Development and analysis of hybrid hydrogels for a new type of 3D *in vitro* model

Traditional drug screening methods, during the early stages, use monolayer (2D) tumor cell cultures, which lack basic features of the tumor complexity. As a result, sophisticated culture platforms were developed to mimic the tumor niche (*e.g.* micro-bioreactors) but which require expertise and complicated maintenance [1,2,3].

As an alternative, hydrogels (3D) have begun to emerge as simpler, time- and cost-saving systems. Although biomaterial-based 3D cell cultures are at first glance seem advantageous, but due to tricky handling and varying lot-to-lot qualities, they are not ideal for high-throughput screening [4]. The use of synthetic materials over natural ones are more advantageous as their qualitative and quantitative composition are precisely controlled, thus reproducibility and variability of their physical properties (e.g. flexibility, nanostructure) are not an issue.

Among the hydrogels, synthetic alkoxysilane-PEG (polyethylene glycol) based ones are promising candidates. These matrices are formed *via* "sol-gel" polymerization in aqueous medium, which allows rapid and easy handling together with control over the incorporated elements.

Nevertheless, the natural cell environment contains insoluble fibrils (e.g., collagen, fibronectin) [5]. Such structures can be mimicked by self-assembling peptides (SAP) [6]. The utilization of SAPs also allows the incorporation of biologically active peptides (e.g. adhesion properties).

The project aimed to utilize such siloxane-PEG hydrogel platform incorporated with SAPs, as a feasible drug screening system, to provide a more complex picture of cellular processes than current 2D *in vitro* studies.

1. Synthesis of PEG-based alkoxysilane hydrogels

Previous works on biocompatible 3D hydrogels used isocyanate group containing alkoxysilanes to incorporate peptides and macromolecules *via* carbamates and carbamides. [7] Such bonds are well-known to break down and not stable enough to be used as medium-term scaffolds under cell culture conditions [8]. In contrast to the carbamates/carbamides, amines are stable, hence a tosylate-S_N strategy has been developed [9].

Such alkoxysilane hydrogels were developed for chondrocyte culturing. It was questionable if such a hydrogel could be utilized as a 3D drug screening model for other cell lines (e.g., tumours, normal),as other cell lines would prefer hydrogels with different mesh-sizes, loosening the polymer network was one of the main focuses of this project.

This aspect can be achieved by using PEG with different Mw (2k-300k) as the gel forming-monomers. The high Mw variants has shown problems with handling and gave nonconclusive analytical results. In contrast, PEG Mw approx. 2000 shown no complication during the synthesis and analysis. Therefore, this compound has been selected for further studies to form bis-mono and bis-tri functionalized monomer building blocks with reactive ethoxy silane groups (bis-mono-alkoxysilane-mPEG; bis-tri-alkoxysilane derivatives-tPEG). The synthetic routes were optimized to several gram scale (*Fig 1*) and were characterised by NMR, HR-MS.

By using different molar ratio of these two monomers, the formed hydrogel's average molecular weight could be increased in a controlled manner.

