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Direct Noninvasive Measurement and Numerical Modeling of Depth-Dependent Strains in Layered Agarose Constructs

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Abstract

Biomechanical factors play an important role in the growth, regulation, and maintenance of engineered biomaterials and tissues. While physical factors (e.g. applied mechanical strain) can accelerate regeneration, and knowledge of tissue properties often guide the design of custom materials with tailored functionality, the distribution of mechanical quantities (e.g. strain) throughout native and repair tissues is largely unknown. Here, we directly quantify distributions of strain using noninvasive magnetic resonance imaging (MRI) throughout layered agarose constructs, a model system for articular cartilage regeneration. Bulk mechanical testing, giving both instantaneous and equilibrium moduli, was incapable of differentiating between the layered constructs with defined amounts of 2% and 4% agarose. In contrast, MRI revealed complex distributions of strain, with strain transfer to softer (2%) agarose regions, resulting in amplified magnitudes. Comparative studies using finite element simulations and mixture (biphasic) theory confirmed strain distributions in the layered agarose. The results indicate that strain transfer to soft regions is possible *in vivo* as the biomaterial and tissue changes during regeneration and maturity. It is also possible to modulate locally the strain field that is applied to construct-embedded cells (e.g. chondrocytes) using stratified agarose constructs.

Keywords

Functional tissue engineering; heterogeneous strains; imaging elastography; agarose scaffold; finite element method

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CONFLICT OF INTEREST

There are no conflicts of interest to report.

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INTRODUCTION

Osteoarthritis (OA) is a debilitating disease of articular cartilage in the joint of humans for which no satisfactory treatment exists, although functional tissue engineering-based strategies have shown considerable potential for repair. Native cartilage exhibits a layered structure with a superficial, middle and deep zone that have different structural and mechanical properties important for its load-bearing and low-friction function (Bevill et al., 2010; Broom, 1984; Hosseini et al., 2013; Mow et al., 1991; Neu et al., 2007). Therefore, it has been postulated that recapitulating such depth-dependent properties in tissue engineered cartilage constructs would improve the quality of the implants, and also the long-term clinical success (for a recent review, see (Kock et al., 2012)). However, regeneration of cartilage with layered properties has been a challenging task. Promising approaches to achieve this include the use of scaffolds with depth-dependent properties (Chawla et al., 2007; Klein et al., 2003; Ng et al., 2009; Ng et al., 2005; Nguyen et al., 2011), and the application of particular loading conditions that result in depth-dependent mechanical stimuli (Khoshgofar et al., 2011; Kock et al., 2013b). In agarose constructs with layers of different stiffness, matrix deposition occurred preferentially in the weaker agarose gels, while in samples with higher agarose concentrations the newly deposited matrix remained in the pericellular area (Ng et al., 2009; Ng et al., 2005). This observation is in agreement with former experimental studies (Buschmann et al., 1995; Dimicco and Sah, 2003; Kock et al., 2013a) and computer simulations (Sengers et al., 2004; van Donkelaar et al., 2011).

The ability to modulate spatial matrix deposition in chondrocyte-seeded agarose constructs by a layered scaffold organization, and using advanced loading conditions, is required to more closely emulate the native function of cartilage. However, the exact way in which the combination of such scaffold and loading conditions would change the distribution of strains in a tissue engineering construct is not easy to measure using experimental techniques, or predict reliably using numerical modeling. Unfortunately, standard experimental measurements in the field are poorly suited to elucidate the complex internal mechanical response of complex layered constructs (Harley et al., 2010). Mechanical testing provides instantaneous and equilibrium properties of the bulk tissue construct, but is not able to reveal layered properties or distributions of the internal mechanical quantities, such as strain, which may be important determinants that improve or even accelerate tissue repair.

Displacements under applied loading MRI (dualMRI) allows for the noninvasive measurement of deformation, i.e. displacements and strains, in soft tissues under cyclic loading conditions (Chan and Neu, 2012; Neu et al., 2005; Neu and Walton, 2008). This technique has previously been demonstrated in a wide range of load-bearing musculoskeletal tissues, ranging from rabbit intervertebral disc (Chan et al., 2011) to human articular cartilage (Chan and Neu, 2012; Griebel et al., 2013a; Griebel et al., 2013b). It has also been applied to uniform engineered tissue constructs comprising agarose and chondrocytes (Neu et al., 2009), but has not yet investigated how layered, stratified constructs of variable stiffness may modulate internal distributions of strain.

