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**Deformation and fracture of echinoderm collagen networks**

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Collagen networks provide the main structural component of most tissues and represent an important ingredient for bio-mimetic materials for bio-medical applications. Here we study the mechanical properties of stiff collagen networks derived from three different echinoderms and show that they exhibit non-linear stiffening followed by brittle fracture. The disordered nature of the network leads to strong sample-to-sample fluctuations in elasticity and fracture strength. We perform numerical simulations of a three-dimensional model for the deformation of a cross-linked elastic fibril network which is able to reproduce the macroscopic features of the experimental results and provide insights into the internal mechanics of stiff collagen networks. Our numerical model provides an avenue for the design of collagen membranes with tunable mechanical properties.

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1. Introduction

Living systems provide a formidable source of inspiration for the design and synthesis of new classes of materials with a potentially wide range of medical and non-medical applications (Huebsch and Mooney, 2009; Wegst et al., 2015). The structural and mechanical properties of animals mostly rely on collagen (Buehler, 2006), one of the main protein constituents of the extracellular matrix in connective tissues and an essential component of mammalian skin, bones and tendons. To achieve remarkable mechanical properties, long collagen molecules self-assemble into hierarchical structures by forming fibrils that can then arrange into bundles, known as fibers, and extended elastic networks. The mechanics of fiber networks has received a wide attention both experimentally (Vader et al., 2009; Lindström et al., 2010, 2013; Münster et al.,...
Fig. 1 - Echinoderm collagen networks: The fibril structure of collagen networks as observed by SEM is reported in panels (a), (c), and (e) for sea urchin (Paracentrotus lividus) (inset of b, photo by Federico Betti) starfish (Echinaster sepositus, (inset of d, photo by Tato Grasso, CC 3.0) and sea cucumber (Holothuria tubulosa (inset of f, photo by Roberto Pillon, CC 3.0). The corresponding diameter distributions are reported in panels (b), (d), and (f).
2013; Licup et al., 2015) and computationally (Qin and Buehler, 2011; Das et al., 2012; Lee et al., 2014; Feng et al., 2015, 2016; Žagar et al., 2015; Depalle et al., 2015) because of its relevance for applications but also as a model system to understand soft and disordered matter in general.

Most of the experimental studies focus on shear deformation of very soft collagen gels which are typically extremely soft at small deformations and become stiffer as the deformation proceeds (Motte and Kaufman, 2013). This peculiar non-linear strain stiffening, observed widely in soft collagen gels under shear (Vader et al., 2009; Lindström et al., 2010, 2013; Münster et al., 2013; Licup et al., 2015) but also present in stiffer skin tissues under tension (Yang et al., 2015), has important mechanical and physiological implications since it provides at the same time stability for large deformations and facilitates soft remodeling at small deformations. The precise origin of non-linear stiffening is still under scrutiny. In biopolymer networks this general behavior is usually attributed to a crossover between bending dominated and stretching dominated elasticity (Onck et al., 2005) or to the strain induced reorientation of the fibers (Vader et al., 2009; Feng et al., 2015). The application of these ideas to collagen is still debated (Licup et al., 2015) and it is not clear if the deformation is dominated by the non-linear response of individual fibers or by collective properties of the network (Gardel et al., 2004).

Marine-derived biomaterials in general — and marine collagens in particular — are promising materials for applications to tissue engineering. The mutable collagenous tissues (MCTs) of echinoderms, widespread in all five extant echinoderm classes, display striking passive mechanical properties (viscosity, tensile strength, and stiffness) that can be actively transformed within seconds by active mechanisms controlled by the nervous system (Wilkie, 2005). Hence, in addition to the mechanical functions usually associated with conventional collagenous structures (i.e. energy storage, transmission and dissipation), MCTs allow for the detachment of appendages or body parts in response to disease, trauma or predator attack (Wilkie, 2005) and for the energy-sparing maintenance of posture (Takekama et al., 2009). As in mammalian connective tissues, most mutable collagenous structures are formed by parallel aggregations of collagen fibrils attached to proteoglycans (Trotter et al., 1994; Sugni et al., 2014). Collagen fibrils are usually surrounded by an elastic network further improving the structural and mechanical properties of the tissue (Wilkie, 2005).

