Preparation of ToLuminate Photogels

1. Introductory Notes

- **Mechanism of gelation**

  ToLuminate Photogels are fabricated from the norbornene-modified polymer dextran (N-Dextran), a crosslinker and the photoinitiator LAP (Lithium-Phenyl-2,4,6-trimethyl-benzoylphosphinat). Upon exposure to the appropriate light the photoinitiator is split into two radicals which will in turn radicalize thiols of the crosslinker. The crosslinker then binds to the norbornenes of N-Dextran, which results in a network of N-Dextran and crosslinker to form the hydrogel. Thiol-modified cell adhesion peptides can be added to the polymer-crosslinker solution before exposure to light to biomimetically modify the hydrogel network.

- **Photoinitiator, light source and times of light exposure**

  The light absorption spectrum of LAP (Fig. 1) determines the wavelength to be used to effectively form radicals. Light sources emitting light at wavelengths between 365 and 405 nm are best suited to activate LAP. To prevent cytotoxic effects by exposure to light, longer wavelengths should be preferred. Light sources which emit wavelengths between 380 to 405 nm are recommended.

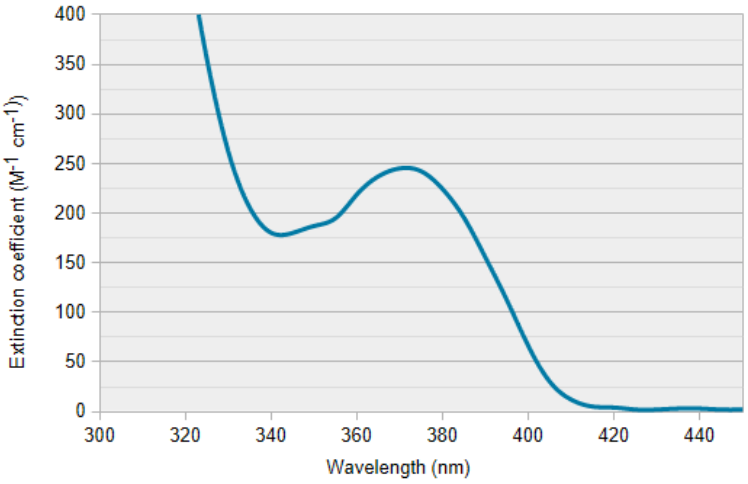

Fig. 1: *Absorption spectrum of LAP .*

When pre-gel solutions are exposed to different wavelengths of light or to different light intensities, the time of exposure to reach maximum stiffness can vary significantly. The extinction coefficient of LAP (Fig. 1) determines the efficiency of its activation to form radicals and, thus, the efficiency of gel formation. Exposure of pre-gel solutions to light at wavelengths corresponding to higher extinction coefficients will accelerate the activation of LAP into radicals at equal light intensity. The recommended exposure times in Table 3 were determined by rheologic measurements using a LED light source emitting light at 405 nm wavelength (bluepoint LED eco, Dr. Hönle AG, Germany). Many suitable light sources are commercially available. The time necessary for completion of crosslinking depends on the intensity of the light source, its wavelength and its distance from the pre-gel solution. Consult the manual or contact the manufacturer to obtain specifics about your light source.

Note: Do not overexpose the pre-gel solution to light. Once the polymerization of the gel is complete, surplus radicals may attack hydrogel bonds and decrease gel stiffness and also react with cellular components leading to cytotoxicity.

- **LAP photoinitiator concentration**

  The concentration of LAP should be at least one third of the concentration of norbornene groups to ensure an efficient crosslinking and attachment of peptides to the polymer N-Dextran. Optionally, a slightly increased concentration of LAP can be applied to enhance the speed of gel formation. The concentration of LAP should not exceed the ratio of 1:2 of LAP to norbornene groups to minimize cytotoxic effects.

- **AgaFloat**

  AgaFloat (Cat. No. A10-3) can optionally be added at 10% of the total gel volume to prevent cells from sedimentation before and during gelation. Gelation is not affected by AgaFloat nor does the addition of AgaFloat change the hydrogel's stiffness significantly. AgaFloat does not interfere with cell spreading and migration.