To better understand the biomechanical function of complex repair tissues using noninvasive measures, the aim of the present study is to establish the limitations of standard mechanical

testing for stratified scaffolds, noninvasively determine the strain fields in layered agarose constructs by dualMRI, and provide a numerical model for comparison. We provide a thorough evaluation of the strains throughout layered agarose constructs under loading with an indenting bar, similar to the loading that was applied during the promising sliding indentation loading regime (Khoshgoftar et al., 2011; Kock et al., 2013b; Kock et al., 2010). These evaluations are also performed using a numerical model, which allows computing different and more difficult conditions where matrix is gradually deposited during prolonged culture. Such a model may also eventually be used to optimize the combination of scaffold properties and loading regime, where experimental (i.e. dualMRI) measures may prove more difficult to achieve.

MATERIALS AND METHODS

Agarose Construct Preparation for MRI

Low-melt agarose (2% and 4% w/v; Bio-rad, Hercules, CA) was mixed with PBS to create uniform and layered constructs for evaluation by MRI. Agarose was plated in a 60 mm cell culture dish to a total depth of 6 mm, and cylindrical (10 mm) diameter plugs were then harvested from this culture dish. For layered constructs, the first (bottom) layer was allowed to gel before the second layer was applied. In total, 6 construct types were tested: 2%, 4%, 2% at 1/3 thickness over 4% at 2/3 thickness (*hereafter termed 2%/4% (1:2)*), 2% at 2/3 thickness over 4% at 1/3 thickness (*2%/4% (2:1)*), 4% at 1/3 thickness over 2% at 2/3 thickness (*4%/2% (1:2)*) and 4% at 2/3 thickness over 2% at 1/3 thickness (*4%/2% (2:1)*).

Bulk Mechanical Testing

To directly compare the bulk construct mechanical properties, and to establish baseline data for use in comparative studies and numerical models of construct mechanics, uniform (homogeneous) and layered agarose constructs were tested in unconfined compression using a servoelectric materials testing system (TestResources, Shakopee, MN) (Figure 1). Constructs ($n=3$) were bathed in $1\times$ PBS and compressed at 5% nominal strain increments at a physiological strain rate of $17.9\% \text{ s}^{-1}$ (Neu et al., 2008) coupled with a 30-s hold (stress relaxation) period. Engineering stress was determined using initial sample geometry and a 1000g load cell (LPM 512, Cooper Instruments, Warrenton VA) at a sampling rate of 100 Hz. Instantaneous modulus was calculated based on the largest (compressive) stress experienced during each strain step, while equilibrium modulus was calculated as an average of 100 data points at the end of each relaxation period, as previously described (Korhonen et al., 2002). Poisson's ratio was measured, in uniform constructs, using a high accuracy ($\sim 20 \mu\text{m}$ resolution in x direction), two-dimensional (2D) laser displacement system (LJ-G080, Keyence, Woodcliff Lake NJ) mounted to analyze the full surface of construct in the vertical axis (Figure 1a). The lateral (maximum) expansion of the mid-height of the construct was tracked during compression. Poisson's ratio was calculated using the maximum displacement in the middle of the construct during each strain step compared to the original position of the construct before mechanical testing. Poisson's ratio was measured at both instantaneous and equilibrium time points. Data was analyzed using a custom MATLAB algorithm (R2010b, Natick MA).

Displacements Under Applied Loading by MRI (dualMRI)

Uniform and layered agarose constructs bathed in PBS were cyclically and intermittently loaded with a cylindrical (4.5 mm diameter) glass indenter at 0.33 Hz until a quasi-steady state load-deformation response was achieved (Neu and Walton, 2008) (Figure 2). A target load of 0.67 N was applied. However, the load varied by ± 0.2 N due to variability in the construct height coupled with a resistive spring required for the vertical test configuration. A 14.1 Tesla MRI system (Bruker Medical GMBH) noninvasively acquired displacement-encoded (phase-sensitive) data through the center of the samples. A DENSE-FISP imaging sequence was used with a displacement encoding gradient area of $2.13 \pi/\text{mm}$ (Chan and Neu, 2012). FISP parameters were: TR/TE = 3.7/1.85 ms; in-plane spatial resolution = $100 \times 100 \mu\text{m}^2$; image matrix size = 256×256 pixels²; number of averages = 4; slice thickness = 1.0 mm; flip angle = 25° . Displacement fields were obtained from MRI phase data using MATLAB (Mathworks) software.