Besides their physiological relevance, MCTs have been recently proposed as a promising source of collagen for applications to regenerative medicine (Di Benedetto et al., 2014; Sugni et al., 2014). In particular, collagen barrier-membranes of mammalian origin are typically used to facilitate proper tissue regrowth in anatomically separated compartments during guided tissue regeneration (Głowacki and Mizuno, 2008). Marine collagen and MCTs in particular could provide an innovative and safer alternative to mammalian ones (Silva et al., 2014; Di Benedetto et al., 2014; Ferrario et al., 2016). Moreover, other new intriguing applications could be derived from the use of collagen derived from echinoderms: for example to investigate cellular contractility as a biomechanics-related readout for phenotypic drug assessment (Zhao et al., 2015).

In this paper, we characterize the elastic tensile deformation and failure of cross-linked collagen networks derived from three echinoderms (Fig. 1): sea urchin (Paracentrotus lividus) and sea cucumber (Holothuria tubulosa). We analyze the stiffening behavior of the networks and characterize sample-to-sample fluctuations in the elastic modulus and tensile strength. We compare our results with numerical simulations for a three dimensional model for cross-linked elastic fibrils with parameters chosen to mimic the microstructure of our samples as revealed by SEM microscopy. Our model is similar in spirit to recent three dimensional models of cross-linked fiber networks (Qin and Buehler, 2011, 2013; Lee et al., 2014; Depalle et al., 2015; Žagar et al., 2015) but its features and parameters are adapted to the stiff collagen networks we study experimentally. In particular, we explicitly endow each cross-linker with bending, shear and torsional rigidities (Žagar et al., 2015). Simulations of the model highlight the importance of cross-linking in determining the mechanical properties of these networks, setting the bases for a computational guided design for this kind of bio-inspired materials.

2. Experimental methods

2.1 Experimental animals

Adult specimens of the starfish Echinaster sepositus, the sea urchin Paracentrotus lividus and the sea cucumber Holothuria tubulosa were collected by scuba divers in Paraggi (Marine Protected Area of Portofino, Ligurian Sea, Italy) and quickly transferred to the Department of Biosciences (University of Milan) in order to be immediately dissected. The aboral body walls (ABW) of the starfish, the peristomial membrane (PM) of the sea urchins and the whole body walls (BW) of the sea cucumbers were dissected and stored at −20 °C for the subsequent collagen extraction protocol.

2.2 Echinoderm collagen extraction

Sea urchin and starfish collagen were extracted from the PM and ABW, respectively, as previously described (Di Benedetto et al., 2014) with only slight modifications for the latter (see below). Briefly, both starfish ABW and sea urchin PM were dissected in small pieces, rinsed in artificial sea water (ASW), left in a hypotonic buffer (10 mM Tris, 0.1% EDTA) for 12 h (RT) and then in a decellularizing solution (10 mM Tris, 0.1% SDS) for 12 h (RT). After several washings in PBS, samples were placed in a disaggregating solution (0.5 M NaCl, 0.1 M Tris–HCl pH 8.0, 0.1 M β-mercapto-ethanol, 0.05 M EDTA–Na). Starfish samples underwent an additional washing step in citric acid (1 mM pH 3–4) between the decellularizing and the disaggregating solutions. All these steps were performed under stirring (rotating) conditions. The obtained collagen suspension was filtered and dialyzed against 0.5 M EDTA-Na solution (pH 8.0) for 3 h (RT) and against dH2O overnight (RT). Sea cucumber BW was dissected in small pieces, placed in PBS and gentamicin (40 μg/mL) and left in stirring condition (RT) for at least 5 days until a homogenous collagen suspension was obtained, which was then subsequently filtered. The
solutions (EDC 30 mM/NHS 15 mM in MES buffer 100 mM pH
branes were then immersed in an EDC/NHS cross-linker

dry overnight at

After several washings with dH2O, they were dehy-
tetroxide in 0.1 M sodium cacodylate buffer (2 h, room tem-
were placed in 5 cm
matrices (Di Benedetto et al., 2014). The collagen suspensions
were centrifuged for 10 min at 50 × g and then for 20 min at
2000 × g. The pellet was re-suspended in autoclaved dH2O
(100 mg/mL) and 8.7 mL of this suspension
were placed in 5 cm × 3 cm rubber silicone molds and left
daynight at +37 °C. The so-obtained collagen mem-
were then immersed in an EDC/NHS cross-linker
solution (EDC 30 mM/NHS 15 mM in MES buffer 100 mM pH
5.5) for 4 h and then washed with PBS, dH2O and ethanol 70%.