2. Protocol

The following protocol describes the preparation of soft ToLuminate Photogels for 3-D cell culture with or without modification with the cell adhesion peptides RGD and GFOGER-3. Please read the full protocol before you start preparing a gel.

2.1 Reagents and materials

Hydrogel Kits	Catalog Number
ToLuminate PEG Photogel	PG90-1
or	
ToLuminate CD Photogel	PG91-1
or	
ToLuminate HA Photogel	PG95-1
or	
ToLuminate CD-HA Photogel	PG96-1
Peptides (optional)	
3-D Life RGD Peptide	09-P-001 or P10-3
3-D Life GFOGER-3 Peptide	P12-1 or P12-3
Accessory (optional)	
3-D Life AgaFloat	A10-3
Related Products	
3-D Life Dextranase	D10-1

Reagents and materials not included in the products:

Cell culture medium, cell culture plate, reaction tubes, pipet tips, micropipets, serological pipets, light source.

2.2 Preparations

Hydrogel reagents:

- Dissolve hydrogel reagents or peptides that are provided in lyophilized form according to the accompanying Product Data Sheets.
- If hydrogel reagents are frozen, thaw all reagents at room temperature. Make sure that salts in the 10x CB are completely dissolved. Do not put 10x CB on ice, this may cause the salts to crystallize.
- If AgaFloat is used:
  - Melt AgaFloat according to the instructions in the accompanying Product Data Sheet. Keep AgaFloat at 37°C until use. Do not keep the molten AgaFloat at room temperature because it will solidify within 30 minutes and will not be pipettable anymore.
  - Prepare a bucket of ice or a similar cooling device.

Notes:

1. Do not expose thiol-containing reagents (RGD Peptide, GFOGER-3 Peptide, CD-Link, PEG-Link, Hylink or CD-Hylink) to air and room temperature longer than necessary to minimize oxidation of the thiol groups. Close cap after each use.

2. Do not expose LAP to intense light to avoid premature radicalization before hydrogel formation is induced.

Biological sample:

Prepare a stock cell suspension or any other biological sample of your choice in culture medium, PBS or in any other physiological solution. In this protocol, the biological sample takes 20% of the total hydrogel volume. Accordingly, the cell density in the gel will be only 20% of the cell density of the stock cell suspension. If you chose to use different volumes of biological samples, the online calculation tool assists you to calculate the correct volumes of all reagents utilized.

2.3 Experimental procedure

The following pipetting schemes describe the preparation of a soft hydrogel with the option of modification with RGD or GFOGER-3 Peptide, or with both peptides in one gel. For most applications peptides concentrations of 0.5 mmol/L RGD Peptide and/or 1.2 mg/ml GFOGER-3 Peptide are sufficient. The volumes of hydrogel reagents required for 1 mL of gel are listed in Table 1 and Table 2. If different volumes of hydrogel are to be prepared, the online calculation tool assists you to calculate the corresponding volumes of reagents.

Table 1: Preparation of ToLuminate PEG-based (PG90-1 or PG91-1) Photogels: *Reagent volumes for 1 mL hydrogel using N-Dextran polymer to be crosslinked with 3 mmol/L SH groups of the crosslinker CD-Link or PEG-Link (3 mmol/L crosslinking strength) with the option of modifications with 0.5 mmol/L RGD and/or 1.2 mg/ml GFOGER-3 Peptide. Compositions are given with or without the addition of AgaFloat.*

