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Group 2
Date of classes: 9, 10, 11th of April 8:30 - 11:30

In vitro testing of biomaterials – Laboratory classes

Report

Aim of the classes: The main objective of the laboratory classes was to familiarize students with fundamental in vitro techniques used in biological evaluation of biomaterials. The classes focused on the handling and maintenance of cell cultures under sterile conditions, preparation of biomaterial extracts, and assessment of cytotoxicity using both metabolic and staining assays. Students gained hands-on experience in key procedures such as cell seeding, extract dilution, viability assessment, and spectrofluorometric analysis, enabling them to evaluate the biocompatibility of materials intended for biomedical applications.

Description of tested materials

For cytotoxicity testing, 4 materials were provided:

- **25C** - A sample of wire made from ZnMg0.004 alloy. The sample was initially extruded to the diameter of 1.8 mm, followed by a drawing process at the temperature of 25°C.
  Diameter of the wire: 0.8 mm.
  Surface area of the sample: 3 cm^2.
- **250C** - A sample of wire made from ZnMg0.004 alloy. The sample was initially extruded to the diameter of 1.8 mm, followed by a drawing process at the temperature of 250°C.
  Diameter of the wire: 0.8 mm.
  Surface area of the sample: 3 cm^2.
- **TWISTED** - A sample of wire made from ZnMg0.004 alloy with same methodology as 250C sample subjected to subsequent twisting at the temperature of 250°C.
  Surface area of the sample: 3 cm^2.
- **Graphene** - sample of UV-curable resin-based nail polish containing 1% graphene oxide. The sample surface was flat and homogeneous.
  Sample mass: 5 g.

Summary of Laboratory Session – Day 1 – 9th April

The first laboratory session focused on the preparation and handling of cell cultures in an in vitro environment, particularly in the context of interactions with biomaterials. The practical exercises aimed to familiarize students with sterile technique, basic cell culture methods, and assay planning for biocompatibility analysis.

1. Preparation Phase

At the beginning of the session, prior to commencing laboratory work, the instructor provided an overview of fundamental techniques and procedures for operating laboratory equipment under sterile conditions, with particular emphasis on the use of the laminar flow cabinet equipped with a HEPA filter. This introduction ensured that all participants were familiar with aseptic workflow, minimizing the risk of contamination during cell culture handling. Then the laboratory classes begin with transferring passaged cells to 96-wells TCPS (which surfaces had been treated with oxygen plasma to introduce hydroxyl groups, improving hydrophilicity and cell adhesion) plates from cell culture flask. At the same time, extracts of biomaterials were prepared to evaluate the effect of the substances released from them into solution on cell viability. A corresponding amount of DMEM (Dulbecco's Modified Eagle Medium) was added to materials of a certain weight or area (described earlier).

2. Cell Detachment and Washing

To detach adherent cells, the culture medium was removed, and the cells were washed with PBS (phosphate-buffered saline). Trypsin, a proteolytic enzyme, was added to cleave adhesion proteins. The process was monitored under a microscope—once approximately 90% of cells appeared spherical, the trypsin was neutralized by adding FBS serum-containing DMEM.

3. Cell Collection and Processing

Detached cells were collected and centrifuged. All instruments and pipettes were sterilized and washed to ensure contamination-free operation.
Practical tip: gently tapping the bottle helps to detach the cells

4. Cell Viability and Counting

Cells were stained with Trypan Blue to assess viability—only dead cells absorb the dye. Automated counting (NanoEutek EVE) was used to determine the concentration of live vs. dead cells. A viability of over 80% was considered acceptable; the session achieved ~90%, indicating excellent culture health.
Calculation: 1×10^6 live cells/mL and 1×10^5 dead cells/mL.

5. Dilution for Seeding

Using cell count data, the cell suspension was diluted to the desired concentration (e.g., 1×10^5 cells/mL). Calculations ensured the correct volume was prepared for both metabolic tests and staining procedures, with multiple concentrations and repetitions included for statistical reliability.

