

## Original Article

# Preparation of laminin/nidogen adsorbed urinary bladder decellularized materials via a mussel-inspired polydopamine coating for pelvic reconstruction

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**Abstract:** Pelvic organ prolapse (POP) is a serious health issue that affects many adult women. A common strategy for POP reconstruction is to use scaffold materials to reconstruct the prolapsed pelvic organ. However, the existing materials for pelvic reconstruction do not meet the clinical requirements for biocompatibility, mechanics and immunological rejection. To address these concerns, urinary bladder decellularized materials (UBDM) was selected due to their good strain-stress resistance. To enhance its biocompatibility, laminin/nidogen was used to modify the UBDM with a mussel-inspired polydopamine coating. We found that the biocompatibility and mechanical properties of laminin/nidogen-Dopamine-UBDM were significantly enhanced and that the degradation rate of laminin/nidogen-Dopamine-UBDM was markedly reduced. Moreover, the expression of CD31 in the laminin/nidogen-Dopamine-UBDM group was higher than that in the normal UBDM group. The laminin/nidogen-Dopamine-UBDM treatment mainly guided M2 type macrophages and led to an inflammatory response. These results indicate that laminin/nidogen adsorbed urinary bladder decellularized materials are promising for use in pelvic reconstruction.

**Keywords:** Pelvic organ prolapse, urinary bladder, decellularization, dopamine, laminin/nidogen, reconstruction

## Introduction

Pelvic organ prolapse (POP), also called urogenital prolapse, is the downward descent of the female pelvic organs, including the bladder, uterus or post-hysterectomy vaginal cuff, and the small or large bowel and results in protrusion of the vagina, uterus, or both. POP is a public health issue. In a multicenter study of 1006 women aged 18-83 years presenting for routine gynecological care, 24% had normal support and 38% had stage I, 35% stage II, and 2% stage III POP [1]. Almost 58% of surgeries to treat POP are done in people under 60 years of age [2], and 13% of the patients who have surgery will need another operation within 5 years [2-4]. Surgical treatments that use scaffold

materials to reinforce healing are a common strategy for pelvic reconstruction [5, 6]. The scaffold materials include natural and synthesized materials. Although the naturally derived materials have good biocompatibility and biodegradation characteristics, most of these natural materials are not recommended for the high-strain environment of pelvic reconstruction because they typically fail to provide sufficient mechanical support as a result of their rapid degradation in vivo, which limits their application in pelvic reconstruction.

The urinary bladder is a hollow muscular organ that collects urine from the kidneys before disposing of it via urination [7]. It has two important functions: storing urine and emptying it.

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Urine storage occurs at low pressure, and emptying occurs at high pressure. The bladder can bear high pressure when the urine is full. It has great potential for pelvic reconstruction, but it cannot be directly used because of immunological rejection. Decellularization is a biomedical engineering process that is used to isolate the extracellular matrix (ECM) of a tissue from the cells that compose it such that an ECM scaffold of the original tissue remains, which can then be used in artificial organ and tissue regeneration [8]. This process creates a natural biomaterial to act as a scaffold for cell growth, differentiation and tissue development.

Physical adsorption and covalent crosslinking are common approaches that immobilize bioactive molecules on the surface of a material [9]. The simplicity and flexibility of physical adsorption have been demonstrated, but a coating is unstable and easy to remove because it is passively adsorbed via electrostatic interactions, which is typically reversible [10, 11]. Recently, a mussel-inspired surface functionalization technique based on dopamine was demonstrated to have a strong adhesive interaction with various material surfaces and, more importantly, to allow secondary modifications such as surface initiated polymerization [12-14]. Moreover, the use of dopamine is not harmful to the human body because it cannot cross the blood-brain barrier [15].

Taking these results together, in this study, we hypothesize that urinary bladder decellularized materials (UBDM) can be used for pelvic reconstruction. To enhance the mechanical properties and biocompatibility of UBDM, we aimed to use EDC/NHS to crosslink it and to further modify it with laminin/nidogen via a mussel-inspired polydopamine coating. In this study, we explored the mechanical properties, degradation rate and biocompatibility of UBDM in vitro and explored its angiogenic effects and inflammatory responses.

### Methods and materials

#### *Preparation of urinary bladder decellularized materials*

A one-year-old male Rongchang swine was sacrificed, and fresh urinary bladder was isolated. The mucosa, serosa, and muscularis were subsequently removed under aseptic conditions.

