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Modification of Materials With Bioactive Peptides

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1. Introduction

Bioactive polymeric materials can be constructed via the modification of bioinert polymers with bioactive peptides such as cell-adhesion peptides or growth factors. Such materials can be designed to induce specific biological responses desired in applications such as tissue engineering and regenerative medicine. For example, transforming growth factor-beta (TGF-β) has been immobilized to polymeric scaffold materials to increase the synthesis of extracellular matrix (ECM) proteins and thus improve the mechanical properties of the engineered tissue ***(1)***, and epidermal growth factor has been immobilized to improve the function of hepatocytes ***(2)***. Cell adhesion peptides have been grafted to polymeric scaffold materials in order to enhance and promote cell attachment and spreading ***(3–5)***. Alternatively, materials can be designed to be cell-selective, and adhesive for only a particular cell type, by modifying cell non-adhesive polymers with cell-selective adhesion ligands. Examples of cell-selective ligands include the peptide REDV, which interacts with endothelial cells but not platelets, fibroblasts, or smooth-muscle cells (SMCs) ***(6)***, and the peptide KRSR, which interacts with osteoblasts but not fibroblasts ***(7)***.

Bioactive materials can be designed in several ways, as outlined in **Fig. 1**. For many applications, surface modification is a good approach, especially given the expense associated with bioactive peptides (**Fig. 1A**). This approach usually involves surface modification to create functional groups, such as primary amines formed after ammonia plasma treatment, followed by covalent attachment of the peptides ***(8)***. However, surface modification is only appropriate for non-porous, non-biodegradable materials, and thus has limited utility in tissue engineering. Alternatively, peptides can be grafted to functional

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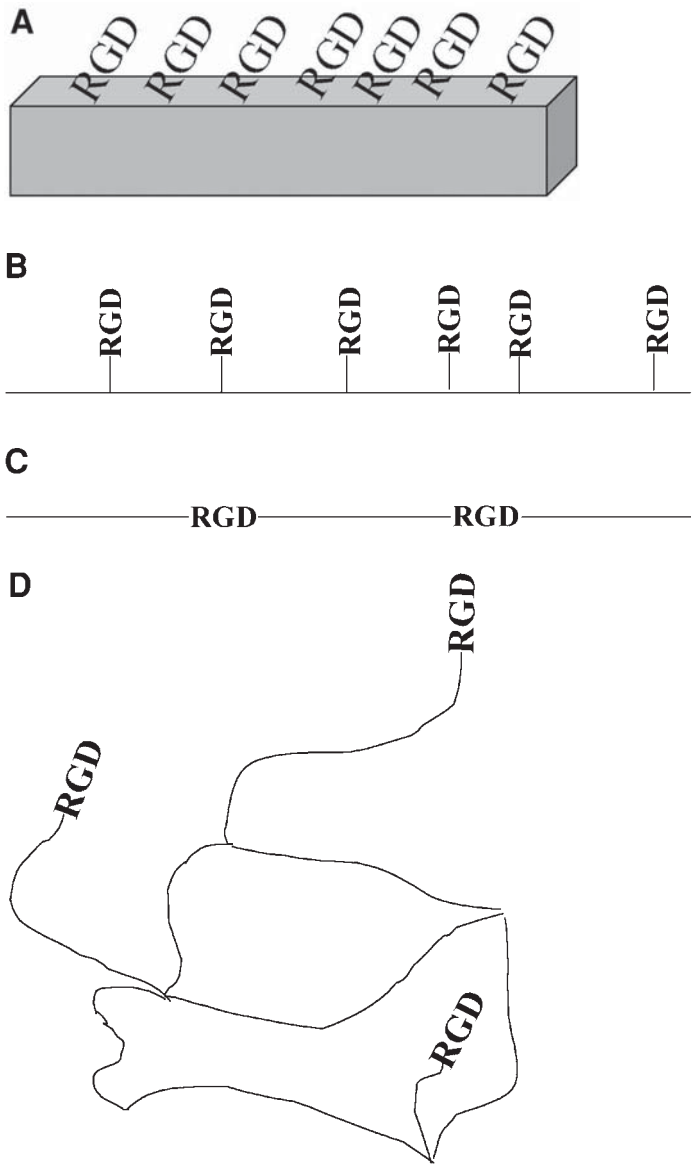