Reagents stock solutions	Volumes for 1 mL gel (µl)							
	w/o peptide		with RGD Peptide		with GFOGER-3 Peptide		with RGD and GFOGER-3 Peptide	
	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float
Water	370	270	311	211	276	176	219	119
10x CB (pH 7.2) Phenol Red-free	80	80	80	80	80	80	80	80
N-Dextran (30 mmol/L norbornene groups)	100	100	117	117	117	117	133	133
RGD Peptide (20 mmol/L SH groups)	-	-	25	25	-	-	25	25
GFOGER Peptide (20 mg/mL)	-	-	-	-	60	60	60	60
Cell suspension	200	200	200	200	200	200	200	200
AgaFloat	-	100	-	100	-	100	-	100
PEG-Link or CD-Link (20 mmol/L SH groups)	150	150	150	150	150	150	150	150
LAP (10 mmol/L)	100	100	117	117	117	117	133	133
Total volume	1000	1000	1000	1000	1000	1000	1000	1000

Table 2: Preparation of ToLuminate HA-based (PG95-1 or PG96-1) Photogels: *Reagent volumes for 1 mL hydrogel using N-Dextran polymer to be crosslinked with 2 mmol/L SH groups of the crosslinker Hyink or CD-Hylink (2 mmol/L crosslinking strength) with the option of modifications with 0.5 mmol/L RGD and/or 1.2 mg/ml GFOGER-3 Peptide. Compositions are given with or without the addition of AgaFloat.*

Reagents stock solutions	Volumes for 1 mL gel (µl)							
	w/o peptide		with RGD Peptide		with GFOGER-3 Peptide		with RGD and GFOGER-3 Peptide	
	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float	w/o Aga-Float	with Aga-Float
Water	386	286	328	228	293	193	235	135
10x CB (pH 7.2) Phenol Red-free	80	80	80	80	80	80	80	80
N-Dextran (30 mmol/L norbornene groups)	67	67	83	83	83	83	100	100
RGD Peptide (20 mmol/L SH groups)	-	-	25	25	-	-	25	25
GFOGER Peptide (20 mg/mL)	-	-	-	-	60	60	60	60
Cell suspension	200	200	200	200	200	200	200	200
AgaFloat	-	100	-	100	-	100	-	100
Hyink or CD-Hylink (10 mmol/L SH groups)	200	200	200	200	200	200	200	200
LAP (10 mmol/L)	67	67	84	84	84	84	100	100
Total volume	1000	1000	1000	1000	1000	1000	1000	1000

The following steps are described for the illumination of pre-gel solution in culture dishes. If the pre-gel solution is used in a bioprinter, follow the instructions provided by the bioprinter manufacturer after completing step 3 (see below). If not indicated otherwise, all steps below are performed in a sterile hood at room temperature:

1. In a reaction tube combine Water, 10x CB (pH 7.2) Phenol Red-free, N-Dextran, Peptide (if applicable) crosslinker, and LAP. Mix well.

   Note: For the viscous HyLink or CD-HyLink crosslinkers the use of low-binding wide orifice pipet tips is recommended.

2. A: If AgaFloat is used:
   - Add the molten AgaFloat and immediately mix the solution.
   - Add the cell suspension and mix gently.
   - Immediately incubate the pre-gel solution for at least 5 min on ice. For larger pre-gel volumes a longer incubation time may be necessary.
   - Gently mix again.

   B: If AgaFloat is not used:
   - Add the cell suspension, if included. Mix gently by pipetting up and down.

3. If the pre-gel solution is used in a bioprinter follow the instructions provided by the bioprinter manufacturer. A guideline for light exposure times at 405 nm wavelength and 30 mW/cm^2 light intensity is given in Table 3.

   Otherwise, continue with step 4.

4. Place the pre-gel solution at its final location and expose it to the appropriate light (for examples, see Table 3). Do not cover the pre-gel solution with any object which blocks light. Gelation can be induced through transparent polystyrene lids if light at 405 nm wavelength is used.

5. Make sure that the gel has completely formed. Optional: test gel formation by carefully touching the gel surface with a pipet tip. The tip should not pull out threads of gel when retracting from the gel surface.

6. Once the gel has solidified, carefully add cell culture medium until the gel is covered.

7. Place the culture dish in the incubator for cultivation of cells.

8. Optionally: Renew medium after 1 hour to further equilibrate the gel with culture medium.

9. Change the medium as needed during cultivation of cells.

3. Variations of Gel Preparations

3.1 Online calculation tool

Reagent volumes for gel variations described below can easily be calculated using the online calculation tool.