The residual tissue was put into 400 mL of chloroform/methanol (1:1, volume rate), gently shaken for 12 hours at 4°C, and washed 3 times with distilled water, for 30 minutes each time. Then, it was put into 500 mL of 0.9% saline solution plus with 0.05% trypsin and 0.05% EDTA to digest for 48 hours at 4°C and washed 3 times with distilled water, for 30 minutes each time. The urinary bladder decellularized materials was called UBDM. Then, UBDM was immersed into 400 ml of distilled water containing 300 mg EDC and 230 mg NHS, gently shaken for 12 hours in 4°C, and washed 3 times with distilled water, for 30 minutes each time. Finally, the EDC/NHS crosslinked UBDM were freeze-dried and stored at -20°C for future use. All procedures were proved by Ethics Committees of the Chongqing Academy of Animal Sciences.

#### *Dopamine coating*

Fifty grams of EDC.NHS crosslinked UBDM was immersed into 400 ml of Tris solution (pH 8.5) supplemented with 800 mg, gently shaken for 12 hours at 4°C, and washed twice with distilled water, for 15 minutes each time. Finally, the EDC/NHS crosslinked and dopamine-coated UBDM (Dopamine-UBDM) were freeze-dried and stored at -20°C for future use.

#### *Laminin/nidogen coating*

Twenty-five grams of Dopamine-UBDM was put into 100 ml of 15 µg/ml laminin/nidogen solution, gently shaken for 12 hours at 4°C, and immersed in distilled water for 1 minute. Finally, the laminin/nidogen-coated Dopamine-UBDM (Laminin/nidogen-Dopamine-UBDM) was freeze-dried and stored at -20°C for future use.

#### *Morphological characterization*

The morphologies of UBDM, Dopamine-UBDM, Laminin/nidogen-Dopamine-UBDM were recorded by a digital camera. The microstructures of UBDM, Dopamine-UBDM, Laminin/nidogen-Dopamine-UBDM were analyzed using field emission scanning electron microscopy (SU8010, Hitachi, Japan).

#### *Cytotoxicity test*

The cytotoxicity of various samples was evaluated according to the SO 10993-5-2009 standard. The UBDM, Dopamine-UBDM, Laminin/nidogen-Dopamine-UBDM samples were steril-

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ized overnight with UV light, and the extracted liquid was collected at a ratio of 0.2 g samples per mL in 10% serum-containing media for 72 h at 37°C. L929 fibroblast cells were subsequently seeded into 96-well plates at a density of  $1 \times 10^4$  cells/well and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C under 5% CO<sub>2</sub> overnight. The culture media was then replaced with 100 ml of the extraction medium from each sample. Fresh DMEM medium was used as a control. The cells were continuously incubated for 48 h, and 10 µl CCK-8 was added at each indicated time point, after which the optical density was measured at 450 nm on a microplate spectrophotometer.

### *Mechanical test*

The UBDM, Dopamine-UBDM, Laminin/nidogen-Dopamine-UBDM samples were first cut into 3 cm × 1 cm pieces, fixed in a hollow cardboard, and mounted on the INSTRON Tensile Tester. The cardboard partitions were cut along discontinuous lines before the fiber was stretched. Six samples were stretched until they failed at a constant rate of 1 mm/min at room temperature.

### *Degradation test*

The UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM samples were cut into 1 cm × 1 cm squares, and the dry weight,  $W_d$ , was obtained. Then, the samples were immersed in 5 ml of Tris-HCl (pH 7.4) solution containing 12.5 U/ml collagenase and gently shaken at 37°C for 7 days. At each indicated time, UBDM, Dopamine-UBDM, Laminin/nidogen-Dopamine-UBDM samples were taken, washed 3 times with distilled water and freeze-dried. The residual mass was determined,  $W_r$ . The degradation rate was calculated using the following equation: Degradation rate (%) =  $((W_d - W_r) / W_d) * 100\%$ .

