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R3-SOP Development of polymer brushes by photocatalyzed SI-ATRP and their application as nano bio-responsive platforms (NanoBioRS)

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We use this protocol and it's working

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Abstract

1. Scope:

This document was created in order to facilitate knowledge transfer between the ER (Mariana Blanco Massani) and the host institution (UIBK) as part of NanoBioRS-MSCA IF action. The transmission of expertise is related to new knowledge acquired at the secondment host (Jagiellonian University, Poland) and was not available at the University in Innsbruck before.

The publication of this document is in line with data sharing as delineated in the data management plan of NanoBioRS (D2.1-ORDP-NanoBioRS).

The methods described here are part of WP1, WP2 and WP4 of NanoBioRS project which results are under review at OA journals at the moment of the creation of this document.

The document presents a method for synthesizing polymer brushes through surface-initiated controlled radical polymerization (SI-CRP), employing a visible-light-mediated process facilitated by the organic photoredox catalyst N-phenylphenothiazine (PTH). This method is proper for application in the biomedical field since the technique is easy to scale up and does not require copper-based catalysts (toxic) (Discekici et al., 2016). Additionally, the monomer scope is not limited by the solubility of PTH in this system.

In contrast to Ir(ppy)₃ and traditional metal-catalyzed ATRP processes, a unique feature of PTH is its highly reducing excited state, which may allow a wider selection of functional groups to be tolerated during the polymerization. This method also allows to perform block and random co-polymerizations (Narupai et al., 2018).

2. Theoretical background.

Mechanistic studies suggested that upon excitation with 380 nm light PTH induces reduction of traditional alkyl bromide-based ATRP initiators via an oxidative quenching cycle. This results in the generation of a propagating radical as well as a deactivating catalyst complex consisting of the radical cation form of the photocatalyst and a bromine anion. This complex subsequently deactivates growing polymer chains through formation of a dormant, bromine-end-capped species and reduction back to the initial ground state PTH (Treat et al., 2014, Discekici et al., 2018).

Compatible substrates are any substrates containing oxygen on their surface: silicon wafers and glass indium tin oxide (ITO), metallic implants. Gold is incompatible with photon mediated catalysis. Within the frame of NanoBioRS project, silicon wafers were used to perform characterization by contact angle and ellipsometry. ITO substrates was used for FTIR-ATR characterization, since this substrate is reflective at IR, and for experiments involving characterization by microscopy.

The objectives of NanoBioRS WP1, WP2 and WP4, involve polymer brushes able to promote cell adhesion, while killing and repelling staphylococci.

We propose the use of polymer brushes with a block copolymer configuration comprising glycidyl methacrylate (GMA) which can be tuned to reach other chemistries of interest in cell adhesion and interaction with antimicrobials, and poly(ethylene glycol) methacrylate (PEGMA) as antifouling agent.

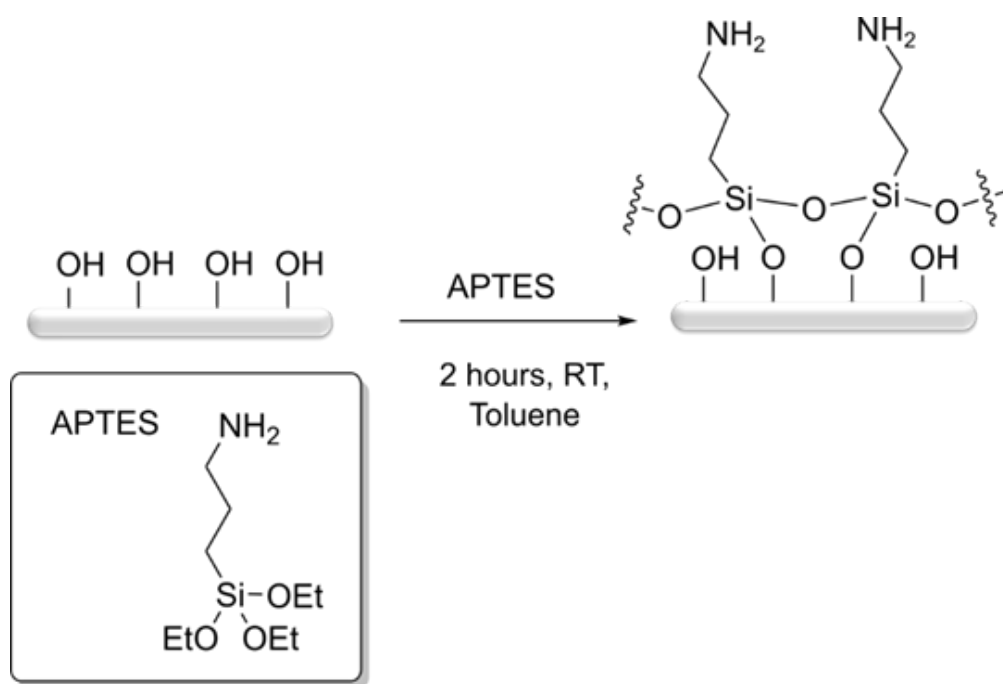
The work described in this SOP is structured into sequential experimental steps, each carried out on a different day to help the trainee organize the setup.

Guidelines

Experimental Methodology and Weekly Workflow Organization.