Membranes of the three different collagen types were prepared
as previously described for sea urchin collagen
Di Benedetto et al., 2014). The collagen suspensions
were centrifuged for 10 min at 50 × g and then for 20 min at
2000 × g. The pellet was re-suspended in autoclaved dH2O
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solution (EDC 30 mM/NHS 15 mM in MES buffer 100 mM pH
5.5) for 4 h and then washed with PBS, dH2O and ethanol 70%.

2.4. Scanning electron microscopy

Substrates were fixed with 2% glutaraldehyde in 0.1 M caco-
dylate buffer for 2 h at 4 °C and post-fixed with 1% osmium
tetroxide in 0.1 M sodium cacodylate buffer (2 h, room tem-
perature). After several washings with dH2O, they were dehy-
hydrated in an increasing ethanol scale and treated with a series
of solutions of HMDS (Hexamethyldisilazane) and ethanol in
different proportions (1:3, 1:1, 3:1 and 100% HMDS), mounted
on stubs, covered with pure gold (Agar SEM Auto Sputter,
Stansted, UK). The samples were observed under a scanning
electron microscope (SEM) (LEO-1430, Zeiss, Oberkochen, Ger-
many). Examples of the observed network structure are
reported in Fig. 1(a), (c), and (e). Images were analyzed using
the ImageJ plugin Diameter-J (Hotaling et al., 2015) to extract
diameter distributions (see Fig. 1(b), (d), and (f)).

2.5. Tensile tests

The collagen membranes were cut into narrow strips by
pressing them under an assembly of evenly spaced razor
blades. The ends of the collagen strips were then glued
between pieces of rough emery paper to facilitate clamping.
The length and width of the exposed collagen surface were
10 mm and 2.25 mm, respectively. Before testing, the thick-
ness of each sample was measured using a LGF-01100L-B
transmission-type photo-electric linear encoder (Mitutoyo)
with 100 nm resolution. Measurements were taken from
three positions and averaged. The average thickness was in
the range 4.6–18.6 µm. We did not find any systematic varia-
tion of the mechanical properties with the thickness of the
sample. The tensile tests were performed under controlled
temperature (21–22 °C) and humidity (64–69%) with a Kamm-
rath–Weiss B.1205. A tensile tester equipped with displace-
ment gauge and DDS3 Controller (Kammrath & Weiss GmbH,
Dortmund, Germany) and a 100 N load cell. After clamping
samples into place, Leibovitz L-15 medium was added on the
surface in order to wet them completely. Additional droplets
of liquid were added every two minutes during the tests to
keep the samples from drying. All samples were tested under
uniaxial tension until failure, with a constant strain rate
(8.35 × 10⁻⁴ s⁻¹). Fig. 2 reports a representative example of a
broken sample visualized under SEM microscopy. We observe
a very smooth fracture surface, indicative of brittle failure.

3. Experimental results

The individual stress–strain curves obtained experimentally
for n equivalent samples are reported in Fig. 3a for sea urchin
(n=14), in Fig. 3b for starfish (n=20) and in Fig. 3c for sea
cucumber (n=7). The curves display a wide variability from
sample to sample. The shape of each curve is, however,
similar in all cases and showing a characteristic stiffening
behavior with an initial non-linear regime, followed by a
linear regime that ends in abrupt failure, in qualitative
agreement with previous experiments on softer mammalian
collagen networks (Roeder et al., 2002). To reveal the typical
behavior of the system, we compute the average stress strain
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(8.35 × 10⁻⁴ s⁻¹). Fig. 2 reports a representative example of a
broken sample visualized under SEM microscopy. We observe
a very smooth fracture surface, indicative of brittle failure.
4. Numerical results