### *In vivo scaffold implantation and Histological observations*

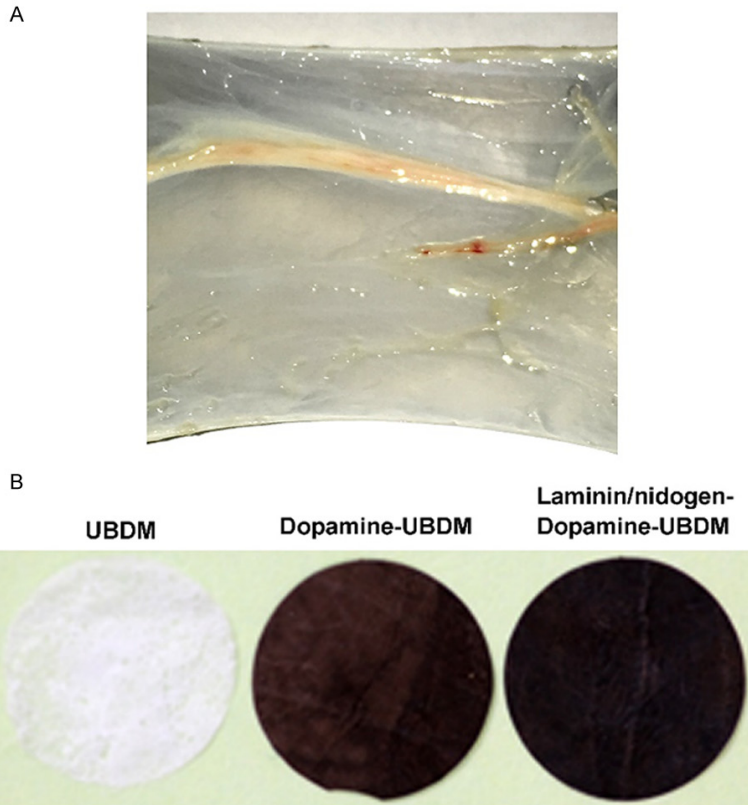
Twelve 12-week-old Sprague-Dawley rats were divided into three experimental groups; the UBDM, Dopamine-UBDM, Laminin/nidogen-Dopamine-UBDM, were separately implanted according to previously described methods [6]. Following the operation, the rats were housed individually. At 6 weeks after implantation, the

rats were sacrificed. The grafts were separated with a small amount (approximately 5 mm) of surrounding native tissue, fixed in formalin and embedded in paraffin. Then, the specimens were deparaffinized with xylene and exposed to a series of ethanol solutions (100%-70%). Sections were subjected to hematoxylin and eosin (H&E) staining for morphological assessment. The primary antibodies used for immunohistochemistry were rabbit anti-rat CXCR3 (T helper cell 1 [Th1] phenotype marker), rabbit anti-rat CCR4 (Th2 phenotype marker), rabbit anti-rat CCR7 (M1 phenotype macrophage marker), rabbit anti-rat CD163 (M2 phenotype macrophage marker) and rabbit anti-rat CD31 (endothelial cell marker), at a 1:100 dilution. The secondary antibody used in this study was goat anti-rabbit immunoglobulin G (IgG), at a 1:100 dilution. All antibodies were purchased from the Beijing Biosynthesis Biotechnology Co, Ltd, and were diluted in filtered PBS (pH 7.4) before use.

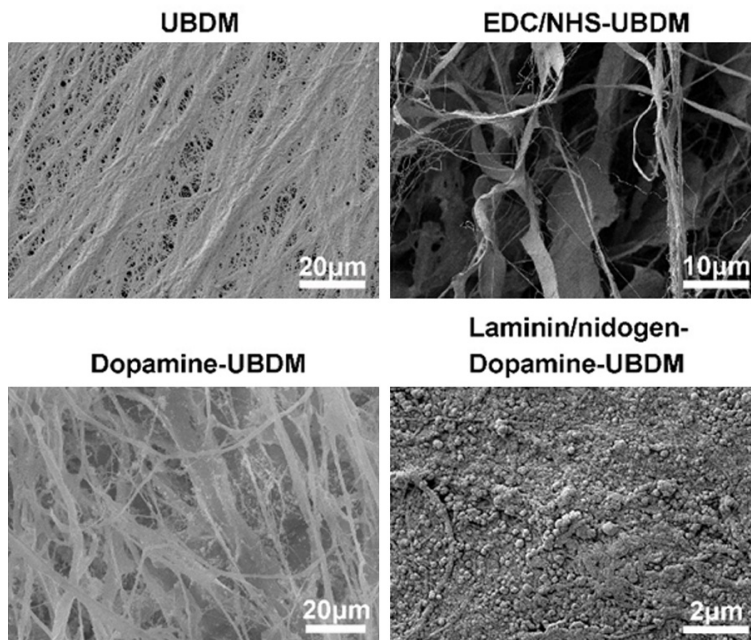
### *RT-PCR*

Total RNA was extracted from tissues using TRIzol and determined its concentration was determined according to the standard. Total RNA (0.5 µg) was used as a template to prepare cDNA (Reverse Transcription System, Promega Corporation, Madison, WI, USA; cat. no. A3500). The mRNA expression of target genes (CCR7, CD163, CXCR3 and CCR4) was quantified using SYBR Premix EX Taq (Takara Bio, Inc., Shanghai, China) on the ABI 7500 sequence detection system (Advanced Biosystem, Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed with the following thermocycling conditions: An initial 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. The thermocycler used in the present study was the StepOnePlus™ Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). The primers sequences were as follows: CCR7, forward 5'-AATGAAAAGCGTGCTGGTGGT-3', reverse 5'-TGTCTCCGATGTAATCGTCCGT-3'; CD163, forward 5'-TCTGTTGGCCATTTCGTCG-3' and reverse 5'-TGGTGGACTAAGTTCTCTCCTCTTGA-3'; CXCR3, forward 5'-CTCACCTAGCTGTAGCAGA-3', reverse 5'-AGGAAGATGAAGTCTGGGAG-3'; CCR4, forward 5'-GAAAGAACAAGGCGGTGAAGAT-3' and reverse 5'-ATGGTGGACTGCGTGAAGATGAG-3'; β-actin, forward 5'-CCTCGCCTTTGCCGATCC-3' and rev-