Finite strain fields (E_{xx} , E_{yy} , and E_{xy}) were generated for each construct (Neu and Walton, 2008). From these strain fields, von Mises strains were additionally calculated (Griebel et al., 2013a; Griebel et al., 2013b) to facilitate a comparison of results by providing a single measure that incorporates the three strains. The von Mises strains were averaged by depth and a cubic spline fit was used to obtain strain values at 10% intervals of depth through the construct. Because the applied loads varied from sample to sample, the strains were normalized to the target load for the depthwise analysis.

Finite Element Simulations

A 3D finite element model consisting of a deformable cylindrical construct sample ($\varnothing = 10$ mm, depth = 6 mm) and a rigid impermeable indenter ($\varnothing = 4.5$ mm) was created in ABAQUS (v11, Pawtucket, RI, USA) (Figure 3). The mesh consisted of 21120, eight-node pore pressure elements. The displacements of the base nodes were restricted in all directions. Only half of the sample was modeled due to symmetry of the defined loading and geometry, with symmetry boundary conditions were applied to the nodes at the symmetry plane. The nodes on the sample periphery and on the top surface of the sample where not in contact with the indenter were prescribed to zero pore pressure to simulate free fluid flow. Frictionless contact was considered between the indenter and the sample. During the simulation, the indenter was only allowed to have vertical (y) displacement. Simulation for each configuration consisted of the prescribed load applied in 1.5 seconds to simulate maximum loading during a 0.33 Hz dynamic compression. Uniform and layered agarose gels (with aforementioned 2% and 4% w/v concentrations) were modeled using mixture (biphasic) theory with moduli measured from bulk mechanical testing experiments. For additional details on the implementation of the material model, and a comparison among biphasic, linear elastic, and neo-Hookean models, please see the Supplemental Material. Both equilibrium and instantaneous moduli were considered in separate simulations. Poisson's ratio of 2% agarose was considered to be 0.17 and that of the 4% agarose was considered slightly lower (0.14). Permeability of 2% and 4% agarose gels were adopted from the literature and were $6.61 \times 10^{-13} \text{ m}^4 \text{N}^{-1} \text{s}^{-1}$ and $6.62 \times 10^{-14} \text{ m}^4 \text{N}^{-1} \text{s}^{-1}$, respectively (Gu et al., 2003).

Statistical Analysis

Bulk mechanical data was analyzed using Minitab v.16 Statistical Software (Minitab Inc., State College PA). A *t*-test was used to compare instantaneous and equilibrium moduli, and Poisson's ratio, for uniform constructs. Poisson's ratio among strain and agarose percentage was compared using general linear model ANOVA with post-hoc Tukey test. Agarose percentage and strain were treated as fixed main effects including all interactions. General linear ANOVA was used to analyze instantaneous and equilibrium moduli of layered constructs where dominant agarose concentration, orientation, and the interaction were considered as main effects. A *p*-value less than 0.05 was considered statistically significant. All values are presented as mean $\pm$ standard error.

RESULTS

Uniform Construct Bulk Mechanical Testing

Higher agarose content (w/v) increased instantaneous and equilibrium modulus, but did not change Poisson's ratio (Figure 1). Instantaneous modulus was increased from 0.75 ± 0.03 MPa to 1.73 ± 0.01 MPa for 2% and 4% agarose, respectively ($p=0.001$) (Figure 1b). Equilibrium modulus was increased from 0.23 ± 0.05 MPa to 1.12 ± 0.01 MPa for 2% and 4% agarose, respectively ($p=0.003$) (Figure 1c). Poisson's ratio was not found to be dependent on agarose percentage ($p=0.321$), strain ($p=0.197$), or the interaction ($p=0.905$). Due to this effect, we pooled across each agarose percentage to give an average Poisson's ratio of 0.17 ± 0.02 mm/mm and 0.14 ± 0.01 mm/mm for 2% and 4% agarose, respectively ($p=0.319$) (Figure 1d). Differences in Poisson's ratio at instantaneous and equilibrium time points was within the error of the measuring system, so for the purposes of this study we report Poisson ratio as a single value for both instantaneous and equilibrium mechanics.