4.1. Model

We construct a three dimensional model of the deformation of cross-linked elastic fibrils. Collagen fibrils are discretized as bead-spring polymers of diameter $d$ with stretching and bending stiffness. The rest length of each discretization segment is $a$ and the elastic energy of a fibril of $L$ nodes can be written as

$$E_f = \frac{1}{2} \sum_{i=1}^{L} k_f (|\mathbf{r}_i - \mathbf{r}_{i+1}| - a)^2 + K \sum_{j=1}^{L-1} \cos(\theta_j),$$

(1)

where $k_f = \pi E d^2 / 4a$ is the stretching stiffness, $K = 3\pi E d^4 / 64a$ is the bending stiffness, and $\theta_j$ is the angle between two fibril segments. The fibrils are assumed to have no torsional resistance. The Young modulus of an individual fibril is chosen to be $E = 10$ GPa, as measured in AFM tests in dry conditions (Wenger et al., 2007). To simulate fibril-fibril mutual hindrance, fibril nodes also interact by a purely repulsive Lennard-Jones (LJ) potential $U(r) = 4\epsilon \left[ (\sigma/r)^{12} - (\sigma/r)^6 \right]$ for $r < \sigma$ and zero otherwise. The parameter $\sigma$ is chosen to represent the typical fibril diameter (e.g. $\sigma = 75$ nm).

To simulate cross-linking, we randomly place links between neighboring fibril nodes. In particular, fibrils are cross-linked with probability $p$ when two nodes are closer than a distance $b$. In order to provide the cross-linkers with stretching, bending and torsional stiffness, we model each link by a set of four springs (see Fig. 5a). Considering two fibrils, $A$ and $B$, a link involves four nodes, two on fibril $A$, $i-1$ and $i+1$ and two on fibril $B$, $j-1$ and $j+1$, which are connected by four springs $i+1-j+1$ (Fig. 5a). We notice that internal stresses are present in real samples (Lieleg et al., 2011), but are not easily quantified in our case. Hence, for simplicity, we assume that initially there is no stress in the system and set the rest length of each of the four link connections to their initial distance $b_{ij\alpha}$ with $\alpha = 1, ..., 4$. The cross-linking energy between fibril $i$ and $j$ is given by

$$E_{\text{link}} = \frac{1}{2} k_l \sum_{\alpha=1}^{4} (|\mathbf{d}_{ij\alpha} - b_{ij\alpha}|)^2,$$

(2)

where $\mathbf{d}$ is the actual length of the link. In most of the simulations, we assume that both fibrils and cross-linking bonds are linearly elastic and do not consider fracture events or the formation of new cross-linkers. We also perform simulations where the cross-linkers display non-linear elastic behavior as recently shown in Depalle et al. (2015). In particular, the cross-linker $k_l$ stiffness in Eq. (2) is increased from

Fig. 3 – Elastic response of collagen membranes: The stress-strain curves measured in collagen membranes for $n$ independent experimental realizations and the corresponding average curves are non-linear and display strain-stiffening. Data reported for (a) sea urchin, (b) starfish and (c) sea cucumber. The Young modulus exhibits an initial strain stiffening and then reaches a plateau value $E^*$ (d). The error bar is the standard error.
4.1 Simulations

We perform numerical simulations of the network model using the parameters reported in Table 1. The evolution of the deformation process is reported in Fig. 5c and d and in video S1. We observe that fibrils are first reoriented in the initial stage and then the sample becomes much thinner. This is quantified in Fig. 6 displaying the evolution with strain of the distribution of the angle \( \theta \) formed by the tensile axis and fibril segments. Fig. 6 shows that as the sample deforms the typical value of \( \theta \) decreases, as shown by the left-ward shift of the peak of the distributions. We checked that the effect is more marked when the sample size is larger.