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**Figure 1.** The preparation of urinary bladder decellularized materials. A. The isolation of urinary bladder tissue from Rongchang swine. B. Photographs of UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM groups.



**Figure 2.** The morphological characterization of original urinary bladder tissue and the UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM groups by scanning electron microscopy (SEM).

erse 5'-GGATCTTCATGAGGTAG-TCAGTC-3'. Housekeeping gene  $\beta$ -actin was used as an internal reference to normalize the results. All experiments were performed in triplicate. Finally, the  $2^{-\Delta\Delta Ct}$  method was performed to calculate the relative expression.

### Statistical analysis

Quantitative data were expressed as the mean  $\pm$  standard deviations. Statistical analysis was performed using a one-way ANOVA, followed by Bonferroni or Dunnett post-hoc tests.  $P < 0.05$  was considered statistically significant.

## Results

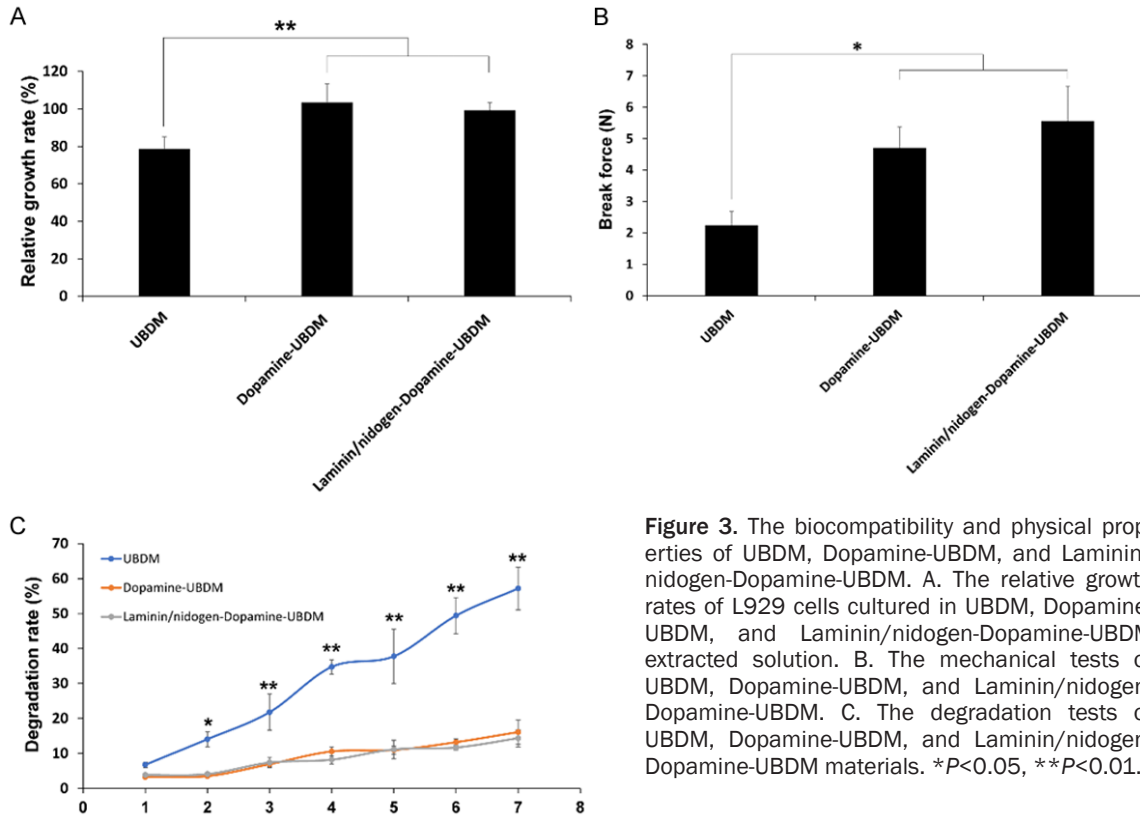
### Preparation of urinary bladder decellularized materials

After the mucosa, serosa, and muscularis were removed, the residual urinary bladder tissue was transparent and water-rich. Moreover, this tissue had strong toughness (**Figure 1A**). The UBDM had a white membrane after decellularization. We used crosslinked the tissue with EDC/NHS, then treated the UBDM with dopamine. The Dopamine-UBDM was black in color, and no significant differences in morphology were observed after using Laminin/nidogen to coat Dopamine-UBDM (**Figure 1B**).