Layered Construct Bulk Mechanical Testing

Bulk mechanical testing was unable to easily discern between layered constructs (Figure 4). Instantaneous modulus was measured to be 0.72 ± 0.01 MPa, 0.76 ± 0.02 MPa, 0.94 ± 0.03 MPa, and 1.02 ± 0.05 MPa for 2%/4% (2:1), 4%/2% (1:2), 2%/4% (1/2), and 4%/2% (2:1) layered constructs, respectively (Figure 4a). Overall, instantaneous modulus was statistically dependent on agarose distribution, where constructs with increased (4%) agarose content (i.e. 4%/2% (2:1) and 2%/4% (1:2)) were significantly higher than constructs with 2/3 2% agarose ($p<0.001$). Further Tukey analysis shows that similar agarose constructs did not show different mechanical properties regardless of layered orientation ($p>0.05$), but did show differences between agarose content ($p<0.05$). Equilibrium modulus was measured to be 0.38 ± 0.01 MPa, 0.41 ± 0.01 MPa, 0.46 ± 0.002 MPa, and 0.57 ± 0.04 MPa for 2%/4% (2:1), 4%/2% (1:2), 2%/4% (1:2), and 4%/2% (2:1) layered constructs, respectively (Figure 4b). Similar to instantaneous results, overall equilibrium modulus was dependent on agarose content ($p=0.001$). Tukey analysis shows that the only 4%/2% (2:1) was significantly different in equilibrium modulus ($p<0.05$).

Displacements Under Applied Loading by MRI (dualMRI)

dualMRI revealed complex and unique patterns of strain that corresponded to the structure of uniform and layered agarose constructs (Figures 5 and 6). Strain magnitudes were generally higher in the regions of constructs containing 2% agarose. Strain was transferred to the lower stiffness (2%) regions, regardless of layering order, which was notably seen in layered 2%/4% (2:1) and 4%/2% (2:1) constructs (Figure 5). When the indenter contacted 2% agarose in layered constructs, E_{xx} and E_{yy} strains remained concentrated near the indenter-agarose interface. In contrast, when the indenter contacted 4% agarose in layered constructs, E_{xx} and E_{yy} strains distributed to deeper (2%) agarose regions, maintaining higher magnitudes through the construct depth. Von Mises strains followed similar patterns, with larger strain magnitudes visible in construct regions containing softer (2%) agarose (Figure 6).

Finite Element Simulations

Consistent with the experimental (dualMRI) measurements, finite element simulations showed a dependency of the transverse, axial and shear strain fields to the stiffness of the layers of the gels in the construct (Figures 7 and 8). When equilibrium moduli were utilized, simulations reveal the highest strain concentrations when 2% agarose contacted the indenter, regardless of layer thickness (Figure 7). The peak transverse, axial and shear strains in these cases were in the range of 10%. In 4%/2% (1:2) constructs, strains were transferred to the 2% agarose, which was particularly apparent in the compressive strain field. This effect was less pronounced when the thickness of the 4% agarose layer was increased in 4%/2% (2:1) constructs. Under identical loading conditions, the use of instantaneous moduli revealed similar trends of variation in the strain fields (Figure 8). However, peak values of the induced strains were decreased (from 10% to 6%) showing the influence of the construct mechanical properties on strain transfer under identical loading conditions.

DISCUSSION

The purpose of this study was to identify the limitations of bulk mechanical testing on stratified tissue constructs, noninvasively determine the strain fields in layered agarose constructs by dualMRI, and compare the results with numerical modeling. Our main findings were 1) bulk mechanical analysis cannot elucidate mechanical differences among or within stratified tissue constructs, 2) dualMRI overcomes this limitation, revealing the complex strain transfer that depends on both construct composition and orientation, and 3) dualMRI results are consistent with a computational analysis based on mixture theory.