1. First day:

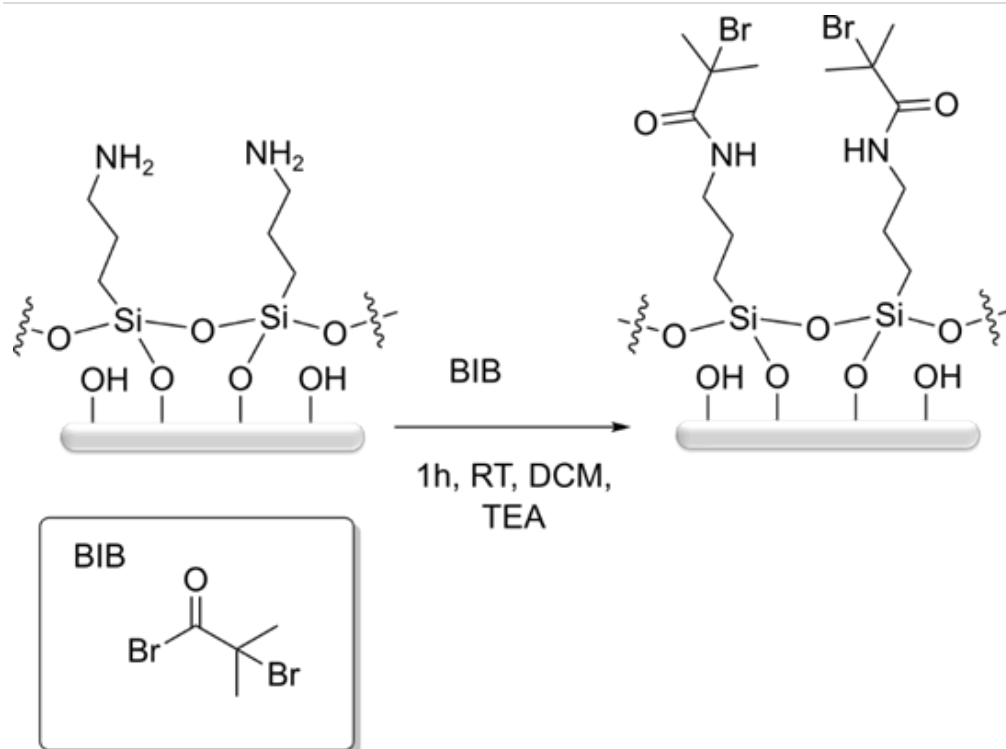
Objective: Generate hydroxyl groups on the substrates to enable the proper chemisorption of linker molecules (**Scheme 1**). The OH groups are generated with the cleaning and oxidation steps and the linker to be used is (3-Aminopropyl)triethoxysilane (APTES). This reaction is taking place in toluene.



Scheme 1. Monolayer produced by chemisorption of APTES on a substrate

2 Second day:

Objective: Functionalize the substrate with an ATRP-initiator monolayer (**Scheme 2**). This reaction is taking place in dichloromethane (DCM) in the presence of Triethylamine (TEA) and the initiator used is 2-Bromo-2-methylpropionylbromide (BIB). TEA is added to create an excess of amine in order to displace the equilibrium to the products. One bromide in BIB is reacting with NH_2 from APTES, the other bromide is the initiator for ATRP.



Scheme 2. Functionalization of and APTES modified substrate with BIB.

Warnings: Samples modified with initiator can be left 1 week under vacuum.

3. Third day:

Objectives: 1-Modify the APTES/BIB substrate with GMA and PEGMA polymer brushes using a molar concentration of each monomer of 1 M and 5% catalyst. Polymerization conditions based on (Ramakers et al., 2017) and (Smenda et al., 2021). 2-Derivatizing the brushes with RGD

Warnings: Polymerization and derivatization can be done on the same or separate days.

Prepare PTH/monomer solutions in advance. At least one day before the reaction.

3.1 Post-polymerization treatment of the brushes

Objective: Modify the epoxy groups from the PGMA containing brushes by nucleophilic ring-opening with arginylglycylaspartic acid (RGD).

Reaction in water under basic conditions

At basic conditions the reaction of the amine group should be beneficial but competition with water is present (Leach et al., 2005).

Warnings: The high reactivity of the oxirane ring of PGMA and the derived hydroxyl group may lead to intermolecular crosslinking when the reaction takes place generating micro/macro gels. This issue could be



avoided by reducing the reaction temperature (55–37°C) and/or by using a large (at least 10-fold) excess of the amine. If using 10-fold concentration is not affordable, overnight incubation at low temperature is recommended.

An adaptation of the method used by (Leach et al., 2005) was used. It is hypothesized that the reaction occurs through the amino group of RGD, and therefore, the brushes present carboxylic acid groups at the surface. This is convenient for the next step taking place at day 4: At acid pH of the M23-PP NPs (Blanco Massani *et al* 2024), the surface is positively charged, and the surface will interact with M23-PP (negatively charged) rendering the polymer brushes with antimicrobial, enzyme-responsive efficacy.

4. Fourth day:

Objective: Rendering the polymer brushes with enzyme-responsive lytic efficacy against *S. aureus*.