6. Seeding Protocol

100 µL of the cell suspension was pipetted into designated wells, avoiding direct contact with the centre of the wells to maintain sterility. Plates were gently agitated to ensure uniform distribution. Edge wells were filled with PBS to prevent evaporation artifacts. The seeded plates were inspected under a microscope and transferred to an incubator (37°C, 5% CO_2).

7. Introduction to Spectrophotometric Assays

The session concluded with a discussion on optical methods used for cell analysis:

- **Absorbance assays** require transparent plates.
- **Fluorescence assays** use black plates to minimize background signal.
- **Luminescence assays** are conducted with white plates to enhance signal reflection and sensitivity.

Summary of Laboratory Session – Day 2 – 10th April

The second day of the laboratory workshop focused on evaluating cell viability after 24 hours of incubation and performing exposure experiments using serial dilutions of biomaterial extracts. This session emphasized sterile technique, proper sample preparation, and understanding visual indicators of culture health.

1. Visual Inspection of Cell Cultures

At the start of the session, cell cultures were inspected visually by assessing the colour of the culture medium:

- **Yellow medium** – indicative of dead or dying cells (acidification due to metabolic waste).
- **Orange medium** – cells are viable, however they used greater part of the nutrients from the media and metabolic waste is present in the media; indicator that medium change is required.
- **Rapid colour change** – likely contamination (bacterial or fungal).
- **Slow colour change** – normal metabolic activity.
- **Magenta hue** – potential presence of Mg^{2+} ions, indicating dead/dying cells or fungal/yeast infection.

Our cells appeared healthy, with no signs of contamination or metabolic stress.

2. Preparation of Extract Dilutions

Media and PBS were pre-warmed to physiological temperature (~37°C). Before applying the extracts to the cells, the necessary dilutions were prepared:

- **Concentrations [%]:** 10 (600 µL) - no dilution, 5 (300 µL) - diluted twice, 2.5 (150 µL) - diluted four times, and 1.25 (75 µL) - diluted 8 times
- **Medium added respectively**: 0 µL, 300 µL, 450 µL, 525 µL

Sterilization procedures included:

- 70% ethanol for surface decontamination of extract bottles, syringes, and filters.
- Filters were attached to syringes with the sterile side facing the media.
- Special care was taken not to touch the sterile side of the filter to any unsterile surfaces (e.g., inside of new falcons).
- Filtered media was transferred from non-sterile sample flasks to new, sterile falcons.

4. Cell Treatment Protocol

Cells cultured the previous day were processed as follows:

1) Medium was carefully aspirated to remove dead cells without allowing the monolayer to dry out.
- Cells were gently washed with PBS to remove remaining residues.

2) Extracts were added in increasing concentration order (from lowest to highest), minimizing cross-contamination.

3) Control Conditions

- **Negative Control:** cells washed with PBS and incubated in fresh medium under identical conditions – serves as a reference for no cytotoxicity.
  a) **Positive Control:** exposure to 1% Triton X-100 in PBS, a surfactant that disrupts cell membranes – serves as a reference for maximal cytotoxicity.

6. Additional Observations

Cells tend to accumulate in the corners of the wells, possibly due to surface tension gradients or spatial preferences during seeding. This behaviour should be considered when analysing distribution patterns.

Summary of Laboratory Session – Day 3 – 11th April

The third laboratory session focused on evaluating the cytocompatibility of biomaterial extracts using two complementary in vitro methods: a metabolic activity assay based on fluorescence (Alamar Blue) and a cell viability staining assay using Crystal Violet.

1. Metabolic Test – Alamar Blue Assay

This assay measures cell metabolic activity as an indicator of viability. The reagent (Alamar Blue) is reduced by metabolically active cells, producing a fluorescent signal proportional to viability.

Preparation of Reagent Mixture:

Each well requires **150 µL** of the reagent-medium mixture.

Total for 60 wells: 150 µL × 60 = **9 mL**, plus excess volume: **13 mL** prepared.

The working solution:

- **5% Alamar Blue** → **650 µL**
- **Complete culture medium** → **12.35 mL**

Procedure:

1) Aspirate remaining extracts from wells.
2) Wash each well with **100 µL of PBS** to remove residue.
3) Aspirate PBS.
4) Add **150 µL of Alamar Blue mixture** to each well.
5) Incubate for 2 hours at 37°C.
6) Measure fluorescence using a spectrofluorometer.

