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Quantitative multiphoton microscopy of murine urinary bladder morphology during in situ uniaxial loading

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ABSTRACT

Urodynamic tests are the gold standard for the diagnosis of bladder dysfunction, and the mechanical compliance of the bladder is an important parameter in these tests. The bladder wall has a layered structure, differentially affected by pathology, so knowledge of the contribution and role of these layers and their constituents to overall bladder compliance will enhance interpretation of these clinical tests. In this study we document the functional morphology of the detrusor and lamina propria of the murine bladder wall using a custom in-situ tensile loading system under multiphoton microscopy (MPM) observation in unloaded state and under incremental uniaxial stretch. Features in the stress-stretch curves of bladder samples were then directly related to corresponding MPM images. Collagen organisation across wall depth was quantified using image analysis techniques. The hypothesis that the lamina propria deformed at low strain by unfolding of the rugae and rearranging collagen fibrils was confirmed. A novel 'pocket' feature in the detrusor was observed along with extensive rearrangement of fibrils in two families at different depths, providing higher stiffness at high stretches in the detrusor. The very different deformations of detrusor and lamina propria were accommodated by the highly coiled structure of collagen in the lamina propria. Imaging and mechanical studies presented here allow gross mechanical response to be attributed to specific components of the bladder wall and further, may be used to investigate the impact of microstructural changes due to pathology or aging, and how they impair tissue functionality.

Statement of Significance

This article reports the first in-situ multiphoton microscopy observations of microstructural deformation under uniaxial tensile loading of ex vivo bladder. We describe collagen rearrangement through the tissue thickness and relate this directly to the stress-stretch behaviour. We confirm for the first time the unfolding of rugae and realignment of fibrils in the lamina propria during extension and the rapid stiffening as two fibril families in the detrusor are engaged. This technique provides new insight into microstructure function and will enhance understanding of the impact of changes due to pathology or aging.

1. Introduction

The function of the bladder is to store large volumes of urine (typically 400–500 ml in humans) at low pressure and void stored contents from the body when at capacity. The bladder wall is a complex multi-layer tissue consisting of an outer thick layer of smooth muscle (detrusor) and a loose, fibrous inner layer (lamina propria), allowing the bladder wall to undergo large deformations with minimal resistance during filling and contraction across a great length when voiding. Measurement of the compliance of the bladder during urodynamic studies is used clinically to diagnose lower urinary tract disorders. Although these disorders may affect different layers differentially, the distribution of mechanical load between anatomical layers and microstructural constituents is poorly understood.

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The lamina propria is thought to play a capacitance role initially during filling with loose collagen and folded rugae allowing large extension [1]. At higher volumes the flattening of the rugae and straightening of collagen fibrils are supposed to limit distension [2]. The detrusor muscle also plays multiple roles: relaxing during filling and contracting to void urine. The majority of the detrusor consists of bundles of smooth muscle in various orientations, interconnected by collagen fibrils [3]. However, direct evidence of these roles from in situ imaging of the fresh loaded tissue is so far lacking.

The function of the bladder is determined by the mechanical response of the bladder wall, and the extra cellular matrix (ECM) constituents of which it is composed. Alterations to the composition of the bladder wall due to pathology or age are known to affect mechanical response, and thus bladder function, often with severe consequences to patients. Collagens I and III comprise the majority of structural matrix proteins throughout the bladder wall. Fibrillar collagen is primarily localised to the lamina propria and surrounding smooth muscle in the detrusor, and arranged in complex coiled microstructures [4]. Chang et al. (1998) speculate that the high compliance of the wall arises from the unfolding and reorientation of multiple 'levels' of smooth muscle before collagen fibrils begin to uncrimp and bear load. However only recently have studies imaged structural proteins in the whole tissue under increments of stretch to observe this directly [5,6].

Second harmonic generation (SHG) and two-photon excitation fluorescence (TPEF) signals from a multiphoton microscope (MPM) provide high resolution imaging of load-bearing proteins collagen and elastin respectively at tissue depths of up to 300 μm. This permits the use of whole, fresh-frozen tissue with no exogenous stains, avoiding mechanical property and geometric changes due to fixing [7,8].

The aim of this study was to visualize the microstructure of load bearing proteins in the wall layers of the murine bladder, quantify their organization and link whole tissue stress-stretch behaviour to microstructural deformation. We hypothesized that the lamina propria would accommodate strains with geometrical rearrangement of first rugae and then fibrils. This aim was achieved using SHG and TPEF MPM imaging, image analysis techniques and in-situ uniaxial mechanical testing of fresh-frozen whole murine bladder tissue together with SHG, TPEF and histological staining of fixed bladder wall cross-sections.

