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BIOMIMETIC MATRICES FOR HISTOLOGICAL PROCESSING

MATRICI BIOMIMETICE PENTRU PROCESARE HISTOLOGICĂ

Alina Elena ȘTEFAN[1),3)], Daniela GOLOGAN[2),3)], S. MUȘAT[3),4),*)], Raluca STAN[2)], Andreea Cristina SANDA[3)], Manuella MILITARU[1)]

ABSTRACT | REZUMAT

In human or veterinary histological diagnosis there is an urgent need to reduce consumable costs by merging biopsies from the same patients to facilitate high-accuracy diagnoses. Our paper aims to examine and demonstrate that BxFrame™ is a medical device that can be used as a support matrix for the incorporation of several biopsies. The "ideal" material must be suited for all preparative techniques employed in routine clinical diagnosis, starting from fresh or fixed biopsies, eliminating the possibility of fragmentation, maintaining orientation, and reducing the need of tissue manipulation. The experiments were based on rheological testing of several materials of different compositions following decalcification with routinely employed methods, as well as by assessing the quality of the resultant histological preparations by means of different dehydration processes (routinely used nowadays in histology laboratories) with different working times. The results obtained after testing were marked with the scores "Very good", "Good", "Sufficient" and "Insufficient".

Keywords: tissue decalcification, biomimetic materials, histology, multiplexing, tissue microarray (TMA)

În diagnosticul histologic uman sau veterinar există o necesitate urgentă de reducere a costurilor de consumabile prin comasarea biopsiilor provenite de la aceiași pacienți, care să faciliteze diagnostice de acuratețe ridicată. Lucrarea noastră și-a propus să urmărească și să demonstreze că BxFrameTM este un dispozitiv medical care poate fi utilizat ca matrice suport pentru încorporarea mai multor biopsii. Materialul "ideal" ar trebui să fie adecvat pentru toate tehnicile preparative folosite în diagnosticul clinic de rutină, începând de la biopsiile crude sau fixate, eliminând posibilitatea fragmentării acestora, menținând orientarea și reducând necesitatea manipulării țesuturilor. Experimentele s-au bazat pe testarea mai multor materiale de compoziții diferite în metodele uzuale de decalcifiere prin efectuarea de măsurători reologice, precum și prin evaluarea preparatelor histologice rezultate prin diferite procese de deshidratare (utilizate în prezent în laboratoarele histologice) cu timpi de lucru diferiți. Rezultatele obținute în urma testelor au fost notate cu calificativele "Foarte bine", "Bine", "Suficient" și "Insuficient".

Cuvinte cheie: decalcifiere tisulară, materiale biomimetice, histologie, multiplexare, tissue microarray (TMA)

Various research studies have shown that tissue matrices containing small biopsies are not always truly representative to the donor paraffin blocks they are coming from, quite often there are losses of valuable diagnostic material and/or cracks in the paraffin block. Moreover, manufacturing a good quality TMA paraffin block requires specific training and mastering precise, lengthy, and sometimes capricious protocols (11,12).

Therefore, we tried to assess the suitability of several biomimetic polymers [previously employed for manufacturing of BxChip™, (9)] for the generation of high-quality TMAs.

1) University of Agronomic Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, Bucharest, Romania
2) Politehnica University, Bucharest, Romania
3) Themis Pathology, Bucharest, Romania
4) Lumea Inc., Lehi, United States of America
*) *Corresponding author: sorin@lumea.net*

The quality of structural preservation of tissue components can be affected, by the choice of reagents, exposure times, temperature, agitation, as well as by the eventual decalcification. When all pre-analytical protocols are complete (all extractable water and eventual hard minerals are removed) the tissue is infiltrated with a support medium that provides sufficient rigidity to allow the tissue to be sectioned without damage or distortion (3). As the biomimetic material was designed to be used throughout these histological processes, alongside the human or veterinary tissue biopsies, it is imperative to make sure the support matrix can withstand the rigors of the harsh decalcification solutions as well as the myriad of dehydration protocols employed in various laboratories.

Therefore, the purpose of this study was to test 15 different recipes of biomimetic material by three main methods: *i)* subjecting the materials to various decal-

cification protocols to determine their resilience; *ii)* rheological measurements (penetrability) to determine their strength; *iii)* and complete histological processing (*i.e.* dehydration, paraffin embedding, microtome sectioning) using a number of conventional protocols as well as histochemical staining of the resulting slides (to check if these materials to not interfere in histodiagnostic).