Materials

- Photocatalyst: 10-Phenylphenothiazine (PTH)
- ATRP initiators: traditional alkyl bromide-based initiators, 2-Bromo-2-methylpropionyl bromide (BIB)
- Substrates (compatible): silicon wafers; indium tin oxide (ITO) glass
- Monomers for block copolymers: glycidyl methacrylate (GMA); poly(ethylene glycol) methacrylate (PEGMA)
- Linker molecule: (3-Aminopropyl)triethoxysilane (APTES)
- Solvents: toluene (distilled and kept under argon for silanization); ethanol; THF; water; dichloromethane (DCM); acetone; methanol; N,N-dimethyl-acetamide (DMAC)
- Cleaning aids: detergent; tap water; distilled water
- Oxidizing/cleaning reagents: piranha solution components — concentrated sulfuric acid (H_2SO_4) and hydrogen peroxide 30% (H_2O_2)
- Light sources for excitation/polymerization: 380 nm; homemade LED reactor ($\lambda_{\text{max}} 400 \pm 5 \text{ nm}$; intensity $18 \pm 2 \text{ W m}^{-2}$); UV fluorescent lamp 365 nm, 1 W m^{-2}
- Diamond tip scribe (for cutting substrates; target size $\approx 1.5 \times 1.5$)
- Tweezers (for handling substrates)
- 12-well plate (for holding samples during processing)
- Sonicator/ultrasonic bath (for ethanol/toluene sonication)
- Small containers/beakers for rinsing and sonication
- Clean beakers (for sonication and rinses)
- Argon gas supply (for drying samples and purging/inert atmosphere during silanization and initiator coupling); balloon for argon overpressure on APTES flask
- Plasma pen/piezo brush (for plasma treatment)
- Ozone source/ozonizer or UV-ozone cleaner; desiccator (for ozonation step)
- Ellipsometer (for SiO_2 layer/ellipsometry measurements)
- Erlenmeyer flask with septum (for silanization and subsequent reactions)
- Small flask and cannula (for transferring and purging during BIB coupling)
- Vacuum box/desiccator (for storing samples under vacuum and overnight degassing)
- Inlet and outlet needles for argon purging
- Syringe (for extracting and dispensing reagents)
- Parafilm (for sealing flasks during reaction)
- Washing vial with septum and solvent mixture (THF/toluene/acetone) for rinsing cannulas/needles
- Purification media: basic alumina (Aluminium oxide 90 active basic, 0.063–0.200 mm; Millipore 1.01076.2000) for inhibitor removal from monomers
- Polymerization setup: three vials with rubber septa; double-tipped needles (cannulas) for interconnection/transfer
- Reagents for derivatization (later steps): RGD; M23-PP nanoparticles (NPs)
- Petri dishes (for holding substrates during polymerization)
- Clean glass cover slips (larger than the substrate; pre-cleaned by sonication in ethanol, argon flushing, and ozone oxidation)
- Waste containers, organic solvents (for toluene, acetone, THF, methanol) separated from chlorinated solvent waste (for DCM)



- Sodium hydroxide (NaOH) 0.5 M (for RGD solution)
- Phosphate-buffered saline (PBS), sterile
- 6-well plate (for cell assays)
- Wound healing insert
- MC3T3 cells (2.5×10^5 cells/ml seeding suspension)
- Live/dead staining kit and OA staining reagents
- Humidity chamber/incubator at 37°C
- PGH-PP nanoparticles (PGH-PP NPs)
- Container: HEPA-filtered isolation box for co-culture experiments
- Incubator: CO₂ incubator for mammalian cells (37°C, 5% CO₂)
- Microorganism of interest for co-culture (2.5×10^5 CFU/ml working suspension)
- Small plates to count bacterial cells (for plating 50 µl aliquots)
- Fluorescence microscope for cell imaging

Safety warnings

- ! - Handle wafers only with tweezers to avoid contamination and damage.
- After cleaning, samples placed in the 12-well plate should not touch any surface.
- Piranha solution is highly corrosive and reactive: prepare fresh each time, manipulate very carefully, and dispose according to university regulations.
- During plasma treatment, no metal should be in contact with the plasma pen/piezo brush; keep any electronic device away from it.
- It is very important to oxidize the surface with both, plasma pen and ozone flushing as the brushes copolymerized by chain extension are detached if ozone treatment is not applied.
- During silanization, add only a single small drop of APTES to the solvent to avoid multilayer formation; avoid adding excess APTES. Do not dispense APTES directly onto the samples; add it to the solvent phase under argon.
- Do not allow samples to dry during the solvent-exchange/rinsing steps; drying will hinder monolayer formation.
- Dispose dichloromethane with chlorinated-solvent waste; discard all organic solvents per institutional regulations.
- During the BIB coupling purge, do not introduce the canula into the BIB solution; bubble argon outside the solution in the small flask and gently in the Erlenmeyer.
- Ensure samples do not mount one over the other during the BIB reaction; keep them separated when sealing with parafilm.
- During post-polymerization epoxy ring-opening on PGMA, there is a risk of intermolecular crosslinking and gel formation; avoid long reaction times. Mitigate by lowering temperature (55–37°C) and/or using a large excess of amine. Use low temperature and overnight reaction as a cost-effective alternative to $\geq 10\times$ amine excess.
- Samples modified with RGD can be kept in the fridge only for 1 day.
- For co-culture assays ("race for the surface"), introduce plates into a HEPA-filtered isolation box to avoid cross-contamination; perform experiments in a BSL2 lab when working with *Staphylococcus aureus* or other BSL2 microorganisms.