Fig. 1. Design of bioactive polymers with peptide modification. Biopolymers can be modified with bioactive peptides in several ways. As shown in **(A)** surface modification can be used for non-degradable, non-porous materials. Grafting peptides to polymer chains, as depicted in **(B)** or inclusion of peptides in the polymer backbone as shown in **(C)** have been used for bulk modification of biopolymers. Tethering approaches **(D)** have also been successfully utilized. The sequence RGD is shown here as an example of a bioactive peptide.

groups throughout the bulk of the polymer structure, as shown in **Fig. 1B**. This has been accomplished for amine-modified polyvinyl alcohol ***(9)*** and poly(lactide-co-lysine) copolymers ***(10)***. This approach provides uniform peptide density throughout the bulk of the material, thus making the bioactive moiety available to cells as they migrate through pore structures or as new surfaces are exposed during polymer biodegradation. A third approach is to incorporate the peptide into the polymer backbone, as shown in **Fig. 1C**. This approach has been used to synthesize polyethylene glycol copolymers with peptides targeted for proteolytic degradation ***(11,12)*** and polyurethanes with cell adhesion peptides ***(13)***. Additionally, crosslinked hydrogel materials have been designed that contain tethered peptides, as depicted in **Fig. 1D**. In this approach, several polymers with reactive groups such as acrylates or methacrylates are mixed in solution and then chemically crosslinked. One or more components of the polymer mixture can be modified with bioactive peptides. If the peptide-bearing polymer has only one reactive group, the peptide can be tethered into the hydrogel structure during the crosslinking process ***(12,14)***. One advantage of this approach is that many hydrogel materials are relatively cell non-adhesive ***(15,16)***, making this platform suitable for the development of cell-selective materials. Furthermore, providing the tethering chain has been shown to improve cellular interactions with the incorporated peptides, presumably by increasing the mobility of the peptide and decreasing steric hindrance ***(14)***.

In addition to the structural design of the bioactive polymer, it is important to consider and characterize the concentration of the incorporated peptide. In many cases, bioactive peptides can have the desired effects at the optimal concentration, but just the opposite at higher or lower concentrations. For example, many cell adhesion peptides can promote tissue formation by enhancing cell attachment and migration but will inhibit cell growth and ECM protein synthesis when presented at high concentrations ***(17,18)***. Another critical consideration is the use of controls for these types of studies. It is crucial to compare results with bioactive polymers, not only to unmodified polymers but also to polymers modified with scrambled amino acid sequences in order to ensure that the observed effects are indeed due to the effects of the bioactive peptide and not simply a change in the surface characteristics of the material.

This chapter describes a model surface—aminated glass—that can be used to investigate the effects of a bioactive peptide on cellular processes such as adhesion, migration, proliferation, and matrix protein synthesis. Using this system, it is easy to vary the peptide surface density, thus making it possible to identify optimal concentrations to use in biopolymer design. Several common chemistries that can be used to modify polymers with bioactive peptides are also described.

2. Materials

2.1. Preparation of Aminated Glass

1. Glass microscope slides.
2. 3:1 (vol/vol) 2 *N* sulfuric acid:2 *N* nitric acid mixture.
3. Acetone, dried with molecular sieves.
4. 100% ethanol, USP-grade.
5. Distilled water.
6. Aminopropyl triethoxysilane.
7. Rotary shaker.
8. Centrifuge.
9. Vacuum oven.

2.2. Evaluation of Amine Content Via the Ninhydrin Assay

1. Ninhydrin reagent solution (Sigma).
2. 0.1 *M* sodium citrate, pH 5.0.
3. 1 m*M* leucine prepared in 0.1 *M* sodium citrate.
4. Hot plate and water dish for boiling samples.
5. UV/vis spectrophotometer.

2.3. Coupling Peptides to Amine Groups Using EDC Chemistry

1. Aminated glass or polymer.
2. Bioactive peptide with amine groups blocked or protected (for example, acetylated via reaction with equimolar acetic anhydride in aqueous buffer, pH 7.4, for 2 h at room temperature).
3. 30 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC).
4. 0.4 mL ethylmorpholine.
5. 20 mL anhydrous dimethylformamide (DMF).
6. 100-mm glass Petri dish.
7. Oven set at 37°C.
8. Distilled water.
9. 4 *M* urea, if modifying glass.
10. Ultrasonic bath, if modifying glass.
11. Cellulose dialysis membrane, mol wt cutoff >500 Da, if modifying an aminated polymer.
12. Lyophilizer, if modifying an aminated polymer.