2. Staining Test – Crystal Violet Assay

This method is used to assess the number of adherent cells and their general morphology. Crystal Violet stains DNA and cellular structures after fixation.

Fixation and Staining Procedure:

1) Aspirate medium from wells.

- Add **100 µL of 2% formaldehyde** solution to each well to fix the cells.

2) Incubate for **10–15 minutes**.
3) Aspirate formaldehyde carefully.
4) Wash wells with PBS, then aspirate the PBS.

- Add **0,5% crystal violet solution in 20% methanol** to each well.

5) Incubate for **30 minutes**.

- Aspirate stain and rinse wells thoroughly with PBS or distilled water to remove excess dye. This action was performed multiple times until rinsing liquid was clear.

After staining, cells were visualized using an inverted microscope AxioVert 40 (Carl Zeiss). For each well and sample, photographs were taken, with a scale bar included for reference in image analysis.

Results

To evaluate the cytocompatibility of the tested biomaterial extracts, two complementary analytical methods were employed: the **Alamar Blue assay**, which assesses cellular metabolic activity, and the **crystal violet staining assay**, which evaluates cell adhesion and density. These assays allowed for both **quantitative** and **qualitative** analysis of cell behaviour after exposure to varying concentrations of extracts. In the **Alamar Blue test**, cell viability was quantified by measuring fluorescence intensity, which correlates with the metabolic activity of live cells. In the **Crystal Violet assay**, fixed and stained cells were analysed **visually under an inverted microscope AxioVert 40**. This dual approach enabled cross-validation of results and provided deeper insight into how the biomaterials affected cellular responses.

Metabolic Test – Alamar Blue Assay

The resazurin reduction test assessing cell viability was carried out for the control samples (positive control – medium with the addition of Triton-X to obtain conditions of high cytotoxicity and negative control – medium without any extracts to obtain conditions of no cytotoxicity) and the samples extracts at concentrations of 1.25%, 2.5%, 5% and 10%, respectively (Fig.1.).

Data are presented as an average (n=3 samples) ± SD (standard deviation). Statistical analysis was obtained using a one-way analysis of variance (one-way ANOVA) followed by the post-hoc LSD Fisher test using OriginLab software. Probability values lower than 0.05: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ were considered statistically significant.

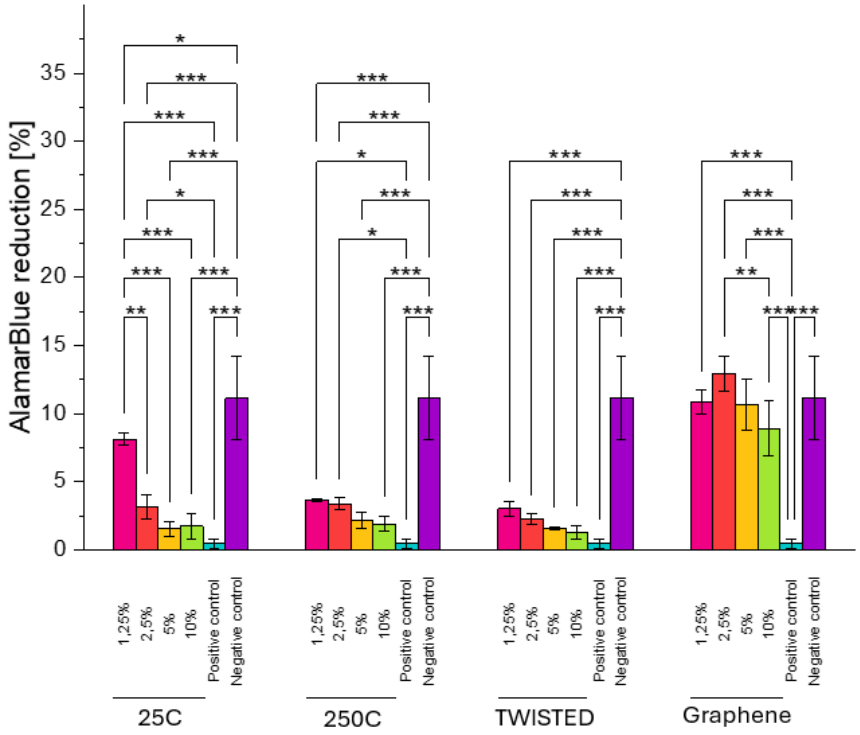

Fig.1. Viability of L929 cells in contact with 1.25%, 2.5%, 5% and 10% extracts from the samples in comparison with positive control and negative control.