Before start

- Prepare Monomers premixed with PTH in DMAC at least one day before the polymerization step.
- Pre-clean glass cover slips and ensure they are larger than the substrates: sonicate in ethanol, flush with argon, and oxidize with ozone.
- Prepare polymerization hardware in advance (petri dishes; UV/LED light source at 400 nm LED or 365 nm UV; argon for drying).
- Coordinate the splitting of the MC3T3 cell line and the activation of *Staphylococcus aureus* in alignment with your co-culture experimental plan, once the enzyme-responsive polymer brushes have been prepared.

1. First day: Silanization

- Objective:** Generate hydroxyl groups on the substrates to enable the proper chemisorption of linker molecules. The OH groups are generated with the cleaning and oxidation steps and the linker to be used is (3-Aminopropyl)triethoxysilane (APTES). This reaction is taking place in toluene.

Time frame: from cutting to washing ca. 2 hours for five samples. From ozonization to silanization 4 hours, this includes ellipsometry measurements. Then overnight vacuum.

Cutting the wafers and cleaning

- Cut the the substrates (silicon wafers, glass) using a diamond tip ca size: 1.5×1.5 mm.
Always manipulate your wafers with tweezers! And place them on a 12 well plate (**Fig. 1**).

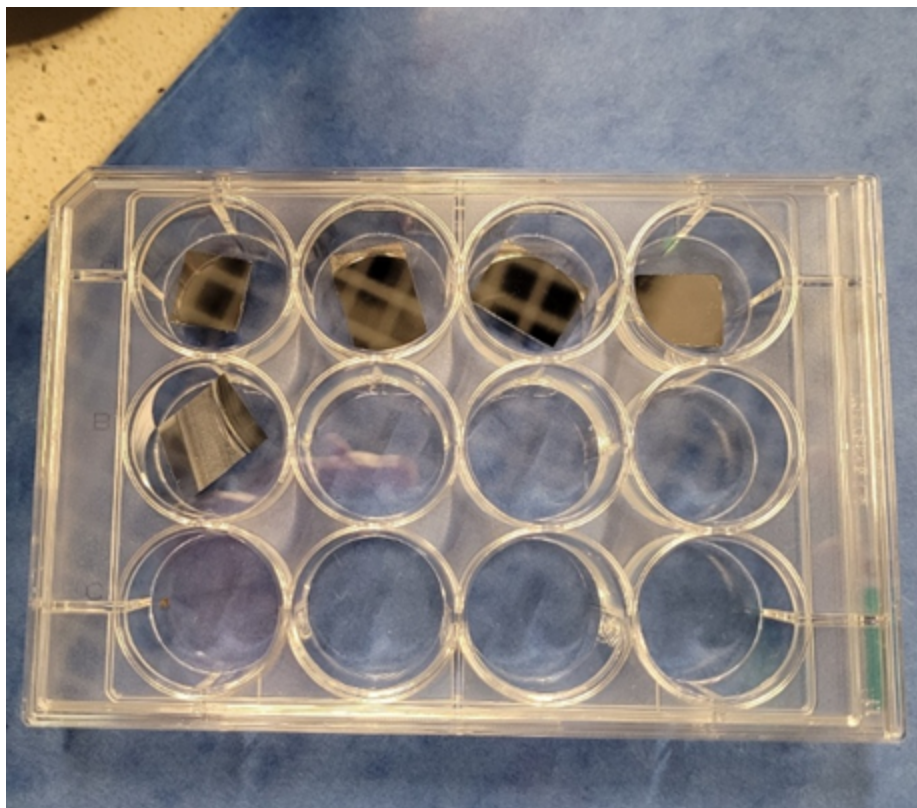


Figure 1. Substrates in a 12 well plate should not touch any surface after cleaning.

- Sonicate the samples for 10 minutes in ethanol, placing them inside a clean beaker.

- 1.3 Rinse with ethanol and apply piranha solution (2× concentrated sulfuric acid (H_2SO_4) and 1× hydrogen peroxide (H_2O_2), 30%) for 10 minutes. Prepare fresh piranha each time this is performed.

Safety information

Manipulate piranha very carefully and dispose according to the chemical disposal regulations

- 1.4 Rinse with water, then with THF, then with toluene (next reaction will take place in toluene). Dry with argon and put the samples inside a 12 well plate.
- 1.5 Apply plasma pen for 1 minute per sample (**Fig. 2**). Depending on the plasma pen model, both metallic and non-metallic samples can be treated, make sure to choose the right accessory according to the manufacturer information.

Note

Keep any electronic device away from the plasma pen!

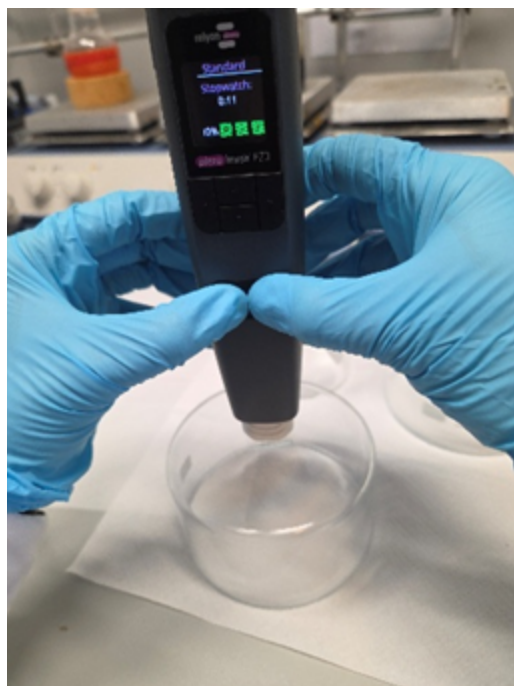




Figure 2. Piezo brush is used for plasma treatment of the surface to increase the oxidation and improve the yield of reaction for APTES monolayer formation.