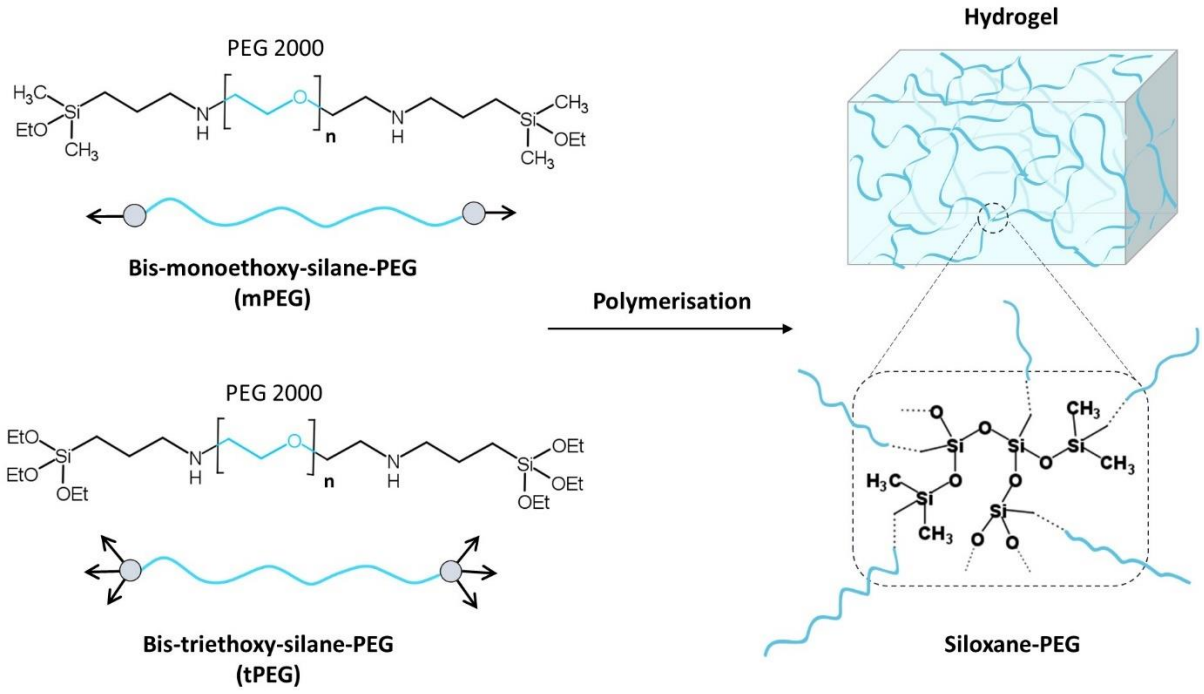

Fig 1. Hydrogels are formed using bis-mono-ethoxysilane- and bis-triethoxysilane-PEG (mPEG and tPEG)

2. Model in vitro system

To test the feasibility of the produced hydrogels, a reference system where cell survival and proliferation can be measured in the hydrogel matrices needed to be selected and optimized. Three cell lines of dermal origin was chosen as a "melanoma model". A tumor and two "quasi normal" cell line was chosen: A2058 (human melanoma), HaCaT (human keratinocytes), and HMEC-1 (human dermal microvascular endothelial cells).

2.1 In vitro protocol

A standardised hydrogel forming and cell seeding protocol was also needed. It was earlier reported, that removal of the hydrogel forming catalyst (NaF) is not a necessary, since it is well tolerated by cells (e.g., chondrocytes, fibroblasts) [7]. After several attempts, however, this has been proven untrue, and a new protocol was developed. The established one was practically not more complicated or time consuming than traditional 2D *in vitro* cytotoxic measurements, which was an important aspect. We also found that classical cell viability essay methods (MTT, Alamar Blue) produced misleading and inaccurate results, despite several reported data, therefore we used CellTiterGlo 3D assay instead [10, 11].

2.2 Viability measurements and the effect of the gel composition

A cytotoxicity assay has been chosen to compare our hydrogels with the traditional 2D system. Daunomycin (Dau), a clinically used chemotherapeutic agent, was used for testing cell survival in the produced hydrogels. After culturing cells in the hydrogels (96h), all cells were treated with Dau at their IC50 value for 24h and then cell viability was measured.

In general, cells have populated the gels, the proliferation behaviour was similar to the 2D cultures. However, atypical behaviour was found at certain gel compositions for A2058 human melanoma cells but not for the "quasi normal cells" (HMEC-1, HaCaT). At certain gel compositions (tPEG/mPEG molar ratio) spontaneous assembly of cells could be detected, which then later organized into tumor-cell spheroids (*Fig 2*).