Bulk mechanical testing can provide a valuable first look into the mechanical behavior of constructs, as well as provide mechanical property estimates of uniform constructs that are useful for computational modeling. However, the data shown here indicate that it is lacking when an understanding of the internal strain field is required. The inability to differentiate between constructs with different orientation of the layers leaves a major void in the evaluation of engineered tissue constructs.

In contrast to bulk mechanical testing, dualMRI nicely fills this void and provides internal patterns of mechanical quantities, including strain. With sub-pixel resolution, the resultant strain fields provide a very detailed look into the complex internal strains resulting from the applied cylindrical indentation. Using dualMRI, we showed that patterns of internal strain fields for cartilage tissue engineering can be modulated by using layered agarose constructs, and is further dependent upon orientation of these layers to the impacted surface. As expected, the largest deformations occur in layered constructs when the indenter contacts the weaker 2% agarose that is placed above a stiffer 4% gel. With increasing thickness of the top 2% layer, strain magnitude increases while penetration depth of the strain decreases. When the stiffer 4% agarose contacts the indenter and is situated above the 2% agarose, the strains are distributed more equally throughout the construct, with on average higher strains in the deeper (2% agarose) regions, compared to constructs that have uniform agarose content.

Finite element simulations utilizing mixture (biphasic) theory correspond well to the dualMRI-generated strain fields. Simulations revealed quantitative (when equilibrium modulus was considered for the gels) and qualitative consistency between the numerical predictions and experimental measurements, supporting the value of numerical modeling in providing systematic insights into the mechanical conditions inside tissue engineering constructs. Furthermore, it is shown in particular that when the overall construct stiffness is increased (i.e., when the instantaneous moduli were used instead of the equilibrium moduli in the simulations), under identical loading conditions, the induced peak strain in the constructs decreases substantially (by approximately 40%). Because we expect that long-term culture of cell-laden agarose constructs would naturally stiffen (e.g. following matrix deposition), our finding that stiffened constructs decrease internal peak strains suggests that the applied loading regime should be adjusted (i.e. increased) to normalize mechanical stimulations of the seeded cells.

Heterogeneous strain patterns observed by dualMRI in stratified agarose constructs suggest that it is possible to modulate locally the strain field that is applied to embedded chondrocytes. These insights may be used to further optimize loading regimes for tissue engineering, or to enhance the interpretation of experimental data. For instance, the higher strains that are induced in a 2% agarose layer, when situated on top of a 4% agarose layer, may explain previous results detailing increased matrix deposition in the weaker superficial layer during mechanical stimulation of constructs (Ng et al., 2009; Ng et al., 2005). In future studies, imaging-based techniques like dualMRI, and the numerical simulations incorporating gradual matrix deposition in engineered constructs, may provide useful insight into the dynamic and complex mechanical microenvironment of the embedded cells, which may further help to achieve optimized culture conditions in tissue engineering studies.

Due to natural inhomogeneities in the agarose, the simulations, which assume a perfectly homogenous material in each layer, present a more uniform and symmetrical strain distribution than what was measured by dualMRI. An additional source of discrepancies between the measurements and calculations could arise from the quasi-steady state loading of the agarose in dualMRI. It is possible that pseudo-plastic deformation has occurred and is not accounted for in the model. Also, the strains measured for the stiffer 4% construct are

higher than the softer 2%. Differences in construct height, combined with limitations with the pneumatic actuator assembly, resulted in a larger applied load on the 4% gel.

This latter limitation helps to illustrate an important point regarding the applicability of our results to agarose constructs fabricated with various concentrations and methods of production. The 2% and 4% agarose concentrations used in this study are consistent with values in the literature. Concentrations of 2% and 3% are most common in cartilage tissue engineering (Buschmann et al., 1992), but lower (down to 0.25% (Kock et al., 2013a)) and higher (up to 5% (Rennerfeldt et al., 2013)) gels have also been used. In addition to concentration, stiffness of agarose gels may vary depending on parameters such as thermal history, average molecular weight, or concentration of chains involved in the gel network. Depending on the variations in such factors, reports of elastic moduli with an order of magnitude difference for gels with same concentrations exist in the literature (Aymard et al., 2001; Buckley et al., 2009; Normand et al., 2000). The conclusions presented herein are valid for gels with larger or smaller elastic moduli provided that the applied loads in the experiments are adjusted accordingly.