- 1.6 Ozonize the samples for 30 minutes (**Fig. 3**). This should be done right before silanization using APTES. Alternatively, samples can be flushed with ozone for 30 minutes in a desiccator. Measure the SiO_2 layer with an ellipsometer (**Fig. 4**) right before modifying with APTES.

Note

It is very important to oxidize the surface with both, plasma pen and ozone flushing as the brushes copolymerized by chain extension are detached if ozone treatment is not applied



Figure 3. Example of ozonizator.

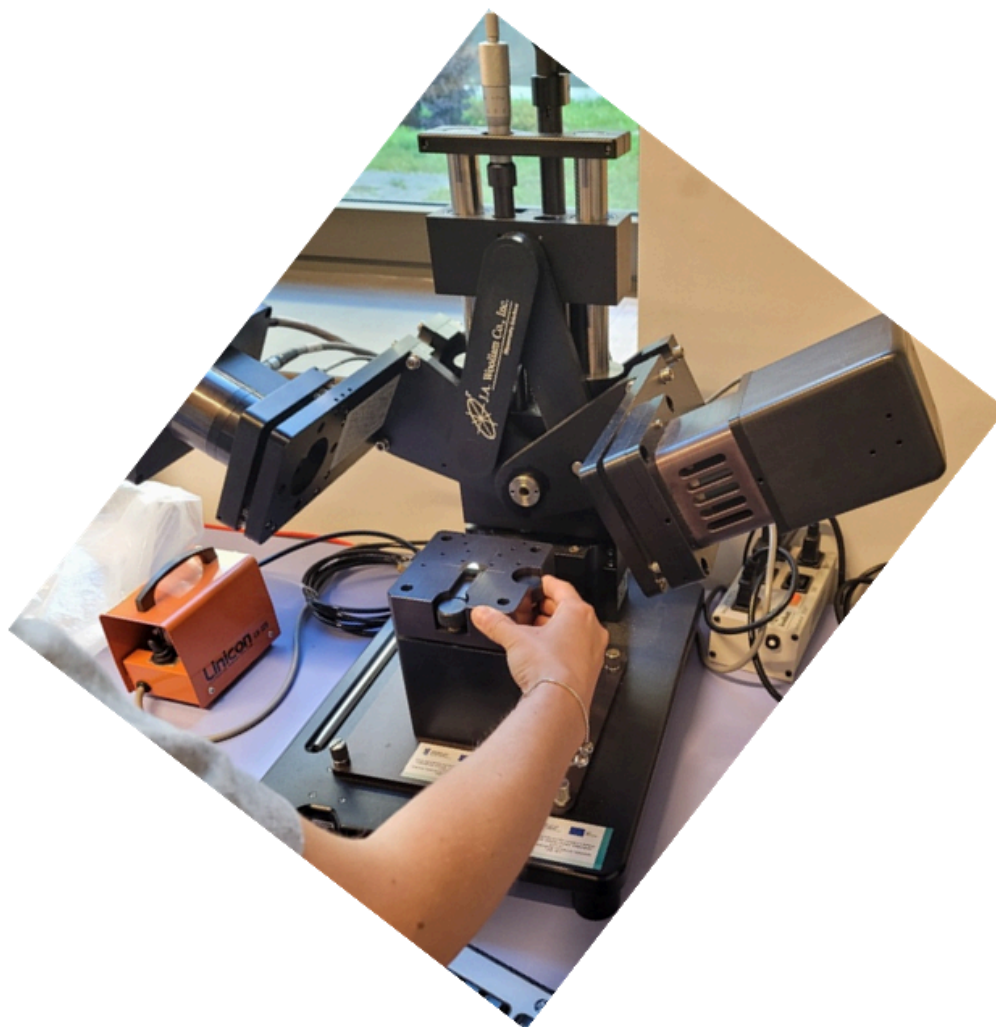


Figure 4. Ellipsometer

1.7 Save the samples under vacuum until further use.

1.2 Silanization

1.8 Place 2 samples of 300 mm² in an Erlenmeyer and close with the septum.

1.9 Fix the Erlenmeyer and insert argon and an outlet needle to purge the system.

1.10 Add 4 ml of toluene, previously distilled and maintained under argon atmosphere.

1.11 Purge the system for 2 min.

1.12 Remove APTES from the flask; for this, place a balloon with argon in the septum of the APTES container (**Fig. 5**).



Figure 5. Setup proposed to remove APTES under argon atmosphere.

- 1.13 Insert a syringe in the septum to carefully extract less than 1 ml of APTES from the flask.
- 1.14 Remove the syringe and apply APTES inside the Erlenmeyer. Make sure to do it fast and accurate, placing only one drop inside and removing the needle after applying APTES. Do not apply APTES directly on the samples but in the solvent.

Note

It is important to avoid adding too much APTES to avoid multilayers.

