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Tissue-engineered medical products — Quantification of sulfated glycosaminoglycans (sGAG) for evaluation of chondrogenesis

Produits médicaux issus de l'ingénierie tissulaire — Quantification des glycosaminoglycanes sulfatés (sGAG) pour l'évaluation de la chondrogenèse

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Foreword

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This document was prepared by Technical Committee ISO/TC 150, *Implants for surgery,* Subcommittee SC 7, *Tissue-engineered medical products.*

Tissue-engineered medical products — Quantification of sulfated glycosaminoglycans (sGAG) for evaluation of chondrogenesis

1 Scope

This document specifies test methods for the quantification of sulfated glycosaminoglycans (sGAG), one of the major extracellular matrix components in articular, meniscal, and elastic cartilages, and tissue-engineered cartilage constructs.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purpose of this document, the following definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
tissue-engineered cartilage
cultured construct obtained by combining living cells such as chondrocytes, or stem cells with or without scaffolds, or other types of biomaterials

3.2
sulfated glycosaminoglycans
long unbranched chains of repeating disacchararide units, which are sulfated at variable positions

EXAMPLE Chondroitin sulfate, keratan sulfate, dermatan sulfate.

3.3
test sample
piece of *tissue-engineered cartilage* (3.1) construct

3.4
parallel test sample
one of three samples divided from a *test sample* (3.3)

Note 1 to entry: The protocol provides a sGAG value from each corresponding parallel test sample.

3.5
cysteine
non-essential and sulfur containing amino acid, used for preparing digestion solutions

3.6
papain
cysteine protease enzyme, used for digesting extracellular matrices of diverse tissues

[SOURCE: Enzyme Commission (EC) Number 3.4.22.2]

3.7
1,9-dimethylmethylene blue
dye that specifically binds to a range of sulfated glyocosaminoglycans (3.2), whose absorbance after binding correlates linearly with the amount of bound sGAG

3.8
chondroitin 6-sulfate
one of the sulfated glycosaminoglycans (sGAG), an important structural component of cartilage and connective tissues

4 General and principle

The quantification of sulfated glycosaminoglycans (sGAG) is ranked as one of the first steps in evaluating chondrogenesis of tissue-engineered constructs, since they are one of the primary extracellular matrix consituents of native cartilage.

sGAG can be quantified by digesting test samples with enzymes to release sGAG into solution, incubating with a sGAG-binding dye, measuring the absorbance, and normalizing to the weight (wet or dry) or DNA content of the test sample.

This document applies to tissue-engineered cartilage constructs even if the amount of sGAG content might be low and offers a dynamic range of 3 µg/ml to 100 µg/ml using a standard curve.

Binding of 1,9-dimethylmethylene blue (DMMB) dye is widely-accepted for quantifying sGAG[21][22][29], and therefore was selected for this document.

The procedure to quantify sGAG in constructs comprises the following steps:

1) Pre-treatment;
2) Extraction of sGAG;
3) Measurement of sGAG content by the DMMB assay;
4) Normalization of sGAG content.

In order to realize the accurate quantification, careful attention should be paid to critical steps, such as measuring of weights and volumes, pipette manipulations, and drawing of standard curves. During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled water or water of equivalent purity (See Annex B). An example of a test report format for sGAG measurement is shown in Annex E.

5 Apparatus

5.1 Clean bench.

5.2 Sterilizer, e.g. autoclave.

5.3 Phase contrast microscope.

5.4 Cell culture incubator, capable of maintaining a humidified atmosphere containing 5 % CO_2 at 37 °C.

5.5 Cell counting apparatus, e.g. hematocytometer.

NOTE If applicable, refer to ISO 20391-1.

5.6 Centrifuge.

5.7 Scales.

5.8 Heating apparatus, capable of maintaining 60 °C. An incubator is preferable so that solutions in tubes can be surrounded with the temperature.

5.9 Spectrophotometer or microtitre plate reader.

5.10 Sterile cutting knife.

5.11 Sterile tubes.

5.12 Sterile tissue culture dish.

6 Preparation of parallel test samples from test samples

6.1 General

Tissue-engineered cartilage and parallel test samples should be assumed to be potentially infectious, and corresponding precautions should be taken according to ISO 13022, where the term “medical products containing viable human cells” should be read as “tissue-engineered cartilage”.