### Morphology characterization

As shown in **Figure 2**, after removing the mucosa, serosa, and muscularis, the UBDM consisted of a mass of non-uniform collagen fibers. After crosslinking with EDC/NHS, the collagen fibers connected into pieces, and many small particles could be seen in the

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**Figure 3.** The biocompatibility and physical properties of UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM. A. The relative growth rates of L929 cells cultured in UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM extracted solution. B. The mechanical tests of UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM. C. The degradation tests of UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM materials. \* $P < 0.05$ , \*\* $P < 0.01$ .

Dopamine-UBDM. Interestingly, many spherical particles were absorbed and completely covered the surface of the Dopamine-UBDM after it was coated with laminin/nidogen.

### Biocompatibility test

As shown in **Figure 3A**, the relative growth rate of the L929 cells in the UBDM, Dopamine-UBDM, Laminin/nidogen-Dopamine-UBDM extract media was  $(78.48 \pm 6.81)\%$ ,  $(103.36 \pm 10.13)\%$ , and  $(99.44 \pm 3.82)\%$ , respectively. The relative growth rates of the L929 cells in these treatments were all higher than 75%, especially in the Dopamine-UBDM and Laminin/nidogen-Dopamine-UBDM treatments, indicating that no significant in vitro cytotoxicity was present according to the cytotoxicity grading criteria [16].

### Mechanical test

**Figure 3B** shows that the break force of the Dopamine-UBDM and Laminin/nidogen-Dopamine-UBDM groups was higher than in the UBDM group ( $P < 0.05$ ). However, no significant difference was apparent between the Dopa-

mine-UBDM and Laminin/nidogen-Dopamine-UBDM groups.

### In vitro degradation test

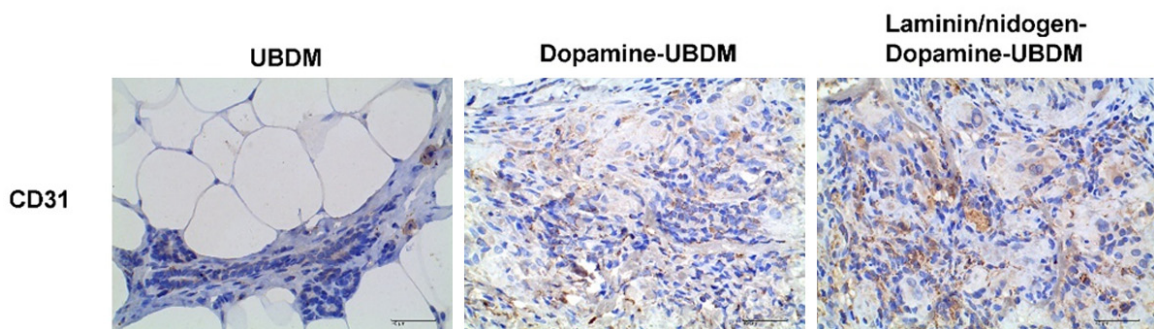
We also investigated the biodegradability of UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM. As shown in **Figure 3C**, the normal UBDM was degraded more rapidly than were Dopamine-UBDM and Laminin/nidogen-Dopamine-UBDM. After 7 days of degradation using collagenase, the degradation rate of normal UBDM reached  $(57.17 \pm 6.13)\%$ . In comparison, the degradation rate of the Dopamine-UBDM, Laminin/nidogen-Dopamine-UBDM groups was only  $(16.1 \pm 3.46)\%$  and  $(14.34 \pm 2.46)\%$ , respectively.

### In vivo scaffold implantation

**Figure 4** shows that at 6 weeks after implantation, the normal UBDM was almost completely absorbed. In comparison, some residual Dopamine-UBDM and Laminin/nidogen-Dopamine-UBDM could be seen. In addition, no fibrous capsule formation occurred among the three treatments.



**Figure 4.** HE staining of the UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM groups at 6 weeks after in vivo implantation.



**Figure 5.** The expression of CD31 in the UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM groups at 6 weeks after in vivo implantation.