- 1.15 Leave purgation for a couple of minutes and let the reaction take place for 2 hours. For this, leave the Erlenmeyer adjusted and protected from light (**Fig 6**).



Figure 6. Setup for reaction of APTES chemisorption.

- 1.16 Rinse your sample to change the solvent, since the next reaction will take place in dichloromethane. This will involve several steps: (i) Remove one sample from the Erlenmeyer and quickly rinse with toluene, place the sample carefully in a small container with toluene and sonicate for 10 minutes (**Fig 7**). (ii) After sonication, rinse the sample again with toluene, and then dichloromethane.

Note

It is crucial at this step to prevent the samples from drying, as drying will heavily difficult to form a monolayer.

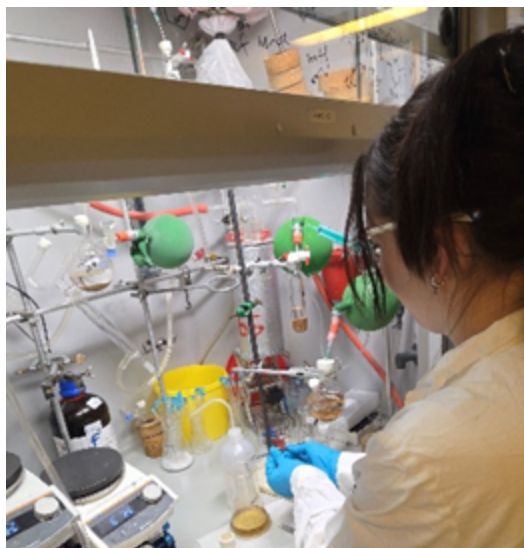


Figure 7. Step 1. remove your sample from the erlenmeyer



Figure 7. Step 2. quickly rinse with toluene

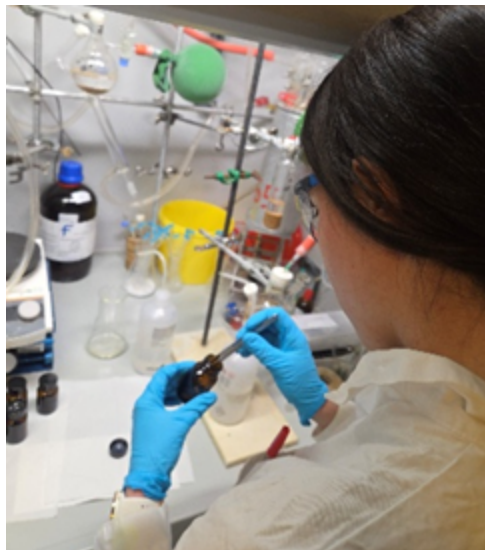


Figure 7. Step 3. carefully in a small container with toluene

Safety information

Consider that dichloromethane should be wasted with chlorinated solvents

- 1.17 After rinsing, dry with argon and apply vacuum overnight to remove any multilayer of physisorbed APTES which will interfere with brushes polymerization.

1.3 Washing your materials

- 1.18 Always wash and rinse all your glass material to have it clean for the next days. Do this using detergent, tap water, distilled water, acetone.
- 1.19 In the case of syringes and needles, rinse them with acetone immediately after application of APTES to avoid formation of crystals.

Safety information

Discard every organic solvent according to the regulations for solvent disposal

2. Second day: BIB

- 2 **Objective:** Functionalize the substrate with an ATRP-initiator monolayer (Scheme 2). This reaction is taking place in dichloromethane (DCM) in the presence of Triethylamine (TEA) and the initiator used is 2-Bromo-2-methyl-propionylbromide (BIB). TEA is added to create an excess of amine in order to displace the equilibrium to the products. One bromide in BIB is reacting with NH_2 from APTES, the other bromide is the initiator for ATRP.

Time frame: 4 h from preparation of setup to clean substrates functionalized with BIB.

2.1 Procedure

- 2.1 Introduce the 2 samples from the previous treatment in a small Erlenmeyer. Fix the system and purge with argon. Add 4 ml of DCM to the Erlenmeyer and 160 μl of TEA, trying not to move the samples to avoid mounting.
- 2.2 Prepare a small flask with 2 ml of dichloromethane and 148 μl of BIB.
- 2.3 Purge oxygen from the system using argon for 10 minutes and a cannula as presented in **Fig. 8**.
In detail: Argon inlet in the small flask, cannula connecting the small flask with the Erlenmeyer, and needle purging out the oxygen from the system. For purging, the cannula should not be introduced in the BIB solution, but outside of it in the small flask. In the Erlenmeyer, the cannula should carefully bubble argon in the solution.



Figure 8. Proposed setup for introducing BIB for substrates modification under argon conditions

- 2.4 Remove argon from the solution in the Erlenmeyer, introduce the canula inside the solution containing BIB in the small flask, and allow argon to push the liquid to the other flask (Drops of the BIB solution will be added to the Erlenmeyer flask by allowing them to fall in gradually).



2.5 Remove the canula and needles covering the flask with parafilm. **Make sure that samples do not mount one over the other.** Let the reaction for 1 hour at room temperature.

2.6 Wash with DCM, rinse, sonicate 5 minutes and rinse again.

2.7 Rinse with methanol

2.8 Dry with argon

2.9 Measure the samples with spectroscopic ellipsometry.

Expected result

After this step a layer not higher than 2 nm is expected.