6.2 Preparation of parallel test samples

Tissue-engineered cartilage and parallel test samples shall be handled with care to prevent contamination. Tools used for cutting tissue-engineered cartilage shall be sterile to prevent contamination.

a) Prepare phosphate buffered saline (PBS) solution (NaCl 8,00 g/l, KCl 0,20 g/l, Na_2HPO_4 1,44 g/l, KH_2PO_4 0,24 g/l, pH 7,4) containing 5 mM cysteine-HCl and 5 mM EDTA-2Na; adjust pH to 6,0 with HCl; sterilize the solution by filtration through a 0,2 µm filter; store the reagent at 4 °C.

b) Cut a full thickness piece of test sample from the tissue-engineered cartilage construct in a bio-safety cabinet.

c) Divide the test sample into three fragments in a morphologically equal manner, measure their wet weights, and transfer the parallel test samples directly into PBS solution.

NOTE 1 The minimum measurable sGAG content in parallel test sample is 0,3 µg (cuvette assay) or 0,06 µg (microtitre plate assay).

NOTE 2 Keep liquid free when weighing the sample.

7 Procedure

7.1 General

Sulfated glycosaminoglycans in tissue-engineered cartilage are quantified by the following four steps: pre-treatment, extraction of sGAG, measurement of sGAG content, and normalization of sGAG content. A flowchart of the overall procedure is shown in Annex A.

7.2 Procedure for pre-treatment of parallel test samples

7.2.1 Procedure for dissection

a) Pick up each parallel test sample from the PBS solution.

b) Using an appropriate blade, mince the parallel test samples into pieces small enough (approximately 1 mm) to be extracted in digestion solutions.

NOTE An alternative procedure (pulverization or lyophilisation; see Annex C) might be used as an option.

c) Put the dissected parallel test samples into tared 1,5 ml tubes, centrifuge and remove the liquid.

d) Calculate the weight of each dissected parallel test sample by subtracting the weights of the empty tubes from the weights of the tubes containing dissected parallel test samples. Prevent parallel test samples from drying in order to weigh precisely.

e) Proceed to the extraction steps.

7.3 Procedure for extraction of sGAG from pre-treated parallel test samples

7.3.1 General

The dissected parallel test samples should be dissociated for extraction of sGAG. For that purpose, papain digestion is recommended because it is an adequate method for digesting diverse cartilage matrices. Depending on the type of scaffold used if any, an alternative procedure (Collagenase digestion, Guanidine salt extraction, or guanidine extraction/pepsin digestion/elastase digestion; see Annex D) might be used as an option. The collagenase digestion might be adopted in cases where scaffolds are composed of collagen gel. Guanidine salt extraction might be adopted in cases that those enzymatic procedures cannot be used. The guanidine extraction/pepsin digestion/elastase digestion is applicable only when neither enzymatic extraction nor guanidine salt extraction is succeeded.

7.3.2 Procedure for papain digestion

a) Prepare extraction solution [prepare PBS solution containing papain (lyophilized powder, comparative activity value: >10 units/mg protein, 125 µg/ml) just before use].

b) Set a heating apparatus to 60 °C before digestion so that the temperature of the solution reaches 60 °C quickly.

c) Digest pre-treated parallel test samples with the papain solution, so that the volume of the solution is 9 times larger than that of the sample, at 60 °C for 6 h or overnight (the volume of each test sample is calculated based on the density being 1 g/ml). An appropriate apparatus such as a shaker (20 r/min to 60 r/min) or a rotator (2 r/min to 10 r/min) shall be used to fully immerse the parallel test samples.

d) Centrifuge the parallel test samples at 5 000 g for 3 min, and transfer each supernatant to a tube of the same type with digestion step.

7.4 Procedure for measurement of sGAG content of parallel test samples by DMMB assay

7.4.1 Principle

sGAG content is measured by using DMMB, a dye that binds to sGAG and which is the most commonly-used reagent for this purpose. The sGAG concentration is determined using a chondroitin 6-sulfate standard curve. The digested parallel test samples might also serve as solutions for measuring DNA amounts, which could be used as an alternative to the wet weights of the samples for normalization.

The procedure for measurement of sGAG content is composed of the following two steps: preparation of 1,9-dimethylmethylene blue (DMMB) reagent and the DMMB assay.

7.4.2 Procedure for preparation of 1,9-dimethylmethylene blue (DMMB) solution

a) Add 16 mg DMMB to 5 ml ethanol, and stir the reagent in a clean dry beaker wrapped with aluminium foil.

b) Add 3,04 g of glycine, 2,37 g of NaCl and 95 ml of 0,1 N HCl.

c) Add 800 ml distilled water and adjust pH to 3,0 with 0,1 N HCl.

d) Bring the volume to 1 000 ml with distilled water.

e) Stir the reagent with a magnetic bar at room temperature for 2 h to 16 h. Protect from light.

f) Filter the reagent with adequate filter paper (e.g. grade 4 qualitative filter paper with pore size of 20 µm to 25 µm) for removing the debris.

g) Store the reagent in a brown bottle at room temperature, and prepare the reagent freshly every three months.

