fig. 2e) showed that the leakage occurred after 24 h in PPAM group while the cross-linked samples did not leak during the experiment due to the reason that the Schiff base crosslinking reaction between the aldehyde group of OQGG and the free amino group of PPAM make the fiber denser and reduce the penetration of water molecules. The water absorption rate of the samples was reduced after cross-linking due to the same reason mentioned above as shown in Fig. S7 (Supporting information).

The antibacterial properties of PPAM and OQGG-PPAM against *S. aureus* and *E. coli* were summarized in Fig. 2f. Clearly, OQGG-PPAM exhibited antibacterial activity against both bacteria species. This was due to the fact that the quaternary ammonium group on OQGG which can destroy the structure and function of the bacterial cell walls and finally result in the destruction of bacteria [31,32] was introduced into OQGG-PPAM through the cross-linking reaction. The positively charged quaternary ammonium groups can interact electrostatically with negatively charged proteins, lipids and carbohydrate residues on the surface of bacteria, disrupting the structure and function of the bacterial cell wall, leading to metabolic disruption or deformation, or even cytoplasmic leakage, ultimately leading to the destruction of the bacteria. With the increase of OQGG's concentration for fixation, the antibacterial activity of OQGG-PPAM was also improved to a certain degree due to the more quaternary ammonium groups introduced by OQGG-fixation. Moreover, the antibacterial activity of OQGG-PPAM against *S. aureus* was stronger than that against *E. coli*, which might be due to the difference in bacterial cell walls, the cell wall of S. aureus has lower lipid content and is more easily invaded by quaternary ammonium groups [33]. Figs. 2g and h demonstrated that *S. aureus* and *E. coli* respectively presented a typical sphere shape or rod shape, and the number of bacteria on OQGG-PPAM was significantly reduced compared with that on PPAM. The introduction of the antibacterial function into the dura mater substitute materials could reduce the intracranial infection after the operation and also reduce the adhesion resulted from the infection.

Fibroblasts that are the main functional cells for dural repair were used to assess the cytocompatibility of the samples [16]. The proliferation of fibroblasts cultured on PPAM, 0.5%-2.5% OQGG-PPAMs and GA-PPAM was measured by CCK-8 assay and shown in Fig. 3a. The results showed that both PPAM and GA-PPAM inhibited the proliferation of L929 cells, while OQGG-PPAM exhibited better cell proliferation-promotion. The cytotoxicity of PPAM and GA-PPAM might be from the continuous leaching-out of small molecule degraded substances from PPAM-degradation or unreacted GA in GA-PPAM. Compared with GA-PPAM, OQGG-PPAM presented a better cytocompatibility. Firstly, OQGG cross-linking improved the ability of PPAM to resist enzyme degradation. Secondly, OQGG is derived from naturally occurring polysaccharide, which shows low cytotoxicity; the molecular weight of OQGG was large enough to hinder itself from diffusing into cells to react with macromolecules inside cells and presented low cytotoxicity. Finally, the unreacted aldehyde groups in OQGG reacted with adjacent hydroxyl groups to form hemiacetals, which significantly reduced the cytotoxicity. In addition, there were differences in the cytotoxicity of 0.5%-2.5% OQGG-PPAMs. When OQGG-concentration reached 1.5%, the OQGG-PPAM showed the best cytocompatibility, and its cell viability reached peak. However, with the concentration of OQGG further increasing to 2% and 2.5%, the cell viability