### Angiogenesis

We investigated the angiogenic effects of UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM. As shown in **Figure 5**, the expression of CD31 in UBDM was markedly lower than that in the Dopamine-UBDM and Laminin/nidogen-Dopamine-UBDM groups. Moreover, the expression of CD31 was significantly increased in the Laminin/nidogen-Dopamine-UBDM group compared with the Dopamine-UBDM group.

### Inflammatory response

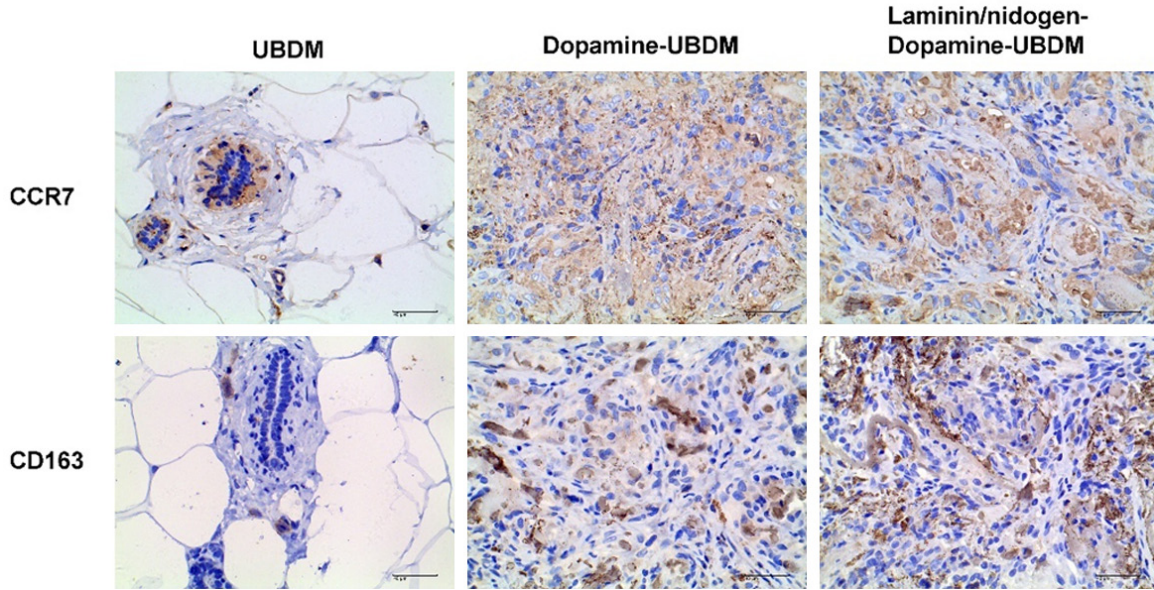
As shown in **Figure 6**, the expression of an M1 type macrophage (CCR7) in the Dopamine-UBDM group was higher than that in the Laminin/nidogen-Dopamine-UBDM group. In contrast, the expression of M2 type macrophage (CD163) in the Dopamine-UBDM group was lower than that in the Laminin/nidogen-Dopamine-UBDM group. Similarly, the expression of Type 1 T helper cells (CXCR3) in the

Dopamine-UBDM group was higher than that in the Laminin/nidogen-Dopamine-UBDM group, and the expression of Type 2 T helper cells (CCR4) in Dopamine-UBDM group was lower than that in the Laminin/nidogen-Dopamine-UBDM group (**Figure 7**). Moreover, the RT-PCR results also showed that both the relative expression of CCR7 and CXCR3 in Dopamine-UBDM group were higher than that in the Laminin/nidogen-Dopamine-UBDM group ( $P < 0.05$ ). On the contrary, the relative expression of CD163 and CCR4 in Dopamine-UBDM group were lower than that in the Laminin/nidogen-Dopamine-UBDM group ( $P < 0.05$ ) (**Figure 8**).