From the simulations, we notice that the amount of cross-linking plays a major role in the mechanical response. This is illustrated in Fig. 7a where we show that the stress–strain curves change substantially by varying the number of cross-linkers \( N_{\text{link}} \). The same curves display features that are in qualitative agreement with experiments (Fig. 3). For each curve we compute the local slope that we fit with an exponential function of the strain \( E(\epsilon) = E_0 + (E' - E_0)(1 - \exp(-\epsilon/\epsilon_0)) \), which allows us to estimate the steady state Young modulus \( E' \).
Fig. 3b. Fig. 7c and d show that the resulting Young modulus $E^*$ increases with the number of cross-linkers $N_{\text{link}}$ and also depends on their stiffness $k_{\text{link}}$. The results reported in Fig. 7c correspond to a set of 750 fibrils, while those in Fig. 7d correspond to 1500 fibrils. Finally, we investigate how the elastic energy is distributed in the system as it is loaded. In Fig. 8a and b, we report the distribution of elastic energies $E_{\text{el}}$ of fibrils (Eq. (1)) and cross-linkers (Eq. (2)). Both distributions decay exponentially with a decay constant that simply scales with the loading strain energy (e.g. $E_{\text{el}} \propto \epsilon^2$) as illustrated in (Fig. 5). Cross-linked fibril model: (a) Schematic of the cross-linking between two fibrils A and B. The four cross-linking springs are depicted in red. (b) The initial configuration of the simulated collagen network. A configuration in the (c) non-linear and (d) linear elastic regimes. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this paper.)

Table 1 – Parameters used in the numerical simulations in SI units. Notice that the parameter ranges (e.g. in $k_l$) represent the range that has been explored in different simulations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>SI value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibril segment length</td>
<td>$a$</td>
<td>150 nm</td>
</tr>
<tr>
<td>Fibril length</td>
<td>$50a$</td>
<td>7.5 μm</td>
</tr>
<tr>
<td>Fibril diameter</td>
<td>$d$</td>
<td>75 nm</td>
</tr>
<tr>
<td>Young’s modulus</td>
<td>$E$</td>
<td>10 GPa</td>
</tr>
<tr>
<td>Bending stiffness</td>
<td>$K$</td>
<td>$3.1 \times 10^{-11}$ Nm</td>
</tr>
<tr>
<td>Stretching stiffness</td>
<td>$k_l$</td>
<td>$294.5$ N/m</td>
</tr>
<tr>
<td>Link stiffness</td>
<td>$k_l$</td>
<td>$13-330$ N/m</td>
</tr>
<tr>
<td>Link distance</td>
<td>$b$</td>
<td>150-450 nm</td>
</tr>
<tr>
<td>Link probability</td>
<td>$p$</td>
<td>0.1-1.0</td>
</tr>
<tr>
<td>Volume fraction</td>
<td>$\rho$</td>
<td>0.03-0.06</td>
</tr>
<tr>
<td>Number of fibrils</td>
<td>$N$</td>
<td>750-12 000</td>
</tr>
<tr>
<td>Repulsion energy</td>
<td>$c$</td>
<td>$1.65 \times 10^{-13}$ J</td>
</tr>
<tr>
<td>Repulsion radius</td>
<td>$\sigma$</td>
<td>75 nm</td>
</tr>
<tr>
<td>Damping parameter</td>
<td>$\gamma$</td>
<td>$2 \times 10^{-14}$ kg/s</td>
</tr>
<tr>
<td>Fibril segment mass</td>
<td>$M$</td>
<td>$10^{-18}$ kg</td>
</tr>
</tbody>
</table>

Fig. 6 – Fibrils reorient during deformation: the distribution of fibril angles $\theta$ as a function of strain for $N=12$ 000 fibrils. The data indicate that the angles between the loading axis and the fibrils decrease with strain.

(Compare Fig. 3b with Fig. 7c). This suggests that geometrical effects due to fibril arrangement and reorientation are not enough to fully account for the experimentally observed stiffening. To overcome this problem, we explicitly include non-linear effects in the cross-linker elasticity following Depalle et al. (2015).
Fig. 9a shows the stress–strain curves obtained for two values of the linear cross-linker spring stiffnesses (i.e. $k_l = 13.3 \text{ N/m}$ and $k_l = 1330 \text{ N/m}$). The curves are then compared with simulations with non-linear cross-linker springs which interpolate between the two linear spring stiffness values. The resulting stress–strain curves display slopes that interpolate between the curves obtained with linear cross-linker springs. The local Young moduli, shown in Fig. 9b, now display a more pronounced non-linearity that is closer to experimental results.