2. Methods

2.1. Tissue preparation

Sixteen, 3–4 month-old male C57/BL6 mice were used in the current study. All experiments were carried out under an approved UK protocol and project license and in accordance with the University of Sheffield Animal Care Committee. Mice were sacrificed by cervical dislocation following isoflurane anaesthesia. Intact bladders were placed in phosphate buffered saline (PBS) and kept frozen at −20 °C in excess of 24 h. The absence of calcium in the storage medium ensured that the bladders contractions were minimized.

Eight bladder specimens were used for mechanical testing and an additional four each used for histology and planar imaging studies. Following thawing for two hours at 23 ± 2 °C rectangular strips of bladders were cut along the circumferential axis, removing the apex and base regions (See Fig. 1). Loose areolar tissue on the outer layer was carefully removed and the specimens were washed in PBS. DAPI stock (Fischer Scientific) was diluted 1:1000 in PBS. Tissue was incubated in DAPI working solution for 10 min in dark area, then washed several times with PBS. Full length strips were cut to measure approximately 12 × 4 mm. Tissue strips were placed in a resting configuration on a microscope cover slip and hydrated with PBS. A second cover slip was placed over the sample to secure the tissue and prevent movement and folding of tissue.

Histology was performed on cross sections excised from the mid-body of bladders (n = 4) aligned with the circumferential axis of the bladder. Tissue was fixed in 10% formalin and processed for paraffin embedding (Leica ASP 300S, Leica, UK). Microtome sections, 10 μm thick were cut through the bladder wall thickness and applied to adhesive slides. Tissue samples chosen for staining were rinsed and equilibrated in PBS at room temperature and incubated in DAPI (Fisher Scientific) and thoroughly rinsed with PBS before imaging. Collagen orientation analysis was performed on z-stack maximum intensity projections across the visible cross section of the tissue to obtain orientation across depth.

2.2. Imaging protocol

A Zeiss LSM 710 inverted confocal microscope (Zeiss, Oberkochen, Germany) fitted with a pulsed femto-second Chameleon laser operating at 860 nm, pulse width 140 fs at peak and repetition rate 80 Mhz was utilized for imaging urinary bladder tissue. Collagen (SHG) and elastin (TPEF) were imaged at wavelengths of 430 nm and 520 nm, respectively, with spectral bins of ±20 nm. Objectives used were Zeiss air 10x Plan-Apochromat (working distance 2 mm, NA 0.45), and 20x Plan-Apochromat (working distance 0.55 mm, NA 0.8). For DAPI stained tissues excitation was at 800 nm and imaging at 460 nm ± 20 nm. Power at the sample with these settings varied from 1500 to 3500 mW. Individual filter channels were overlaid into a single image. Planar images with resolution 1024 × 1024 were acquired over a 5 × 12 tile region and imaged from both inner and outer surface perspectives. A fixed number of optical slices were acquired across different z-stack depths resulting in a z-resolution of between 1 and 5 μm, depending upon layer imaged and penetration depth. A maximum image height of approximately 200 μm was achieved. Cross section images were taken as a tiled region of 5 × 5 images. Bladder wall thickness and width were measured using ImageJ (W. Rasband, NIH, USA).

2.3. Uniaxial tensile testing protocol

Mechanical testing data was obtained using a custom in-situ uniaxial system with a linear actuator (±1 μm, Zaber T-NA08A25; Zaber, Vancouver, Canada) and 5 N load cell (±0.5 mN, Interface, Scottsdale, USA). Rectangular tissue strips (n = 8) were loaded in the device using custom-made clamps, aligning the bladder circumferential axis to the axis of extension. Pre-testing using specimens marked with ink showed no slipping at the clamps for the forces applied. Clamps were submerged in a bath of PBS at ambient temperature and adjusted to achieve a tare load of 0.02 N after tissue was clamped, and specimen thickness measured with an optical micrometer (mean of five points, Keyence, UK, precision 10^{-5} m).

Bladder strips were subjected to three preconditioning cycles to a stretch of 1.5 with 3 min between cycles [9]. Using first Piola-Kirchhoff stress, stress-strain curves were obtained by applying displacement using a triangular ramp function at 1% s^{-1}. This was repeated 3 times and results averaged. After the mean stress-strain curve had been recorded, tissue was imaged during uniaxial stretch applied at increments of 0.1, to a maximum stretch limit of 2. Ex vivo inflation experiments [10] suggest this corresponds approximately to the filling of the bladder from rest to maximum capacity. Stretch was maintained for 2 min by which time load cell output had reached a steady value at each step before imaging. Following imaging from one side of the

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