2.4. Coupling Peptides to Amine or Hydroxyl Groups Using DCC Chemistry

1. Amine or hydroxyl-bearing polymer, such as polyethylene glycol.
2. Bioactive peptide with amine groups blocked or protected (for example, acetylated via reaction with equimolar acetic anhydride in aqueous buffer, pH 7.4, for 2 h at room temperature).
3. 2 500-mL round-bottom flasks.

4. Dicyclohexyl carbodiimide (DCC).
5. Anhydrous dichloromethane (DCM).
6. Anhydrous DMF.
7. Anhydrous pyridine.
8. Ethyl ether.
9. Magnetic stir plate and stir bars.
10. Vacuum manifold.
11. Argon or nitrogen gas.
12. Vacuum oven.

2.5. Coupling Peptides to NHS Groups

1. N-hydroxysuccinimide (NHS) functionalized polymer, such as polyethylene glygol-NHS (PEG-NHS, Shearwater Polymers).
2. Bioactive peptide (to couple at N-terminus only, any side-chain amine groups should be protected, to couple at both ends of the peptide, an amine-bearing residue such as a lysine should be added to the C-terminus of the peptide sequence).
3. 0.1 *M* sodium carbonate, pH 8.5.
4. Orbital shaker.

2.6. Coupling Peptides to Isocyanates or Isothiocyanates

1. Isocyanate or isothiocyanate modified polymer, such as a prepolymer formed during polyurethane urea synthesis or polyethylene glycol isocyante.
2. Bioactive peptide (to couple at N-terminus only, any side-chain amine groups should be protected, to couple at both ends of the peptide, an amine-bearing residue such as a lysine should be added to the C-terminus of the peptide sequence).
3. 0.1 *M* sodium carbonate, pH 9.0.
4. Refrigerator.

3. Methods

3.1. Preparation of Aminated Glass

1. Clean glass slides with the sulfuric acid:nitric acid mixture in a chemical fume hood. Rinse thoroughly with acetone, then ethanol, then distilled water. Allow to dry.
2. In a graduated glass cylinder with a stir bar at the bottom, combine 95 mL ethanol and 5 mL distilled water. Stir well. Remove and discard 2 mL.
3. While stirring, add 2 mL aminopropyl triethoxysilane. Stir for 5 min.
4. Pour the ethanol:aminopropyl triethoxysilane mixture over the slides in a glass tray. Place on a rotary shaker and agitate for 10 min.
5. Replace the ethanol:aminopropyl triethoxysilane mixture with pure ethanol. Rinse slides thoroughly with the ethanol.
6. Place slides in a suitable centrifuge holder and spin at 260*g* for 10 min.
7. Place slides in a vacuum oven at 50°C overnight.

3.2. Evaluation of Amine Content Via the Ninhydrin Assay

1. Boil water on hot plate.
2. Put 15 mL of the sodium citrate buffer in a test tube. Add your modified glass or polymer.
3. Prepare serial dilutions of the leucine stock solution with the sodium citrate buffer in test tubes. An appropriate concentration range is often 0, 0.1, 0.05, 0.025, and 0.0125 m*M*.
4. Add 15 mL of ninhydrin reagent solution to each test tube.
5. Place tubes in boiling water and incubate for 15 min.
6. Remove tubes from boiling water and allow to cool to room temperature.
7. Read the absorbance of each sample at 570 nm.
8. Perform linear regression on the readings from the standards. Use this curve to calculate the amine content of your samples. This assay can be performed before and after peptide conjugation to determine the peptide density.

3.3. Coupling Peptides to Amine Groups Using EDC Chemistry

1. Place 30 mg EDC in Petri dish.
2. Add 0.4 mL ethylmorpholine.
3. Add 20 mL DMF.
4. Add an appropriate amount of the bioactive peptide (based on desired material design). Swirl dish to mix.
5. Place in an oven for 30 min.
6. Add the glass or polymer to the Petri dish. Continue to incubate at 37°C for another 3.5 h.
7. If modifying glass, remove slides and rinse with distilled water. Place slides in 4 *M* urea solution and sonicate for 10 min. Rinse again in water and allow to dry.
8. If modifying a polymer, add 20 mL of distilled water. Dialyze against distilled water for at least 12 h. Lyophilize the product.
9. Characterize the remaining amine content of your material using the ninhydrin assay described in **Subheading 3.2.** to determine the peptide density.