MATERIALS AND METHODS

Decalcification solutions

Themis Pathology provided for this study a variety of materials (15) based on a biomimetic gel with tunable properties. It is the basis for the manufacture of the BxChip™ medical device with utility in the collection and processing of cylindrical (tru-cut) biopsies. It is commercialized in different gauges to cover the various types of biopsy needles/tissues (*e.g.*: prostate - 18G, kidney, liver -16G, breast - 14G, bone marrow - 12G). Since certain tissues require processing in decalcification solutions (because they might have bony tissue within them, either as a normal characteristic of the tissue or because of certain pathologies), it is imperative to assess the suitability and resilience of the matrix biomimetic material for the harsh decalcification procedures (8, 10).

Table 1

Materials composition

MATERIAL	Hydrophobic Component A, (%)	Hydrophobic Component B, (%)	Water (%)
I	100	0	0
II	0	100	0
III	70	30	0
IV	50	50	0
V	30	70	0
VI	100	0	15
VII	0	100	15
VIII	70	30	15
IX	50	50	15
X	30	70	15
XI	100	0	30
XII	0	100	30
XIII	70	30	30
XIV	50	50	30
XV	30	70	30

We received 15 experimental materials from Themis Pathology SRL with different compositions for assessment of resilience using a penetrometer (Wagner Force Test FTK 100, Wagner Instruments, Greenwich, USA) as well as their mechanical strength following routine during histological techniques (processing, embedding, and sectioning) used in diagnostic laboratories and compared the results with the properties of various types of veterinary biopsies.

The following tissues were harvested from pigs made available by Apollo slaughterhouse (Apollo, Afumați, Ilfov, Romania): spleen, liver, skin, brain (cerebellum cortex), kidneys (cortical region), heart (anterior wall of the left ventricle), lung. The 15 different test materials varied in their composition in terms of water content as well as in the particular mix of hydrophobic components (Table 1).

Hard tissue specimens, such as bone or tooth, cannot be sectioned routinely with a regular rotary microtome without prior softening (decalcification) after the fixation stage, by removing the substances that are responsible for their hardness. The decalcification process is the chemical dissolution of insoluble calcium salts with a suitable acid (organic - formic, ascorbic, citric; inorganic - hydrochloric, nitric, etc.) or a chelating agent (EDTA) (4, 7). Acid-based decalcification solutions have a recommended duration of action on tissues from 3h (55°C) to a maximum of 4-5 days for softening a 5mm cube of dense bony tissue and run the risk of decreasing severely stainability downstream. In the case of EDTA-based solutions (which are gentler and fully compatible with basically all topographical stains as well as with immunohistochemistry, in situ hybridization, etc.), the time required can reach several weeks (1, 5).

Samples of the experimental materials were placed in 3 different decalcification solutions (20/1 ratio. vol/wight) with slight agitation on a rocking table (20 cycles per minute) at room temperature and removed at different time intervals for rheological measurements:

a) 10% formic acid (pH=2.5);10 samples of each material (I-XV) were immersed in this solution for 5 days. Every day 2 samples were taken from each type of material.

b) 5% hydrochloric acid (pH=2.5); 10 samples of each material (I-XV) were immersed in this solution for 5 hours. Every hour 2 samples were taken from each type of material.

c) 10% EDTA (pH=7.46); 10 samples of each material (I-XV) were immersed in this solution for 14 days. Every two days 2 samples were taken from each type of material.

Rheological evaluation

Rheology is the science of measurement of deformation and/or up to penetration. Virtually all materials deform in response to an imposed stress. Physical scientists refer to forces acting on materials in terms of stresses, or force per unit area. The response is either quantified in terms of the amount of deformation, or the rate of deformation. It is known that certain polymers can produce materials with high rheological pro-

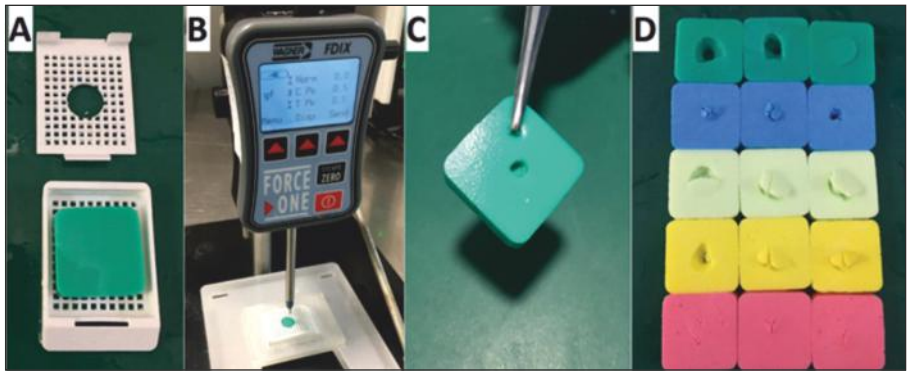

Fig. 1. Rheological measurements (penetrability): (A) Specially designed support for holding the materials; (B) Wagner Force Test FTK 100 rheological device; (C) Sample of material penetrated by the rod (diameter 3 mm) of the device; (D) All materials were subjected to the rheology test

perties such as specific strength, stiffness, and very good workability (6,13).