An interesting trend was observed across all wire samples (25°C, 250°C, and TWISTED), whereby increasing extract concentrations correlated with a decreased efficiency of resazurin reduction. Nevertheless, the values obtained at the lowest extract concentrations remained significantly lower than those of the negative control, particularly for the 250°C and TWISTED samples. Furthermore, for extract concentrations of 2.5%, 5%, and 10%, the measured values were comparable to - or slightly exceeded - those of the positive control. This indicates impaired cell proliferation and confirms a cytotoxic effect induced by the extracts. The most plausible explanation for this phenomenon is the release of cytotoxic ions or particles into the culture medium during sample incubation, which subsequently impairs cell adhesion and proliferation.

Conversely, the sample containing graphene demonstrated an opposite trend. For nearly all tested concentrations of extracts from this sample, the results were comparable to those of the negative control. Only at the highest extract concentration (10%) there was a

marginally lower value observed. These findings suggest that extracts derived from the graphene-containing sample do not exhibit cytotoxic properties.

Staining Test – Crystal Violet Assay

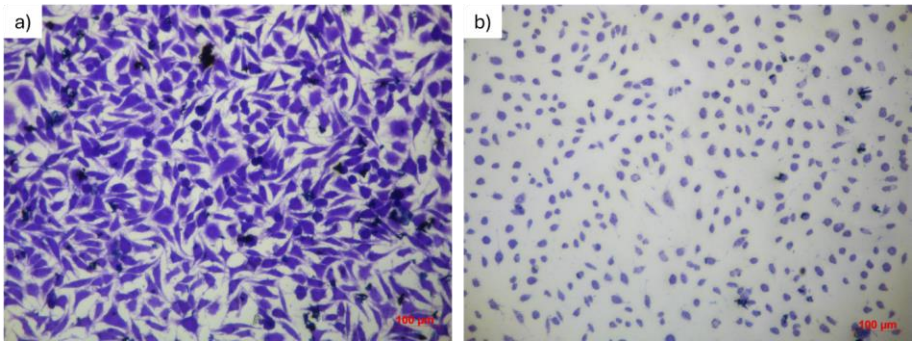

Fig.2. Reference samples: a) negative, b) positive.

The negative and positive sample provide a reference point for further observations. For the negative sample, we see purple stained cells spread and attached across the surface, whereas in image (b) we see round cells detached from the surface. It is worth noticing, that number of cells is significantly lower in case of positive control.