It should be noted that while this analysis focused on concentration-induced differences in material stiffness, the influence of agarose concentration on fluid transport must be considered. Different mechanical and fluid transport properties inherent in different agarose concentrations may lead to enhanced diffusion of growth factors, nutrients or newly formed matrix, further impacting regenerative processes (Buschmann et al., 1995; Dimicco and Sah, 2003; Kock et al., 2013a; Sengers et al., 2004; van Donkelaar et al., 2011). For instance, it was shown that newly formed matrix molecules may be extruded from the agarose as a result of the loading conditions applied (Kock et al., 2013a), which occurs earlier in the low-agarose gels (Kock et al., 2013b) that have higher diffusion coefficients (Snieskers and van Donkelaar, 2005).

This study demonstrates an improved understanding of the biomechanical function of complex tissue engineered constructs using noninvasive experimental and computational measures. Moreover, it demonstrates the far-reaching and complimentary utility of dualMRI and finite element modeling in the tissue engineering field. This powerful imaging technique can be deployed in any of a number of water-based constructs and biological tissues to evaluate mechanical performance under a wide variety of loading conditions. The information obtained via dualMRI could aid in preliminary construct design, monitor healing and integration of the construct during animal (both *in vivo* and *ex vivo*) and clinical trials, and also serve as an excellent validation of the many complex finite element models of engineered tissues. In addition to complex mechanics due to concentration differences, dualMRI has the potential to examine the mechanical response attributed to other modulations of construct mechanics, including fibril alignment or additional scaffold components (e.g. hyaluronic acid) in both bulk and stratified constructs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. In uniform constructs, agarose w/v percentage increases with the instantaneous and equilibrium moduli, but had no effect on Poisson's ratio

Agarose constructs were tested in unconfined compression via stress relaxation. Poisson's ratio was measured using a high-accuracy 2D laser displacement system.

Figure 2. Displacements under applied loading by magnetic resonance imaging (dualMRI) was used to noninvasively determine internal agarose deformation

Agarose constructs were placed in the bore of an MRI system, and image data was acquired of the undeformed and deformed construct during cyclic compressive loading by a cylindrical indenter, shown in the cross section in the image panels.

Figure 3. Experimental and numerical modeling configuration was designed to match experimental conditions

A 3D finite element (mixture theory) model consisted of deformable cylindrical construct and a rigid impermeable indenter. Due to symmetry, only half of the sample was modeled.

Figure 4. Layered agarose constructs showed no mechanical difference based on testing orientation

(a) Agarose constructs containing different layered concentrations showed statistically different instantaneous moduli ($p < 0.05$). Similar agarose contents were statistically similar regardless of orientation. (b) Equilibrium modulus shows less difference, where only 4%/2% (2:1) constructs were significantly different ($p < 0.05$).

Figure 5. Two dimensional internal strain fields for each construct were measured by dualMRI. Transverse, axial, and shear strains are shown for each of the 6 construct configurations studied. It is apparent that the orientation of the lower stiffness 2% gel in relation to the higher stiffness 4% gel dramatically influences the complex internal strain field.

Figure 6. von Mises strains varied as a function of depth through agarose constructs
Strain magnitudes were typically highest in the 2% agarose regions of stratified constructs.

Figure 7. E_{xx} , E_{yy} and E_{xy} strains depended on the configuration and concentration of stratified agarose layers

Equilibrium moduli were used for 2% and 4% agarose as 0.23 MPa and 1.12 MPa, respectively. In layered configurations, the strains were generally transferred to the 2% agarose layer, especially pronounced when the 2% gel was placed on top of the 4% gel.

Figure 8. Use of the higher instantaneous moduli reduced strain magnitudes but preserved relative patterns

E_{xx} , E_{yy} and E_{xy} strains were calculated using instantaneous moduli for 2% and 4% agarose (i.e. 0.75 MPa and 1.73 MPa, respectively) under identical loading conditions. The reduction in strain compared to the less stiff constructs in Figure 7 indicates that during engineered tissue development, it will be important to adapt loading to the stiffness of the changing structure.