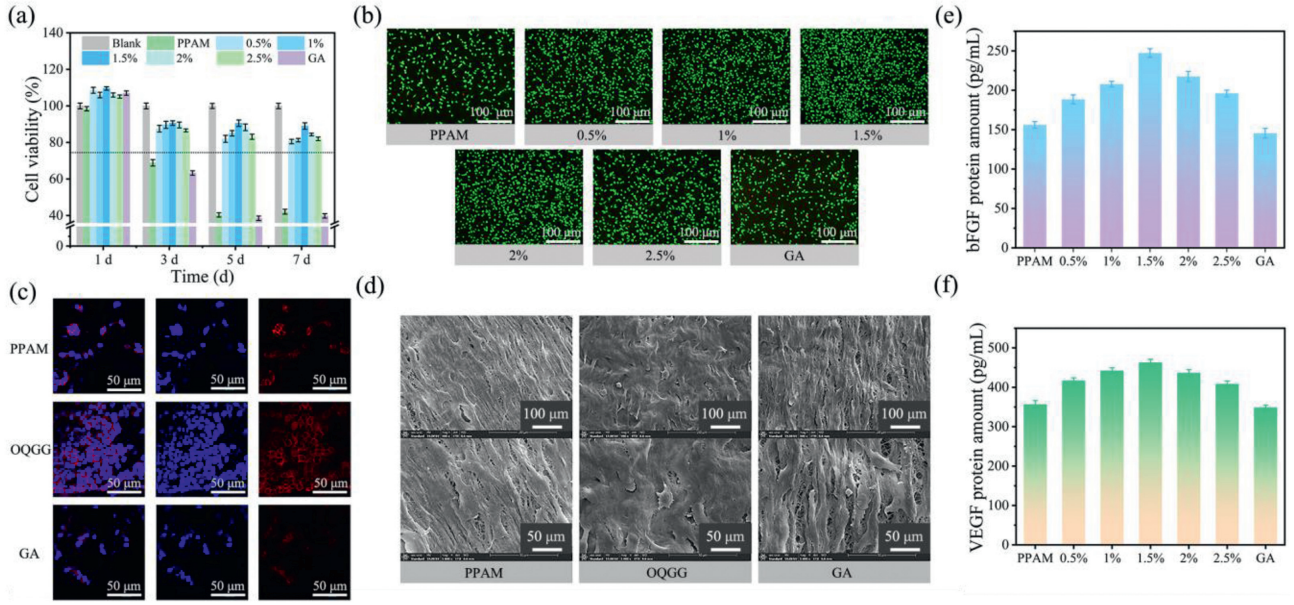

Fig. 3. (a) Cell viability of L929 fibroblast cell co-cultured with PPAM, 0.5%-2.5% OQGG-PPAMs and GA-PPAM at 1 d, 3 d, 5 d, 7 d ($T = 37$ °C); (b) Calcein AM/PI fluorescence staining images of L929 fibroblasts cultured for 4 d on PPAM, 0.5%-2.5% OQGG-PPAMs and GA-PPAM ($T = 37$ °C, the live cells fluoresce green and dead cells fluoresce red); (c) Fluorescence microscopy images of L929 fibroblasts co-cultured for 4 d with PPAM, 1.5% OQGG-PPAM and GA-PPAM ($T = 37$ °C); (d) SEM photographs of L929 fibroblasts cultured for 4 d on PPAM, 1.5% OQGG-PPAM and GA-PPAM ($T = 37$ °C); The effect of PPAM, 0.5%-2.5% OQGG-PPAMs and GA-PPAM on (e) bFGF and (f) VEGF secretion from L929 fibroblasts.

of samples decreased. This could be attributed to the large number of OQGG-introduced quaternary ammonium groups which can inhibit the cell proliferation to certain degree. In view of this, 1.5% OQGG was selected as the optimal concentration for the biological cross-linking agent. The result of calcein/PI live/dead viability/cytotoxicity assay was shown in Fig. 3b. Fig. 3b indicated that the number of live cells on the OQGG-fixed samples was large, and there were only a few red dead cells on them. The number of red dead cells on the GA-PPAM sample and PPAM was significantly higher. The results demonstrated that the OQGG-fixed groups showed a higher cell survival rate than PPAM and GA-PPAM.

The growth of L929 fibroblasts cultured on the surface of various samples for 4 d was also examined by fluorescent staining with DAPI/rhodamine. The results observed by confocal were as shown in Fig. 3c. The number of cells observed on 1.5% OQGG-PPAM was significantly more than that on PPAM and GA-PPAM, indicating the good cytocompatibility of 1.5% OQGG-PPAM. SEM was also utilized to observe the morphology and spreading of cells on various tested specimens (Fig. 3d). Similarly, a lot of spreading-L929 cells with their characteristic spindle-shaped morphology were observed on the surface of 1.5% OQGG-PPAM, and grew better and nearly reached the continuous cell layer. bFGF and VEGF are two cytokines that play an important role in dural repair [34]. bFGF that widely presents in dural tissue mainly manifests its biological effect on dural repair though inducing the formation of tissues and angiogenesis *in vivo* and *in vitro*, and its expression runs through the whole process of dural healing [35]. VEGF is another important cytokine which widely presents in the dura mater. It could promote angiogenesis which participate in blood supply to the skull by inducing endothelial cell proliferation [31,36,37]. The secretion of bFGF and VEGF protein was measured by ELISA assay and presented in Figs. 3e and f. The amount of bFGF and VEGF secreted from cells cultured on the 0.5%-2.5% OQGG-PPAMs was obviously higher, compared to the other counterparts in PPAM and GA-PPAM groups. When OQGG-concentration increased to 1.5%, the amount of bFGF and VEGF secreted from cells reached peak. The reasons for this result may be related to following: Firstly, due to the lowest cytotoxicity, 1.5% OQGG-PPAM could obviously stimulate cell proliferation and growth; thus, there were more fibroblasts on samples that could secrete bFGF and VEGF. Secondly, bFGF and VEGF secreted into medium have synergistic effects. In short, OQGG-PPAM could promote the growth of L929 fibroblasts resulting in increased production of bFGF and VEGF, which is very beneficial to dural repair.