The Wagner Force Test FTK 100 device was used to obtain rheological values. Each sample of material was placed in the holder specially designed for an exact fixation then pierced with a 3 mm diameter rod descending with a constant speed (Fig.1). After performing all measurements, two samples of each material were kept for testing with a 9-hour dehydration protocol, then sectioned at microtome, and at the end qualifiers were assigned.

The scoring of the results obtained after the dehydration process of the materials was made by applying qualifiers as follows:

- "Very good": easy sectioning of the material, without damaging the microtome blade, perfectly stretched section on water bath;

- "Good": relatively easy sectioning of the material, without damaging the microtome blade, the section on water has folds or increases its size;

- "Sufficient": the sectioning of the paraffin block is performed with difficulty, the microtome blade can be scratched due to the hardness of the material, the section on the water bath disintegrates or has folds;

- "Insufficient": the paraffin block cannot be sectioned.

Dehydration protocols

To observe the shrinking following dehydration of the studied materials, they were subjected to various dehydration protocols. Five dehydration protocols with different durations were chosen and are commonly used in diagnostic laboratories (Table 2). All protocols were performed at 37°C (graded ethanols and transition solvents) with alternating positive pressure/vacuum while paraffin embedding was performed at 60°C. The automated processor used was Sakura VIP 2000 (Miles Scientific, Newark, Delaware, USA). Also, for these five dehydration protocols, qualifiers were given for each material analyzed after microtome sectioning.

Table 2

Protocols used in the dehydration process

SOLVENTS	DEHYDRATION PROTOCOLS				
	Protocol 1, (4h30min)	Protocol 2, (5h50min)	Protocol 3, (7h)	Protocol 4, (9h)	Protocol 5, (13h)
	Minutes/step	Minutes/step	Minutes/step	Minutes/step	Minutes/step
Neutral buffered formalin 10%	15	30	30	30	60
70% Ethanol	20	30	40	50	60
80% Ethanol	25	30	40	50	60
95% Ethanol	25	30	40	50	60
95% Ethanol	20	25	40	50	60
100% Ethanol	25	30	40	50	60
100% Ethanol	20	25	40	40	60
Xylene	25	30	30	50	60
xylene	20	30	30	50	60
Paraffin	10	15	15	30	60
Paraffin	10	15	15	30	60
Paraffin	25	30	30	30	60
Paraffin	25	30	30	30	60

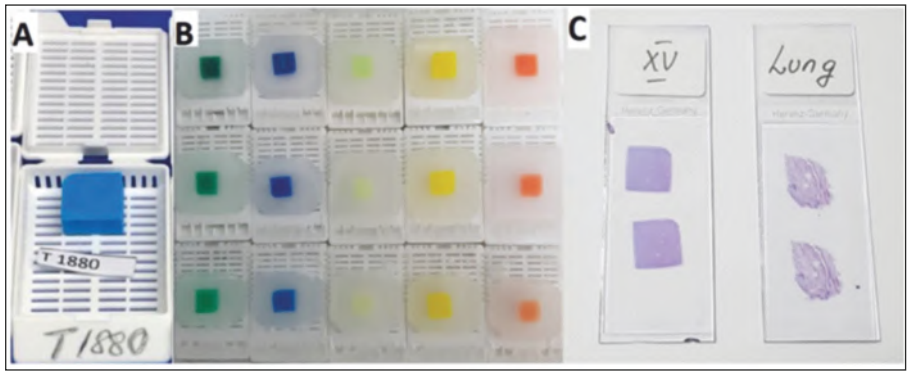

Fig. 2. Histological process:
(A) Material XII in histological cassette;
(B) Paraffin blocks for all 15 materials;
(C) Hematoxylin-Eosin histological slides (material XV and lung)

RESULTS AND DISCUSSION

Decalcification solutions

Material samples immersed in 10% formic acid and EDTA solutions (15 samples for the 15 types of materials) processed adequately after the 9-hour dehydration protocol. Thus, the scores after sectioning the materials at the microtome were "Very good" for 87% of the samples and "Good" for the remaining 13%. In the case of samples immersed in 5% hydrochloric acid solution (15 samples) they sectioned even easier, and the scores were 93% "Very good" and 7% "Good".

Rheology

The basal rheological values (penetration force) for test samples, varied between 500 and 1400 g/cm^2. The initial consistency of the materials varied from rubbery – lower values around 600 g/cm^2, to brittle consistency – higher values around 1300 g/cm^2. Interestingly, regardless of the decalcification solution in which they were immersed, all test samples maintained these values throughout the test, without any change in consistency.