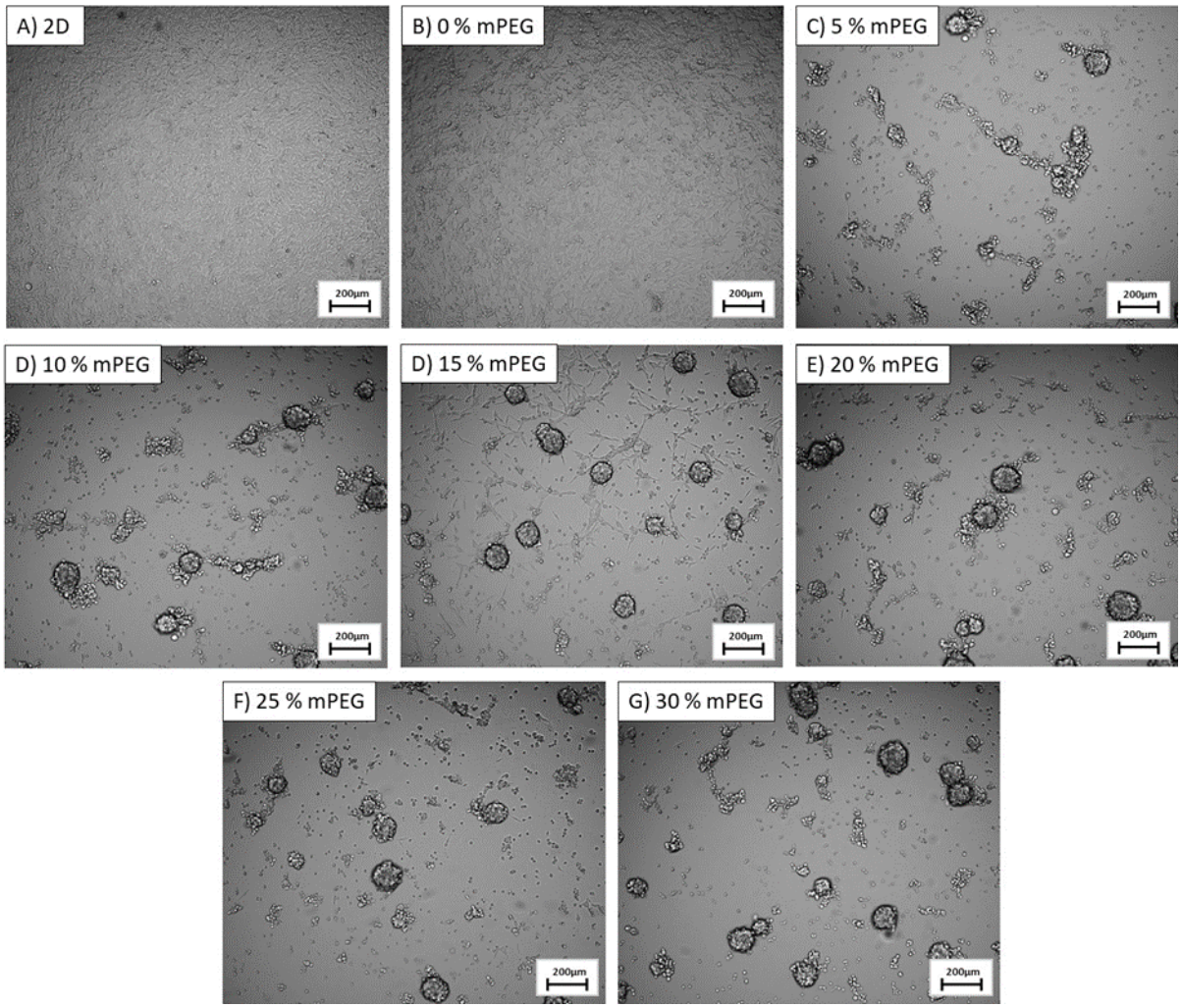

Fig 2. A2058 cells in different hydrogels 96 h after seeding. Images were captured by Celldiscoverer7 (5 × Plan-Apochromat λ/0.35 NA objective with 2 × tube lens), the scalebar is 200 µm. A) 2D control; B) 0% mPEG; C) 5% mPEG; D) 10% mPEG; E) 15% mPEG; F) 20% mPEG; G) 25% mPEG; H) 30% mPEG.