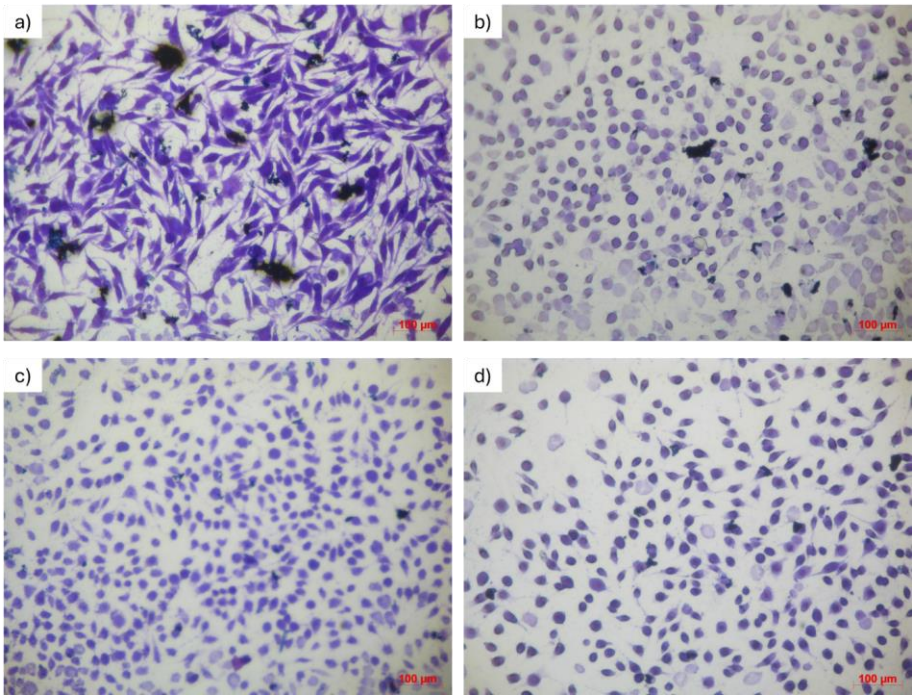

Fig. 3. Crystal violet staining for sample 25C for different concentrations of extracts: a) 1.25%, b) 2.5 %, c) 5.0%, d) 10.0%.

In the case of the 25C sample, a clear presence of adherent viable cells is observed exclusively in image (a). As the extract concentration increases, cell spreading progressively diminishes, and the cells adopt a more rounded morphology, with only minimal deviation from the morphology observed in the positive control. Nevertheless, the overall cell number in all tested samples exceeds that of the positive control and exhibits a slight decline with increasing extract concentration. These observations are coherent with the results obtained from the Alamar Blue assay.

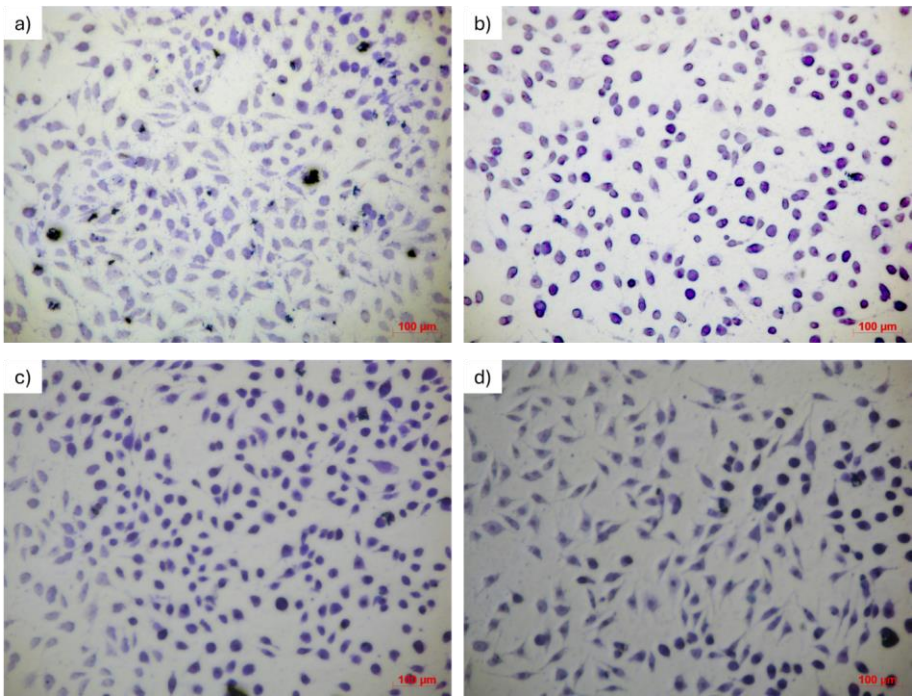

Fig. 4. Crystal violet staining for sample 250C for different concentrations of extracts: a) 1.25%, b) 2.5 %, c) 5.0%, d) 10.0%.

In the case of the 250C sample, the observed results indicate a more pronounced cytotoxic effect compared to the 25C sample. Notably, even at a high dilution (1.25% extract concentration, corresponding to an eightfold dilution), the majority of cells exhibited a rounded morphology, which is a clear indicator of poor adhesion to the substrate. This morphological change is also reflected in reduced cell viability. Microscopically, the number of adherent cells in the 250C sample appeared comparable to that observed in the positive control, which is consistent with the quantitative findings from the AlamarBlue assay.