5. Conclusions

In this paper, we investigate experimentally and computationally the mechanical properties of collagen networks. We focus on echinoderm-derived collagen networks, an alternative to the most widely used collagen of mammalian origin, and show that their mechanical properties are very similar to the latter. We notice that for the three types of collagen networks the resulting mechanical properties are similar, with Young moduli in the 100–500 MPa range and tensile strength in the 20–50 MPa range, values that are remarkably close to what is measured in the human skin (Gilchrist et al., 2012). This feature is possibly relevant in order to use marine collagen in biomedical applications (Di Benedetto et al., 2014; Sugni et al., 2014). Slight quantitative differences exist for starfish collagen that appears to be stronger and stiffer than the other two. This could be attributed to different cross-linking properties due to differences in the sample preparation and extraction. Furthermore, as in other fiber networks studied in the past (Vader et al., 2009; Lindström et al., 2010, 2013; Münster et al., 2013; Licup et al., 2015), our samples also display non-linear stiffening and sample-to-sample strength fluctuations typical of systems with a heterogeneous microstructure (Alava et al., 2006).

To clarify the microscopic origin of the experimentally observed behavior, we develop a computational model for the deformation of cross-linked elastic fibril networks. The model allows us to reproduce some key features of the experimental results such as the non-linear stiffening behavior. If we use linear-elastic cross-linkers, we find non-linear stress–strain curves induced by a reorientation of the fibrils. The resulting non-linear strengthening is, however, weaker than the one measured experimentally suggesting that other mechanisms are playing a role. When we include non-linear elastic cross-linkers in the model, as suggested by Depalle et al. (2015), we obtain a more pronounced stiffening. Additional sources of non-linearity may come from non-linear elastic deformation of the fibrils themselves (Buehler, 2006) and from internal stresses due to sample preparation (Lieleg et al., 2011). Furthermore, our model highlights the crucial role played by the amount of cross-linking in determining the mechanical response of the
sample. This suggests an interpretation from the experimentally measured sample-to-sample fluctuations in the stress-strain curves displaying large variations (around 50%) in the Young modulus. We have performed simulations of the model using the same parameters but different initial arrangements for the fibrils. When we do this we find much smaller variations of the resulting Young modulus (around 5%). A possible explanation is that the experimental variations reflect internal fluctuations in the cross-linking probability or in their stiffness, quantities that we have held constant in the simulations but have shown (Fig. 7) to have a crucial influence on the resulting Young modulus.

Our modeling strategy allows us to obtain an agreement with experimental results and suggests mechanisms for the observed non-linear elasticity. The current version of the computational model is presently not able to simulate fracture, since simply breaking overstretched cross-linkers or fibrils leads to discontinuities that are difficult to deal within the present framework. A possible solution would involve quasi-static approaches where the network deformation is obtained by energy minimization as for other models for fracture (Alava et al., 2006). These types of models pave the way for a computationally based design of bio-inspired materials where the mechanical properties can first be explored in silico to guide the production of materials with desired mechanical properties.

Fig. 8 – Elastic energy distributions: The distribution of elastic energies for (a) fibrils and (b) cross-linkers as a function of strain. (c) The distributions in (a) and (b) can be simply rescaled in terms of $\varepsilon^2$. (d) The fraction of elastic energy carried by fibrils increases as a function of strain.

Fig. 9 – Role of the non-linear elasticity of cross-linkers: (a) Simulated stress-strain curves for a model with non-linear cross-linker springs are compared with the ones obtained with linear cross-linker springs. (b) The corresponding strain dependent Young moduli.
Authors contributions

L.G., L.L., M.S. prepared samples. C.F., M.S. performed SEM experiments. A.M., M.O. performed tensile tests. M.O. analyzed data. Z.B., C.D.B., S.Z. designed the model. Z.B. wrote the code. M.O. performed numerical simulations. C.A.M.L.P., S.Z. wrote the manuscript. C.A.M.L.P., M.J.A., S.Z. designed and coordinated the project.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jmbbm.2016.07.035.

References