Dehydration protocols results

It is widely accepted that tissue processing protocols have to be optimized/chosen depending on the type of tissues and also according to the thickness of the sample (small gastrointestinal biopsies have protocols of about 3 hours, biopsies of maximum 5mm can have protocols between 4 and 9 hours, while thicker specimens (up to 1-2 cm) and brain biopsies or degraded tissues - autopsies - can have protocols up to 24-48 h). After performing all five dehydration protocols, the selected materials and biopsies were included in paraffin, then sectioned at 5 µm with a Leica 2235 rotary microtome (Fig. 2). All materials and biopsies subjected to the five protocols were measured for length, width, and height (thickness) before and after each processing, resulting in variable shrinking depending on the type of material/tissue. For all 15 materials analysed the average shrinking for all five dehydration protocols was approx. 30%, and that of biopsies (spleen, liver, skin, brain, kidney, heart, lung) was around 20%. It is thus obvious that the biomimetic material, which has a supporting role, will be able to hold in place the biopsies during the entire dehydration process, it will tighten evenly around them and the assembly matrix/tissue biopsies will behave like a single entity at the paraffin embedding step. The biopsies encased in a biomimetic matrix seemed to section much easier than a composite paraffin block containing multiple biopsies but without a supporting matrix.

In the case of the dehydration **Protocol 1**, the scores granted to the studied materials during microtomy are mostly "Insufficient" (Table 3). This protocol is too short to obtain paraffin blocks with good sectionability. However, the observed shrinking of materials was surprisingly uniform, except for materials II and VII which have reduced their size by almost 50%. On the other hand, after protocol 1, the skin samples decreased in size by only 10%.

Table 3

Scores offered for each dehydration protocol

SCORE	Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5
	n = 30	n = 30	n = 30	n = 30	n = 30
Very good	-	27%	40%	60%	53%
Good	6%	33%	40%	40%	27%
Sufficient	7%	13%	20%	-	20%
Insufficient	87%	27%	-	-	-

n = number of analyzed materials

Dehydration **Protocol 2** shows the beginning of a differentiation in terms of positive outcome (higher score) between the 15 different materials tested, correlated with hydrophobic component A, hydrophobic component B and water composition in each material (best for V, X, XIV, XV; worst for I, VI, XI, XIII). Following **Protocol 3**, the materials with a preponderant or integral hydrophobic component B had the scores "Very good" and exhibited better sectionability than the spleen, heart, and lung tissues. **Protocol 4** proved to be uniformly associated with the best scores ("Very good" and "Good" qualifiers) for all materials tested as well as for the 7 tissues employed.Not surprising, it is the most widely used in diagnostic laboratories. As reported in the literature, the duration of dehydration protocols is recommended to be 5 to 10 hours in order to obtain sectionable tissues and without having a negative impact on immunohistochemical assays (2). All materials were sectioned without difficulty, in only a few cases the histological sections showed folds and uneven stretching on the water bath. **Protocol 5** was associated with excessive dehydration for materials I and VI. All materials with 30% dilutions, regardless of the hydrophobic components A and B ratio, received "Very good" ratings.

Since there are clear differences between the performance of the different materials during the chosen protocols, we tried to compare them only according to the "Very good" grade. In Table 4 the protocols 2, 3, 4 and 5 were centralized, because only in their case the score "Very good" (perfect sectionability) could be granted for any of the processed materials. As such, protocol 1 was eliminated from this comparison. The only materials with "Very Good" scores (+) for all 4 protocols were V and XV formulations (both have in their composition 70% hydrophobic phase B). Coming close were materials VII, X and XII, by achieving good results in 3 out of 4 protocols. This may indicate their potential suitability for some types of biopsies and/or uniquely stringent dehydration protocols.

CONCLUSIONS

All biomimetic materials in this study retained surprisingly well their resilience and strength during decalcification regardless of the chosen protocols and were not severely affected in terms of sectionability. The formulations containing hydrophobic phase B in proportion of 70%, with or without the addition of water seem to be the most versatile achieving good scores after the largest range of processing protocols.

Table 4

Dehydration protocols comparison

Material	Protocol 2	Protocol 3	Protocol 4	Protocol 5	Total
I		-	-	-	0/4
II	-	-	+	+	2/4
III	-	-	-	-	0/4
IV	-	-	+	+	2/4
V	+	+	+	+	4/4
VI	-	-	-	-	0/4
VII	-	+	+	+	3/4
VIII	-	+	-	-	1/4
IX	-	-	+	-	1/4
X	+	+	+	-	3/4
XI	-	-	-	-	0/4
XII	-	+	+	+	3/4
XIII	-	-	-	+	1/4
XIV	+	-	+	+	2/4
XV	+	+	+	+	4/4

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