Viability measurements showed, that in certain PEG-based hydrogels A2058 melanoma cells - but not “normal” cells - increased resistance against Dau. Compared to 2D models, viability of A2058 tumour cell spheroids in hydrogels were approx. 30% higher (15 mPEG mol% hydrogels). Further microscopic studies and analysis – using an an AI-based application developed by us to help the process of large number of images- also confirmed that this enhanced resistance was correlated with the formation of cellular aggregates (100-200 μm diameter) [9].

This phenomenon is a good indication of the advantageous usage of 3D hydrogels as a complement to traditional 2D assays, as the developed gels are a better model to investigate tumor cell survival.

2.3 Analysis of microscope images

Image analysis softwares (Image J) failed to process the images recorded in hydrogel culturing conditions (e.g., poor ability to discriminate small cell aggregates and spheroids among debris). Although it was not included in the original plan of the project, it became necessary to develop a target image analysis software as well. The AI-aided program (artificial intelligence) was developed with the assistance of En-Co Software Zrt. can distinguish between debris/spots/uncommon shapes and small spheroids on microscopic images in our hydrogel system and lets to quantify the changes in the number and the dimensions/extent of spheroids after drug treatment (*Fig 3*) [9]. The main advantage of our application is that no high-throughput technologies (e.g. Z-stacking, confocal-microscopy) is needed to quantify the aggregate-formation. This kind of image analysis gives albeit approximate, but a rapid image evaluation, which is especially advantageous in screening assays.