Animal experiments were performed in accordance with the Experimental Animal Administrative Committee of Sichuan University. The 1.5% OQGG-PPAM with the best cytocompatibility was selected for the next animal experiment. The retrieved implants and surrounding tissues were embedded in paraffin and sectioned for H&E staining to perform histopathological analysis. The results of H&E staining analysis at the 30^{th} and 90^{th} day after implantation were presented in Fig. 4a. After 30 d post-implantation, although neovascularization was observed in PPAM group, the rapid degradation in a large area occurred, which was not conducive to providing enough time for dural repair. In the GA-PPAM group, a large number of inflammatory cell infiltration was observed. For OQGG-PPAM group, the rapid degradation and severe infiltration of inflammatory cell in the retrieved tissues were not observed, and the obvious neovascularization was visible within implant. 1.5% OQGG-PPAM show good histocompatibility. After 90 d post-implantation, the infiltration of inflammatory cells in all samples was reduced. However, most PPAM-samples were significantly degraded and the inflammatory response of GA-PPAM was still more severe than that of OQGG-PPAM. These results still confirmed the excellent histocompatibility of 1.5% OQGG-PPAM. The immunohistochemical staining of TNF-α also showed good histocompatibility of 1.5% OQGG-PPAM (Fig. S8 in Supporting information).

The ideal dural repair materials should not adhere to the surrounding tissue. The adhesion property of the sample-implants was assessed by the rat abdominal cavity duraplasty model. As shown in Fig. 4b, after 21 d of implantation, the PPAM group curled and adhered severely, the GA-PPAM group adhered but did not curl, and the OQGG-PPAM group exhibited only slight adhesions,

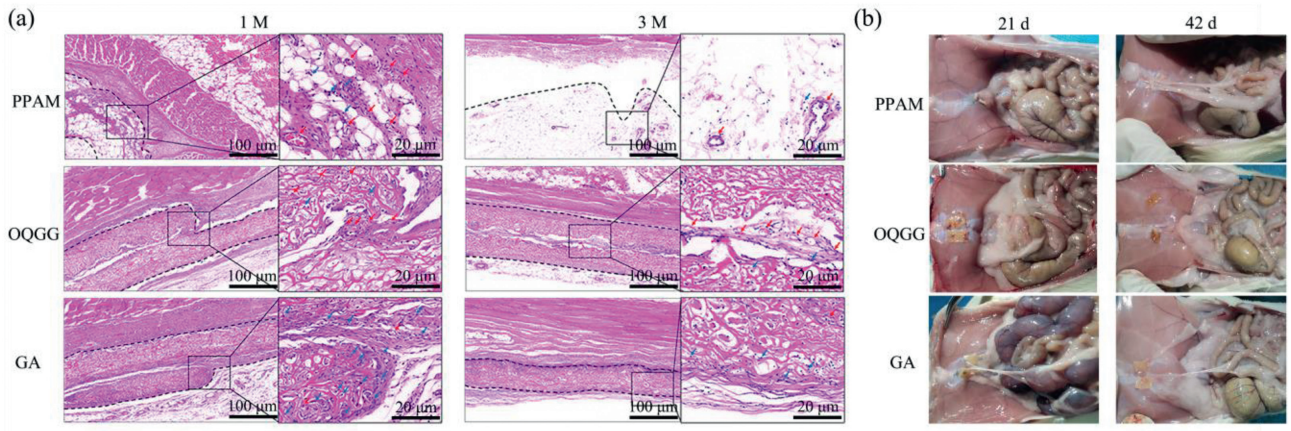

Fig. 4. (a) After 1 M and 3 M post-operation, H&E analysis of various implanted samples (red arrow: blood vessel; blue arrow: inflammatory cells; the black dotted circle: implant); (b) Images of sample-adhesion.

which may be the result of the advantages of being resistant to water swelling after modification which reduces the chance of adhesion by reducing contact between samples and organs, as well as its excellent biocompatibility with little inflammation, which is one of the causes of adhesions. After 42 d of implantation, adhesions were reduced in the all groups due to the inflammation decreased, and the OQGG-PPAM group was essentially free of adhesions, indicating the good anti-adhesion property of 1.5% OQGG-PPAM.

In summary, we designed a new type of antibacterial cross-linking agent (oxidized quaternized guar gum, OQGG) obtained by grafting GG the quaternary ammonium with CHPTAC and then undergoing oxidation reaction with $NaIO_4$, and prepared a novel dura mater substitute using a biologically derived porcine peritoneal cell matrix (PPAM) as a matrix to be bio-functionally chemically modified with OQGG. For the preparation of OQGG, the results showed that OQGG obtained by using the feeding ratio 1:1.5 (QGG: $NaIO_4$) possessed appropriate oxidation degree, almost no cytotoxicity. 1.5% OQGG (1:1.5 (QGG: $NaIO_4$)) was used as a cross-linking agent to fix PPAM to prepare the dura mater substitute and exhibited good cross-linking characteristics. This novel 1.5% OQGG-PPAM as dura mater substitute exhibited suitable mechanical strength, flexibility and excellent tear strength as well as good thermal stability and resistance to enzymatic degradation. It also found that OQGG-PPAM possessed antibacterial activity against both *S. aureus* and *E. coli* and good anti-leakage ability. Moreover, 1.5% OQGG-PPAM not only significantly improved the adhesion and proliferation of L929 fibroblasts but also significantly stimulated the secretion of bFGF and VEGF from seeded cells which was convenient for dural remodeling. It also exhibited the excellent histocompatibility and good anti-adhesion property *in vivo*. This study provides the experimental basis for OQGG as a new cross-linking reagent for fixing natural tissues and 1.5% OQGG-PPAM as a potential candidate material for dura mater substitute.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.06.014.

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