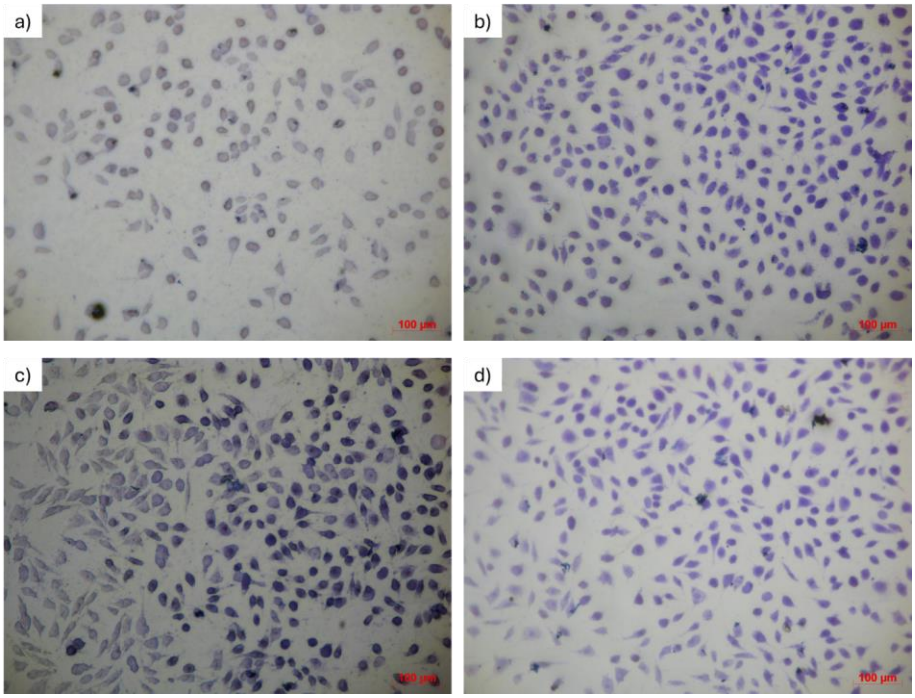

Fig. 5. Crystal violet staining for sample TWISTED for different concentrations of extracts: a) 1.25%, b) 2.5 %, c) 5.0%, d) 10.0%.

For the TWISTED sample, none of the tested extract dilutions rendered it non-cytotoxic. Notably, at the 8-fold dilution shown in image (a), the lowest number of cells is observed, and they exhibit weak staining intensity. These observations are confirmed by the results of the Alamar Blue assay.