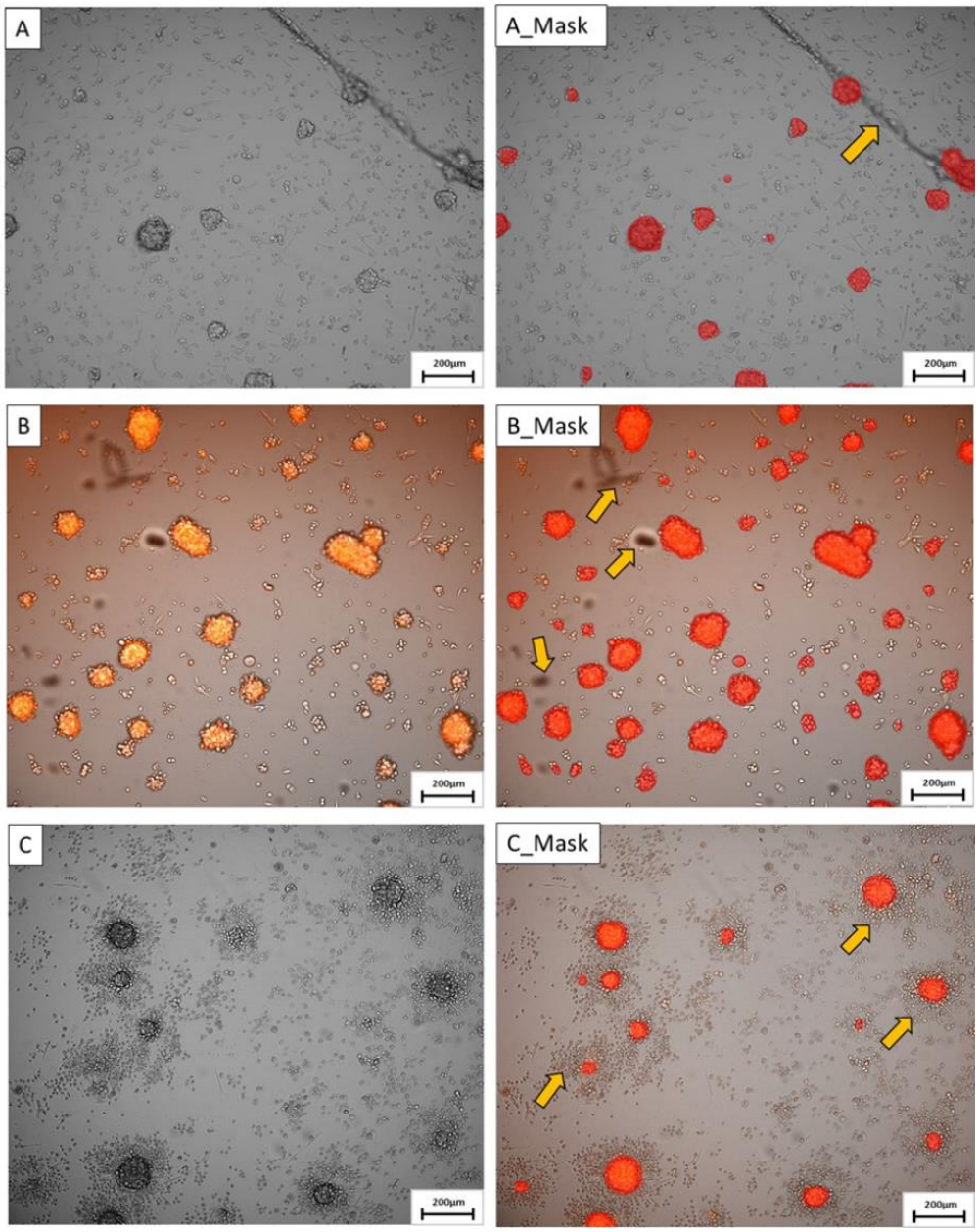

Fig 3. Example of spheroid recognition assisted by AI. Complicated images can be processed (e.g. spots, debris, etc). Spheroids partly out of picture, small aggregates and cell debris (indicated by yellow arrows) can be excluded. A)-C) Original microscopic images of control or Dau-treated A2058 cells; A_Mask)-C_Mask) AI processed pictures, accordingly. Scale bar refers to 200 µm

I reported these findings on the Hungarian Peptide Cometee's conference winning the Excellence Award (Medzihradszky Kálmán award Balatonszemes, 2021).The publication summarising our result was submitted almost one year ago, currently it is accepted for publication, and undergoing minor revisions (FEBS OpenBio). The paper can be reached on preprint server (http://dx.doi.org/10.2139/ssrn.4387672)

2.4 Modelling stent implants

The hydrogel-dependent behaviour of the tested cell lines attracted much interest from our collaborators. We are currently use the developed hydrogels to examine the re-endothelisation process of stent implants. It was found that, while the question cannot be investigated effectively with the use of 2D cell culture methods, the hydrogel system gave useful and reliable results. Several new experiments were initiated to utilise it and new grant applications are submitted (e.g., OTKA, UNKP) to further study this phenomenon.

3. Incorporation of peptide sequences into the hydrogels

Through a systematic literature search, the following peptides were selected for incorporation into the hydrogels to create more native-mimicing environment:

-integrin specific peptide for cell adhesion: cyclo[RGDfK];

- ECM protease Cathepsin-K sensitive peptide: MGPSGPW;

-ECM protease, general MMP selective sequence: VPMSMRGG;

-ECM protease plasmin sensitive sequence: EGTKKGHK;

Peptides were incorporated into the hydrogel sandwitched in-between two triethoxy-silane linkers. Because this functional group is sensitive to H_2O, a new strategy was needed to attach such sensitive group to peptides. To achieve this, -SH containing triethoxy-silane and maleimido-modified peptide derivatives were synthesised and coupled together. The synthesis proved to be reproducible, the products are well characterized and found to be stable for several month under standard conditions.