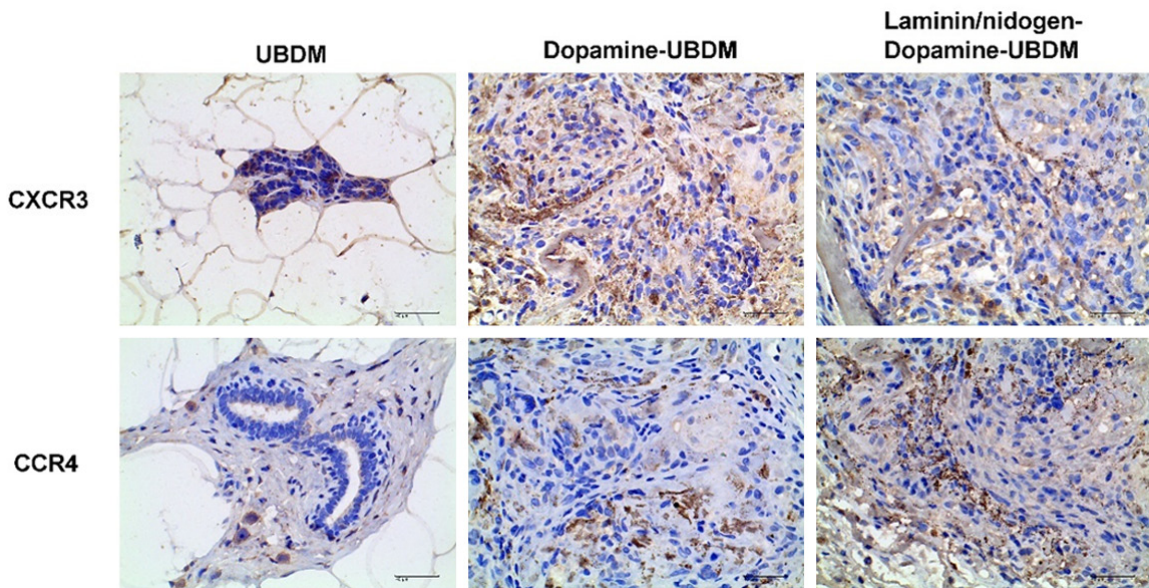
### Discussion

In this study, urinary bladder decellularized materials were chosen as the substrate for pelvic reconstruction. However, normal UBDM degraded rapidly, and they had weak mechanical properties. We found that UBDM was composed of a mass of collagen fibers; thus, to retard its degradation and enhance its mech-

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**Figure 6.** The expression of CCR7 (M1 macrophage) and CD163 (M2 macrophage) in the UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM groups at 6 weeks after in vivo implantation.

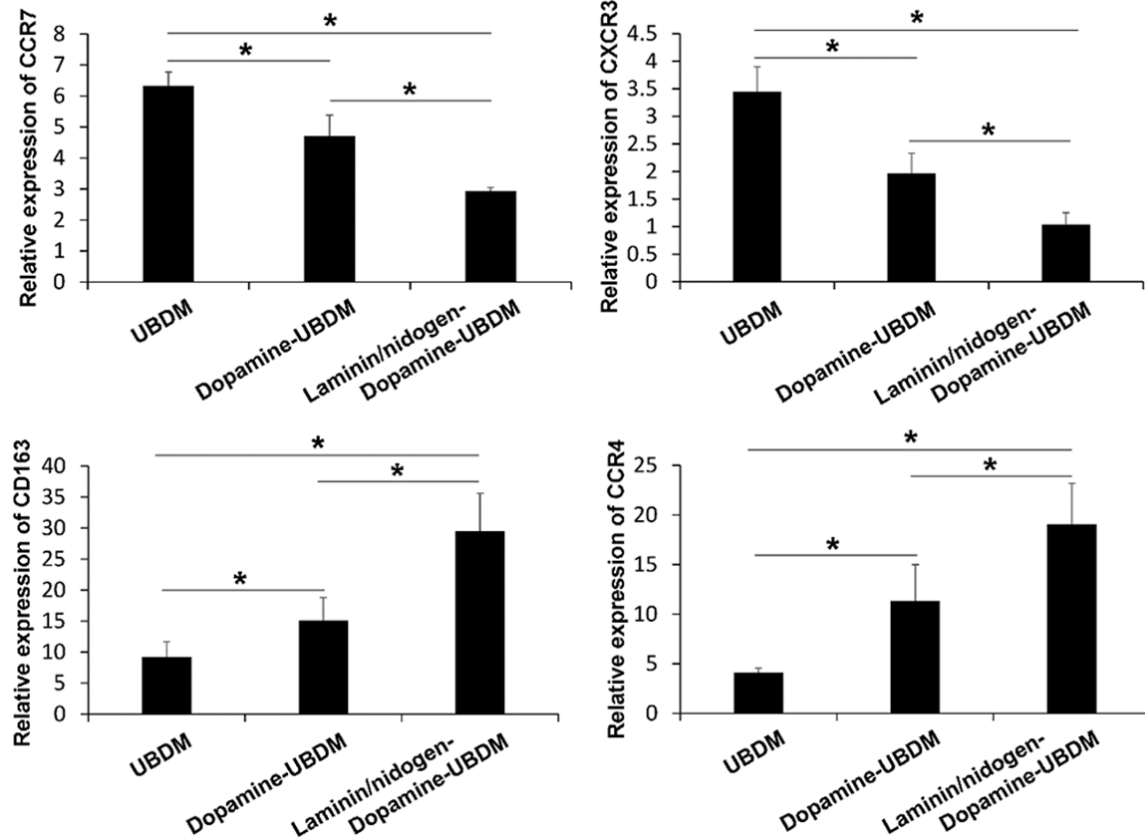


**Figure 7.** The expression of CXCR3 (Th1) and CCR4 (Th2) in the UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM groups detected by immunohistochemistry at 6 weeks after in vivo implantation.