Note: If you compose your hydrogel without the online calculation tool please note that for optimal hydrogel formation the molar LAP concentration should be one third (1/3) of the molar concentration of norbornene groups used in the gel. If you wish to speed up gelation you can increase the ratio of LAP to norbornene groups to 1:2. You can also adjust the molar LAP

concentration to your own settings in the calculation tool.

3.2 Preparation of gels of different stiffnesses

If a crosslinking strength of 3.0 mmol/L (crosslinking with PEG-Link or CD-Link) or 2 mmol/L (HyLink or CD-HyLink) turns out to be not adequate for your application, gel compositions for softer or stiffer gels can be calculated using the online calculation tool. Table 3 lists examples of crosslinking strengths and resulting hydrogel stiffnesses of N-Dextran crosslinked with the indicated crosslinkers. Stiffnesses were determined by rheometric measurements and must not correspond exactly the stiffness of gels prepared by other means. The pre-gel solution was exposed to the light of a LED pen at 405 nm and a light intensity of 30 mW/cm^2. The exposure time indicates the time necessary to reach the maximum stiffness of the gel. Gel stiffness will decrease slightly once immersed in solution (e.g. cell culture medium) due to swelling.

Table 3: *Crosslinking strength and resulting hydrogel stiffness of N-Dextran crosslinked with the indicated crosslinkers. Light exposure times are given for reaching maximum hydrogel stiffness.*

Crosslinker	Crosslinking strength (mmol/L N-groups reacting with crosslinker)	Approximate Stiffness (Pa, shear modulus G*)	Exposure time (sec) at 405 nm and 30 mW/cm^2
PEG-Link	3	700	100
	4	1800	100
CD-Link	3	900	300
	4	1700	300
HyLink	2	700	150
	3	2400	150
CD-HyLink	2	1000	200
	3	2800	200

3.3 Preparation of gels with different concentrations of adhesion peptides

If gels of different concentrations of adhesion peptide are to be prepared, please consult the online calculation tool for calculating volumes of reagents.

> **Note:** If higher concentrations of GFOGER-3 peptide are used, be aware that GFOGER-3 has crosslinking properties. The crosslinking may interfere with cell spreading and migration because GFOGER-3 does not contain a MMP (matrix metalloproteinase) cleavage site.

3.4 Preparation of plain gels (without cells) or embedding of other specimens

For encapsulation of tissues or preparation of plain gels, replace the volume of cell suspension with PBS or other physiologically compatible solution of your choice. Alternatively, use the online calculation tool and keep the component „Biological Sample“ blank or enter "0".

3.5 RGD Peptide replacements for control experiments

Instead of the RGD-Peptide, Thioglycerol (Catalog Number T10-3) can be added to a gel. In this case the gel does not provide cell attachment sites and can be used as a control for peptide-modified gels. Cellendes also offers a scrambled version of RGD Peptide (Cat. No. 09-P-003) for control gels to be compared with gels containing RGD Peptide.

3.6 Addition of AgaFloat

AgaFloat at a concentration of 10% keeps cells suspended in the pre-gel solution.

If bigger biological specimens than single cells are used, the AgaFloat concentration can be increased up to 20%. AgaFloat concentrations above 20% are not recommended as the pre-gel solution will not be pipettable anymore.

3.7 Dissolving ToLuminate Photogels with Dextranase

Live or chemically fixed cells can be recovered from N-Dextran Hydrogels crosslinked with PEG-Link or CD-Link by the enzymatic digestion of the gels with Dextranase (Catalog Number D10-1). For a detailed protocol see Technical Protocol 1 (TP-1). Dextran hydrogels crosslinked with HyLink or CD-HyLink cannot be dissolved by dextranase.

The addition of AgaFloat (10% of total gel volume) to hydrogels does not interfere with hydrogel degradation by dextranase. If a higher percentage of AgaFloat is added to the hydrogel (>15%), the digestion of hydrogel by dextranase will be impaired.