However, incorporating such peptide derivatives into hydrogels proved to be difficult. As the gels were formulated as 10% w/w%, thus this also meant an unusually concentrated peptide solution, which limited due to solubility. Because of this, peptide content of most hydrogels could only reach to 5 mol% (of dry content). In case of hydrogels containing enzyme labile sequences (MGPSGPW, VPMSMRGG, EGTKKGHK), this did not have any effect on cellular viability.

In case of cell adhesion peptide cyclo[RGDfK], a higher content could be reached (25 mol%). Our result shoved that from 10 mol% peptide content, significant cellular behavioural changes occur. We found that if the integrin specific peptide was incorporated into the hydrogel system, cells were more mobile, and the formation of A2058 aggregates were heavily supressed, but still partially retained its drug resistance. In contrast the “quasi normal” cell lines seem to be more sensitive to the effects of Dau.

4. Synthesis of peptide amphiphiles

Four known peptide amphiphiles that self-assemble into fiber like structure were selected for this part of the project (RADA-II, EAK16-II, KLVFFA, Fmoc-FF).

During the synthesis of the aggregating, amphiphilic peptides - which were supposed to be incorporated into hydrogels - some turned out to be unexpectedly toxic. Also, one of the non-toxic peptides proved to be unpractical, as started hydrogelatination in an unrealistically high concentration only (40 mM). Therefore, EAK-II was chosen for further studies.

However, during the synthesis of self-assembling peptides (SAP), a cumbersome methodological problem arouse: handling and purification of peptides with good aggregation ability are problematic and laborious.

To overcome this phenomenon a two-stage protection group/system was developed. In the first step, with a photolabile protecting group, O^{t}Bu-p-hydroxy-phenacyl (pHP(OtBu)) the aggregating site of the SAP is masked. The tBu protecting group enables compatibility during solid phase peptide synthesis SPPS. During the cleavage of the peptide from the solid support, the tBu protection is removed, but not the p-hydroxy-phenacyl moiety. The protective group continues to prevent aggregation and peptide is easily purified and handled. The use pHP group is of highly advantageous as i) helps with water solubility ii) bulky enough to supress aggregation iii) UV photocleavage of the group results in an intrinsic/ non-toxic metabolite.

In vitro studies confirmed that there are no main differences between the hydrogels formed from the photoactivated peptide and the native SAP. Several smaller aggregates (~50 μm) could be detected in the hydrogels in case of A2058 cells which showed moderately resistance (110% viability) to Dau then 2D (*Fig 4*).