anical properties, the UBDM had to be cross-linked. A crosslinking reaction was performed using EDC in the presence of the nucleophile NHS. The addition of NHS increases the rate and degree of collagen crosslinking [17]. In contrast to other crosslinkers such as glutaraldehyde, hexamethylene diisocyanate and acyl azide, the EDC/NHS crosslinker is a “zero-

length” crosslinker, which indicates that no chemical residues remain after the reaction, resulting in better biocompatibility [18]. After crosslinking, the mechanical properties of the EDC/NHS crosslinked UBDM were clearly better than those of normal UBDM, and coating with dopamine or laminin/nidogen absorption did not affect the mechanical properties. In

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**Figure 8.** The relative expression of CXCR3 (Th1) and CCR4 (Th2) in the UBDM, Dopamine-UBDM, and Laminin/nidogen-Dopamine-UBDM groups detected by RT-PCR at 6 weeks after in vivo implantation.

addition, the degradation rate of EDC/NHS-crosslinked UBDM was significantly reduced, and coating with dopamine and laminin/nidogen absorption did not affect degradation.

After the EDC/NHS crosslinking, we further enhanced the biocompatibility of UBDM by modifying it with laminin/nidogen. Before using laminin/nidogen to modify the UBDM, we coated its surface with dopamine. Because dopamine contains catechol and amine groups, it forms a thin film on various material surfaces as a result of its excellent adhesive properties at a basic pH. Furthermore, thin dopamine films may serve as an extremely versatile platform for secondary modifications; thus, it enables a substrate material to be tailored for various functional uses [6].

Subsequently, we used laminin/nidogen to further modify the dopamine-coated UBDM. It is well known that laminin is a ubiquitous component of the basement membrane, which medi-

ates a wide range of interactions, including the modulation of various cellular activities and the binding to other matrix components [19]. This multi-domain protein possesses a cross-shaped structure that appears to be well suited for carrying a multitude of biological activities. Among the various potential supramolecular complexes, laminin combined with nidogen, another basement membrane protein, seems to be particularly stable [20, 21]. Dziadek et al. reported that the presence of nidogen may affect certain biological activities of the laminin. Studies using cell attachment assays demonstrated a distinctly better activity of the complex compared to laminin alone [22]. In this study, after we used laminin/nidogen to treat the UBDM, the proliferation of the L929 cells in the Laminin/nidogen-Dopamine-UBDM group was significantly higher than that in the normal UBDM group, and the relative growth rates of L929 cells in Laminin/nidogen-Dopamine-UBDM group were higher than 100%, indicating no in vitro cytotoxicity according to the



cytotoxicity grading criteria [16]. Moreover, our study showed that the expression of CD31 in the Laminin/nidogen-Dopamine-UBDM group was higher than that in the Dopamine-UBDM group, which indicated the laminin/nidogen modified UBDM has better angiogenic effects. We speculate that these effects are due to laminin/nidogen, which may promote the aggregation of endothelial cells and the formation of blood vessels.

Furthermore, we detected T helper cells (Th) and macrophage (M1 and M2) reactions during implantation via immunochemical analysis, and these cells can be characterized as Th1 or Th2 phenotype, M1 or M2 phenotype based on their receptor expression and production of cytokines and effector molecules. These cells play critical roles in determining immune tolerance or immune rejection functions [23, 24]. In general, M1-activated macrophages produce high levels of inducible nitric oxide synthetase (iNOS), secrete toxic reactive oxygen, and are inducer and effector cells in Th1-type inflammatory responses (cell-mediated rejection). M2-activated macrophages produce arginase (ARG) in place of arginine, subsequently produce ornithine and polyamines, and are involved in polarized Th2 reactions to facilitate tissue repair and regeneration [25, 26]. In our study, we found that the expression of CCR7 (M1 macrophage) and CXCR3 (Th1) was higher in the Dopamine-UBDM group than in the Laminin/nidogen-Dopamine-UBDM group but that, in contrast, the expression of CD163 (M2 macrophage) and CCR4 (Th2) in the Dopamine-UBDM group was lower than that in Laminin/nidogen-Dopamine-UBDM group. These results suggest that laminin/nidogen can affect the immune response via the M2 type macrophage-dominated immune response, which functions in tissue regeneration.

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### Disclosure of conflict of interest

None.

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