7.4.3 Procedure for DMMB assay

a) Prepare a 0,5 mg/ml chondroitin 6-sulfate solution by dissolving 10,0 mg chondroitin 6-sulfate (shark, purity > 95 %) with 20 ml extraction solution or modified extraction solution. Mix for several minutes to fully dissolve.

The extraction solution should be selected according to the procedure for extraction of sGAG in 7.3. The extraction solution is to be papain solution when papain digestion is selected.

The solution for making the standard curve should be as similar as possible to the test sample extraction solution. This will help control for potential interference with DMMB-sGAG binding by molecules contained in the test sample extraction solution, allowing more precise quantification.

b) Prepare a series of chondroitin 6-sulfate solutions in the extraction solution so that the chondroitin 6-sulfate concentrations are 0 µg/ml, 3,125 µg/ml, 6,25 µg/ml, 12,5 µg/ml, 25 µg/ml, 50 µg/ml, and 100 µg/ml.

c) Switch on a spectrophotometer or a microtitre plate reader.

d) Add 0,1 ml of each extract or standard solution to 1 ml DMMB reagent in a cuvette (cuvette assay), or mix 0,02 ml of each extract or standard solution with 0,2 ml DMMB reagent in a 96-well microtitre plate (microtitre plate assay) and mix with a disposable transfer pipette.

Samples should be diluted so that the sGAG concentrations in the samples fall within the linear range of the standard curve.

e) Measure immediately (within 3 min after mixing) the absorbance of the mixtures at 530 (range 525 to 535) nm with a spectrophotometer (cuvette assay) or a microtitre plate reader (microtitre plate assay). Waiting longer than 3 min can cause condensation and prevent the mixtures from being measured accurately.

The assay needs to be done in triplicate (three parallel test samples) to calculate SD in order to quantify the variances in total procedure steps on a single test sample.

It should be confirmed that materials extracted from the scaffold do not interfere with the assay.

It is well recognized that hyaluronan or undersulfated GAGs could form complexes with DMMB under certain conditions, even if they do not have sulfate groups. In the case that precise measurement of sGAG content is required, the protocol should be modified, referring to the reference[19].

7.5 Normalization of sGAG contents

The precision of the normalized sGAG content measurement strongly depends on the reliability of the standard curve. Linear regression analysis may be used to evaluate the standard curve.

Dilute parallel test samples with PBS solution depending on the estimated sGAG content in the parallel test samples.

a) Convert the absorbance to sGAG concentration (µg/ml). As noted previously, take care that the absorbance values for unknowns fall within the linear range of the standard curve, and dilute samples if needed to ensure this.

b) Normalize the sGAG concentrations (µg/ml) to sGAG content (µg/mg) by dividing by the wet weight of the parallel test samples.

$$\text{sGAG content (µg/mg)} = A \times B \times C/D$$

where

A is the sGAG concentration (µg/ml);

B is the dilution factor;

C is the volume of added digestion solution (ml) + the volume of the parallel test sample (ml; the volume can be calculated from the weight so that the density of parallel test sample is to be 1 g/ml.);

D is the wet weight of the parallel test sample (mg).

NOTE sGAG content is normalized by the wet weights of the parallel test samples. Note that samples can also be normalized by DNA content.

8 Test report

The test report shall contain the following items to allow an independent assessment of the results. The results shall be written as means and standard deviations based on the normalized sGAG content of three parallel test samples. An example of a test report format for sGAG measurement is described in Annex E.

a) Test sample description

The test samples are to be described so as to identify them in terms of cell source, cell seeding concentration, scaffold type, start date of culture, and culture duration.

b) Pre-treatment of test samples

The choice of pre-treatment (dissection, pulverization, or lyophilisation) and wet weight of parallel test samples is to be described.

c) Extraction of sGAG from pre-treated parallel test samples

The choice of extraction of sGAG (papain digestion, collagenase digestion, guanidine salt extraction, or guanidine extraction/pepsin digestion/elastase digestion) and the volume of solution(s) to be added is to be described.

d) DMMB assay

The format of the DMMB assay (cuvette assay or microtitre plate assay) and the correlation coefficient (r value) for the standard curve are also to be described.

e) Normalization of sGAG contents

The measured values of each component (A, B, C, and D) are to be described according to the formula as shown in 7.5.

9 Evaluation

The measured values should be evaluated for chondrogenesis of tissue-engineered constructs, taking into consideration that thresholds of the values are variable.