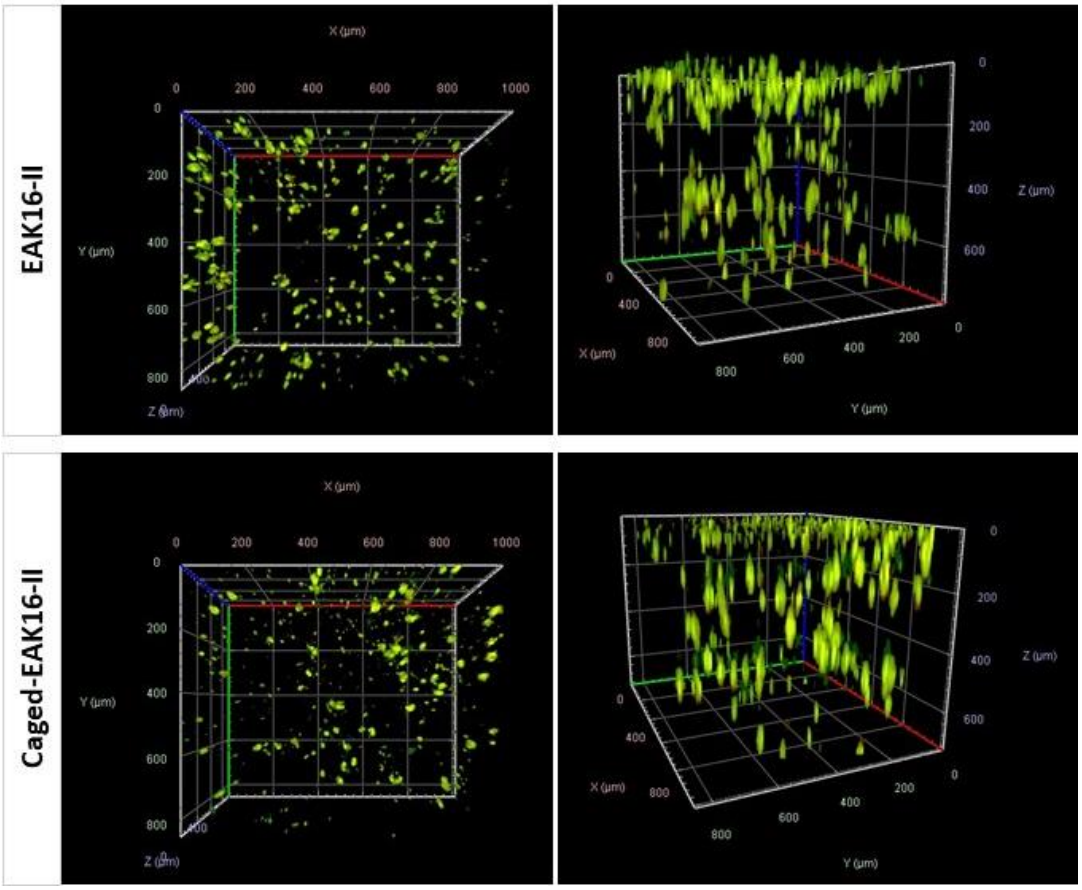

Figure 4. *3D view of cellular arrangements in hydrogels. Cells were stained with acridine orange (0.1 mM). The cellular aggregates were imaged by Zeiss Celldiscoverer 7 using 10x magnification and acquisition of Z-stacks with 10 µm distance between individual planes.*

These findings were presented at the Hungarian Peptide Cometees conference (Balatonszemes, 2023) and the publication summarising our result is currently under review (ACS Omega) and can be reached on preprint server [12]

The application of the pHP(O^{t}Bu) "double" protecting-group and the novel solid phase peptide synthetic strategy we developed may find its utility in the practical synthesis of complicated, hardly soluble, aggregating peptides. We are currently searching collaborating partners for this application.

4.1 SAP derivatives

Known adhesion enhancing sequences from fibronectin (YIGSR, PHSRN) laminin (IKVAV) and elastin (VGVAPG), were chosen to be incorporated into SAPs. In case of melanoma cells, hydrogels using this SAP derivatives moderately improved cellular viability (10-20%).

4.2 Hybrid hydrogels

Incorporation of the developed photoreactive SAP into the PEG-based siloxane hydrogels showed that the effect of the self-assembling "part" is dominant. Cellular aggregates could only be detected in case of melanoma cells. Spheroids size (50-70μm) and survival was the same as in the case of hydrogels formed from SAP.

Reacent results shown, that incorporation of adhesion moiety containing SAPs into siloxane-hydrogels results in aggregates different from previous experiments (size, shape). To analyse the microscopic images we need to improve our AI-based application. We plan to publish an other paper from these results soon.

5. Collaborations

We developed a software that effectively analyse microscopic images, and flexibly detects aggregation in collaboration with En-Co Software Zrt. This software is currently under development to accommodate more complex aggregate-forms as well. This program is a unique solution for the analysis of such cell cultures without using very sophisticated microscopic techniques. The software can be freely downloaded (it was accessed more than 100 times already). We hope, that it may help the scientific community in the future.

A possible further utilisation of light-induced aggregating peptides in the field of 3D hydrogel cell culture (tissue engineering) can be reached with 3D printing technology. To initiate such experiments, we started a collaboration with 4GOLD Kft., a company with many years of expertise in the field of 3D printing. This cooperation agreement between ELTE and 4GOLD Kft. is valid with September 2023. Based on this, we hope that the implementation of the methodologies developed under the funding of this project will continue fruitfully.

6. References

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