3.4. Coupling Peptides to Amine or Hydroxyl Groups Using DCC Chemistry

1. In a round bottom flask, dissolve 16 mmol of the peptide in 10 mL DCM and 2 mL DMF.
2. Add 8 mmol DCC. Stir for 30 min under argon or nitrogen at room temperature.
3. Filter to remove the dicyclohexyl urea that has formed.
4. In another round-bottom flask, dissolve the polymer in DCM.
5. Add the filtered peptide solution to the polymer solution. React for 12 h under argon or nitrogen at room temperature.
6. Precipitate the polymer with cold ether. Filter and wash with additional ether. Dry overnight in vacuum oven.
7. If a removable protecting group was used, the amine groups may be de-protected and further chemical modification can now be conducted.

3.5. Coupling Peptides to NHS Groups

1. Dissolve peptide in buffer at 1 mg/mL.
2. Dissolve polymer in buffer at appropriate concentration based on desired design.
3. Add polymer to peptide solution in a dropwise fashion. Place mixture on an orbital shaker and allow to react for 2 h at room temperature.

3.6. Coupling Peptides to Isocyanates or Isothiocyanates

1. Dissolve peptide in buffer at 1 mg/mL.
2. Dissolve polymer in buffer at appropriate concentration based on desired design.
3. Add polymer to peptide solution in a dropwise fashion. Mix well.
4. Incubate for 8 h at 4°C.

4. Notes

1. When preparing aminated glass, use of high-quality solvents and water is crucial to the success of the procedure.
2. Quantification of peptide density on modified material surfaces is imperative. Techniques such as ESCA/XPS can be used, but quantification of peptide density is generally easier using chemical assays such as the ninhydrin assay. Radiolabeling of peptides—for instance, by iodination of tyrosine residues—can also be used.
3. Glass slides fit well in 50 mL centrifuge tubes. These can be placed in a pyrex dish filled with boiling water during the ninhydrin assay.
4. In designing peptide sequences, performance will often be improved by placing spacer residues between the biologically active sequence and the material, whether it is polymer or glass. Several glycine residues are often used for this purpose.
5. Modified polymers should be characterized using NMR and GPC. FTIR, contact-angle analysis, and reverse-phase HPLC (RPHPLC) may also be useful in characterization. The unmodified base polymer should also be characterized by these methods for comparison.
6. Control peptides should always be used to ensure that any observed changes in cell behaviors are caused by the biological activity of the peptide and not simply a change in surface chemistry. Control peptides may be scrambled amino acid sequences or peptide sequences with one amino acid substitution. In cell adhesion studies, control groups may also be treated with soluble peptide to evaluate the competitive inhibition of cell attachment and spreading.
7. To create biopolymers that allow adhesion of only a particular cell type, a cell-selective peptide sequence can be attached to a cell non-adhesive polymer. Polyethylene glycol- and polyvinyl alcohol-containing materials have shown promise for this type of application.
8. To evaluate the activity of cell adhesion peptides after covalent modification, cell attachment and spreading should be evaluated using the desired cell type. The cell type used must have the receptor for the cell adhesion peptide employed.

In performing these experiments, FlexiPerm membranes can be used to create cell-culture wells on the surface of the modified material. Cell attachment can be assessed by determining the percentage of adherent cells at predetermined time-points over the first 24 h after seeding. Cell spreading can be evaluated by counting the number of spread cells and measuring the spread cell areas (via digital image processing) over the first 24 h after seeding.

9. When modifying materials with growth factors or other proteins, avoid exposure to organic solvents. Good bioactivity has been observed using both NHS and isothiocyanate protocols. Bioactivity of immobilized growth factors should be directly compared to the soluble, unmodified growth factor using a bioassay appropriate to the particular growth factor. For example, fibroblast proliferation may be assessed for materials modified with basic fibroblast growth factor (bFGF), and collagen production is a better bioassay for materials modified with TGF-β.

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