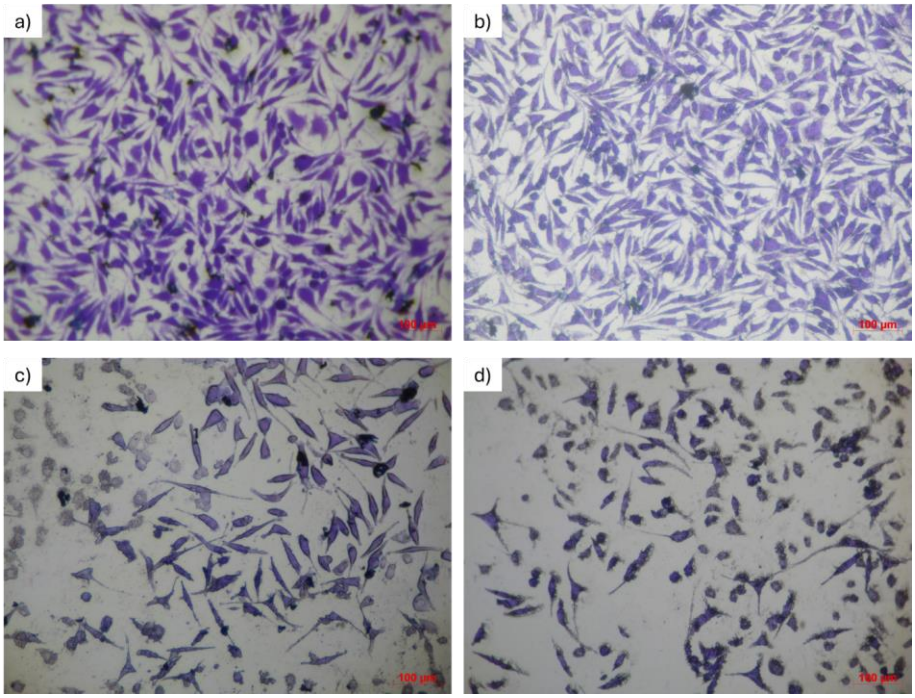

Fig. 5. Crystal violet staining for sample Graphene *for different concentrations of extracts: a) 1.25%, b) 2.5 %, c) 5.0%, d) 10.0%.*

The graphene-containing sample demonstrated the most favourable results. At 1/8 and 1/4 dilutions (corresponding to 1.25% and 2.5% concentrations, respectively), a substantial portion of the surface is covered by well-adherent cells. At higher concentrations, the number of cells is markedly reduced, and some exhibit a rounded morphology; nevertheless, isolated cells remain attached to the surface. The observations for the 1.25% and 2.5% extracts are consistent with the Alamar Blue assay, confirming the relatively low cytotoxicity of the Graphene material. However, the images obtained for the 5.0% and 10.0% concentrations do not align with the Alamar Blue results, indicating the need to repeat the test once again to reassess obtained results.

The aim of the research was to investigate the influence of ZnMg0.004 texture on its cytotoxicity. Previously studied materials have demonstrated a strong correlation between grain orientation and cytotoxicity, however results obtained for ZnMg0.004 do not show such correlation. In the course of our investigation, these samples exhibited the highest levels of cytotoxicity, surpassing the positive control to a slight extent. Cellular proliferation was markedly impaired, as demonstrated by both AlamarBlue and crystal

Z komentarzem [KW1]: Tu pojawia mi się pytanie - a dokładnie jaką zależność powinniśmy zaobserwować

Z komentarzem [KW2R1]: Tak w jednym zdaniu opisać co powinno być, ja już dodałam to wyszło w trakcie labów

violet staining assays. Furthermore, cells failed to adhere to the substrate and retained a rounded morphology The TWISTED sample exhibited cytotoxicity levels comparable to those of the 250C sample. The only indication of a potential texture-related effect was observed at extract concentration of 1.25%, where the cold-drawn sample (25C) showed reduced cytotoxicity compared to both hot-drawn samples (250C and TWISTED). Considering prior findings that clearly demonstrated a correlation between texture and cytotoxicity in other materials, further crystallographic texture and surface roughness investigation is required in order to verify whether observed results are material-related or stem from human error during pipetting or the use of separate well plate for Alamar Blue staining.

The study of the nail polish sample containing graphene oxide (GO) aimed to confirm its non-toxicity. Graphene/graphene oxide itself is a chemically passive form of carbon that generally does not react under physiological conditions of the human body. However, due to the nanoscale size of the graphene oxide flakes, there were concerns about a potential negative impact on cells. As expected, the study confirmed the absence of toxicity or the inability of GO flakes to migrate from the epoxy matrix into the extract. AlamarBlue assay proved that for almost all the concentrations there were no signs of cytotoxicity, which was also confirmed by crystal violet staining.

Summary

During the three-day laboratory course, students carried out a series of experiments designed to assess how different concentrations of biomaterial extracts impact the viability and morphology of cultured cells. The first day focused on establishing cell cultures and preparing extract dilutions. On the second day, the students applied the extracts to the cells and observed early morphological and metabolic responses. The third day involved conducting two key assays: the Alamar Blue metabolic assay, which provided quantitative data on cell viability, and the Crystal Violet staining assay, which allowed for visualization and qualitative assessment of cell adhesion and distribution. Throughout the course, students practiced essential laboratory techniques, including sterile pipetting, use of laminar flow cabinets, filtration, proper handling of reagents, and operation of spectrofluorometric equipment. The exercises emphasized the importance of experimental design, reproducibility, and the interpretation of results in the context of biomaterials testing.