2.10 Store the samples under vacuum until the next experiments.

Note

Samples modified with initiator can be stored 1 week under vacuum.

Washing your materials

3 To rinse cannula and needles use the following setup (**Fig 9**) containing a mixture of solvents (THF, toluene, acetone) in the washing vial with septum



Figure 9. Setup proposed to rinse needles and cannulas

3.3 Third day

- 4 **Objectives:** 1-Modify the APTES/BIB substrate with GMA and PEGMA polymer brushes using a molar concentration of each monomer of 1 M and 5% catalyst. Polymerization conditions based on (Ramakers et al., 2017) and (Smenda et al., 2021). 2-Derivatizing the brushes with RGD

Time frame: Mixing reagents 1-2 h. Polymerization (1-2 h). Preparation of RGD 15 minutes, derivatization of brushes overnight (16-24 h).

4.1 Mixing reagents for polymerization

- 4.1 Assemble a setup of three vials sealed with rubber septa, interconnected via double-tipped needles (**Fig 10**).



Figure 10. Setup proposed to prepare monomers and catalyst mixture under argon conditions.

- 4.2 Add pure N,N-dimethyl-acetamide (DMAC) to the first vial to saturate the system with solvent vapors.
- 4.3 Purify the monomers using basic alumina to remove inhibitors of polymerization (Aluminium oxide 90 active basic, 0.063–0.200 mm, Millipore, 1.01076.2000).
- 4.4 Place a solution of monomer in DMAC in the second vial (1 M for GMA and 1 M for PEGMA, Table 1).
- 4.5 Purge argon on the two vials for 10 minutes.
- 4.6 Transport the DMAC-monomer solution via the double-tipped needle to a third vial containing PTH in a 5% concentration with respect to the monomer (Table 1).



	A	B	C	D	E
	Monomer name	Concentration of Monomer (M)	Volume of monomer (μl)	PTH (mg)	DMAC (μl)
	GMA	1.020049244	40	4	240
	PEGMA	1.044238683	100	3.9	170

Table1. Amounts used for the preparation of each monomer in a 5% molar ratio respect to PTH.

Note

Pay close attention to maintaining the catalyst-to-monomer ratio at approximately 5%, especially if any modifications are made to Table 1.

4.7 Purge the resulting mixture with argon for 5 additional minutes.

4.2 Polymerization and post-polymerization with RGD

4.8 Place the substrate modified with macroinitiator on a petri dish (**Fig 11**).



Figure 11. Setup for polymerization

- 4.9 Deposit about 5 μL of PEGMA/PTH solution or 10 μL of GMA/PTH onto the ATRP initiator-functionalized substrate.
- 4.10 Quickly place a clean glass cover slip on the substrate to uniformly spread the solution and provide a vertical barrier to oxygen diffusion. Additionally a beaker can be placed to give stability to the setup (**Fig 11**).

Note

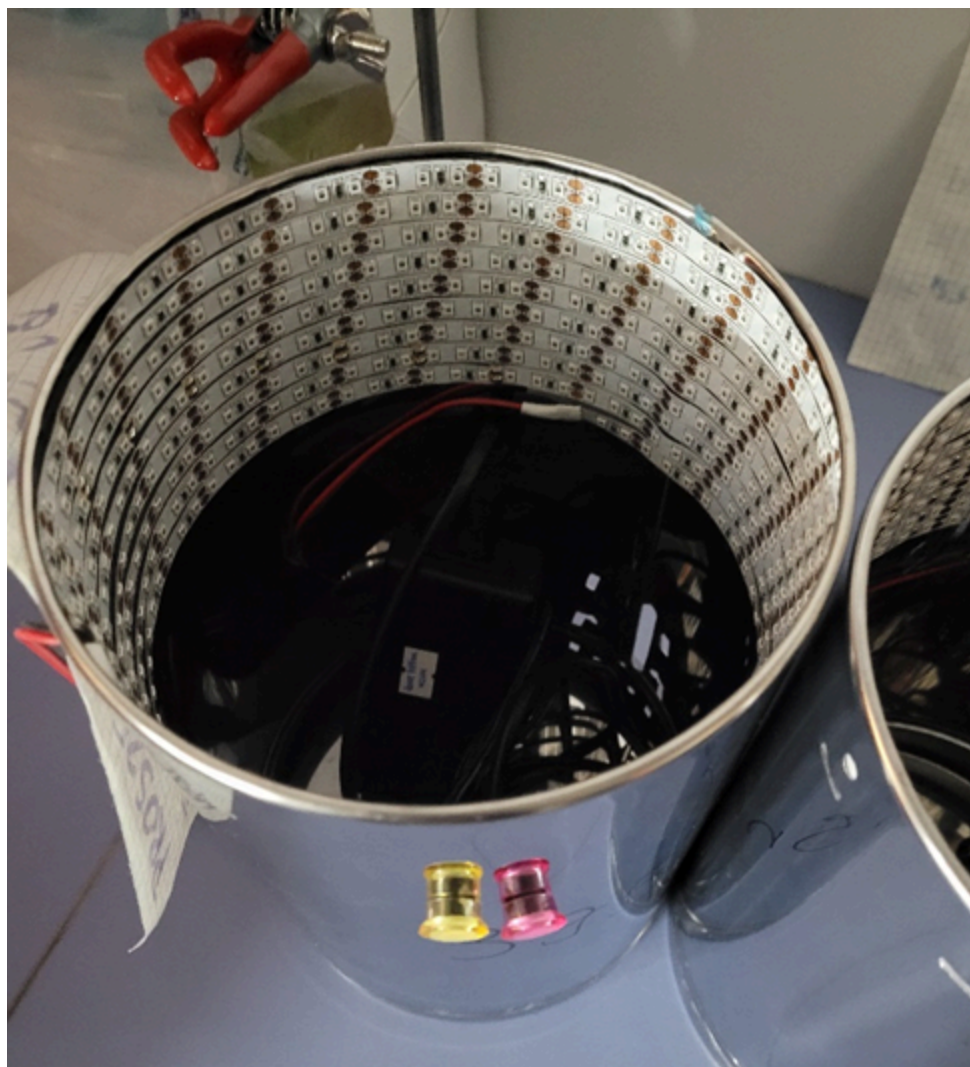
It is recommended that the cover slip is bigger than the substrate and is pre-cleaned with sonication in ethanol, argon flushing, and oxidation with ozone. Make sure that the glass used to cover the samples is transparent to UV light.

Note

Polymer brush growth proceeds without the need for degassing and can be regulated by adjusting either the concentration of PTH or the physical size of the cover slip. Using larger cover slips (relative to the wafer size) and higher PTH concentrations enhances the scavenging of both the initially dissolved oxygen in the solution and any oxygen that diffuses in from the edges of the cover slip or silicon wafer during polymerization (Narupai et al., 2018).

- 4.11 Irradiate with UV light under ambient conditions (10 min for PEGMA, 1 h for PGMA).

- 4.12 Remove the samples from the reactor and wash with DMAC (sonicating for 10 min), then rinse with toluene and dry with argon.
- 4.13 Store the samples under vacuum.
- 4.14 Lamps previously used: homemade LED reactor (Fig 12) (light intensity of $18 \pm 2 \text{ W m}^{-2}$ and a λ_{max} of $400 \pm 5 \text{ nm}$) and TLC UV fluorescent lamp (light intensity of 1 W m^{-2} , and 365 nm). Light excites the PTH, which acts as scavenger to remove dissolved oxygen as well as a catalyst for brush polymerization.



- 5 Place the substrate inside a 6 well plate. Add 100 μ l of RGD solution, place a glass cover slip on top and incubate overnight at 37°C under humidity conditions.
- 6 After incubation, remove the non-bound RGD by incubating 1 h with PBS at RT and then rinsing once more with sterile PBS. Reserve in the fridge at 4°C the samples for the next step.

Note

Samples modified with RGD can be kept in the fridge only for 1 day

Proof of concept: Brushes promoting MC3T3 adhesion.

- 7 **Objective:** Study cell adhesion, migration and proliferation compared to a cell treated well plate

Experiments with MC3T3

- 7.1 Place a wound healing insert on the sterile modified surface.
- 7.2 Seed the cells directly on the substrate according to the instructions provided by the manufacturer of the insert (70 μ l of 2.5×10^5 cells/ml per well, Culture-Insert 2 well by Ibidi).
- 7.3 Check the attachment and proliferation of the cells under the microscope after overnight incubation at 37°C, in 5% CO₂ atmosphere.
- 7.4 Remove the insert, take pictures of the wound gap, and incubate overnight at 37°C, in 5% CO₂ atmosphere.
- 7.5 Measure the wound closure
- 7.6 Stain with live/dead according to the manufacturer instruction
- 7.7 Induce and characterize bone development according to (Blanco Massani, *et al. under review*).



4. Fourth day

8 **Objective:** Rendering the polymer brushes with enzyme-responsive lytic efficacy against *S. aureus* to promote the competitive adhesion of MC3T3 cells to the implant surface in co-culture conditions.

8.1 Prepare M23-PP NPs according to (Blanco Massani et al 2024)

8.2 Incubate your modified substrates with 500 μl of the particles at RT during 1 h.

8.3 Remove the particles.

8.4 Irradiate the samples with UV light for 5 minutes.

8.5 Attach a wound insert to the surface.

8.6 Fill each well with 35 μl of 2.5×10^5 CFU/ml of the microorganism of interest.

8.7 Add 35 μl of 2.5×10^5 cells/ml of MC3T3.

Note

Controls of bacterial cells and MC3T3 only have to be included.

8.8 Incubate overnight in an incubator for mammalian cells at 37°C, in 5% CO₂ atmosphere.

Note

The plate should be introduced in a box isolated with a HEPA filter to avoid cross contamination. This experiment should be performed in a BSL2 lab for co culture with *Staphylococcus aureus*.



- 8.9 Plate 50 μ l of the content of each well using small agar plates to count bacterial cells (according to DOI: [dx.doi.org/10.17504/protocols.io.4r3l212wxg1y/v1](https://doi.org/10.17504/protocols.io.4r3l212wxg1y/v1)).
- 8.10 Incubate the agar plates overnight at optimal conditions
- 8.11 Remove the insert from the substrate, fix the cells, and stain for fluorescence microscopy as detailed in (Blanco Massani et al under review)
- 9 Inspect for bacterial growth after overnight incubation of the agar plates.

Protocol references

RGD pl/Mw tool: https://web.expasy.org/compute_pi/

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