Macrocyclization and Separation of Cyclo-RGDfK:

A Sustainable Peptide Manufacturing Process

Figure 1: Cylco-RGDfK

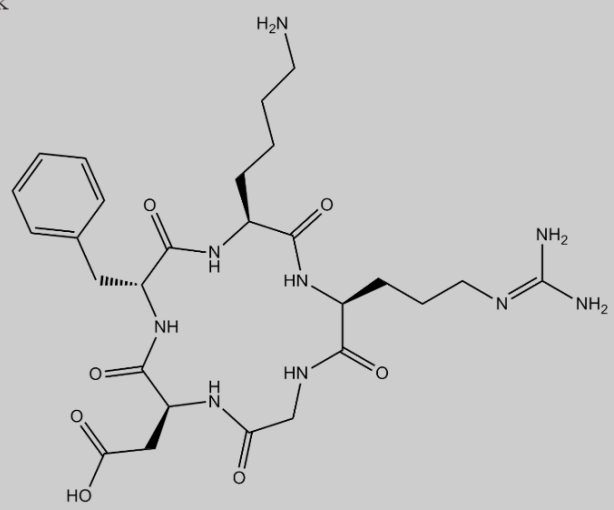


Figure 2: Microscopy Imaging of Radio-labeled Cardiomyocytes

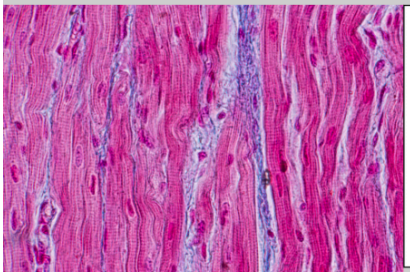
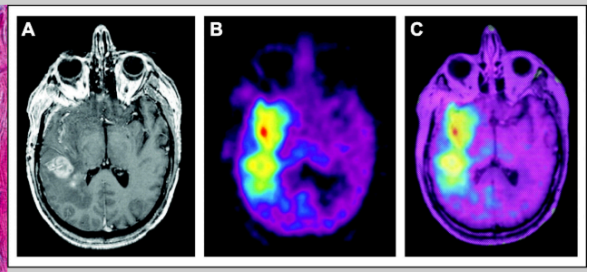


Figure 3: Contrast-enhanced magnetic resonance imaging (MRI) and methionine positron emission tomography (PET) study of a patient with recurrent anaplastic astrocytoma



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ChE 343 Final Project

Green Chemistry & Traditional Peptide Synthesis:

From a fundamental framework, Green Chemistry, introduced by Anastas and Warner in the late 1990s, has become the foundational philosophy of modern chemical engineering process design. The concept's principles emphasize waste reduction, atom economy, minimizing hazardous reagents, using safer solvents, enhancing energy efficiency, and designing inherently sustainable synthesis pathways. As demand for pharmaceuticals, sustainable energy solutions, and materials with highly specific properties increases, so does the responsibility of chemical engineers to redesign processes that minimize environmental impact and reduce public risks. [1-3]

Peptides, defined as short chains of amino acids that function as the building blocks of proteins and as critical signalling molecules across biological systems, are a particularly crucial target of green chemistry. Traditional manufacturing methods are particularly waste-intensive, highly dependent on large amounts of highly hazardous reagents and solvents, and an overall safety risk; an ironic contrast to the growing regulatory and societal expectations to utilize benign substances in pharmaceutical manufacturing.

Introduction of Cyclo-RGD Peptides & Cyclo-RGDfK:

Among manufactured bioactive peptides, cyclo-RGD molecules are cyclic derivatives of the Arginine-Glycine-Aspartate motif and are among the most widely used molecular tools in biotechnology, biomedical research, and medicine.

Cyclo-RGDfK consists of five amino acids: Arginine (R), Glycine (G), Aspartic Acid (D), D-Phenylalanine (f), and Lysine (K). These amino acids are connected in a cyclic backbone via a peptide bond between the N-terminus of Arginine and the ε-amino side chain of Lys. Cyclo-RGDfK is primarily purchased by research laboratories, biotechnology companies, and pharmaceutical development companies. Although purity and formulation of the purchased peptides vary, research-grade cyclo-RGDfK is typically sold in milligram quantities at about $100 per milligram. Annual production and volume sold of specific peptides is not currently publicly available data as cyclo-RGDfK is a high value specialty peptide, however, the global cyclic peptides market was recently estimated to be valued at US ~$3.16 Billion in 2024 and furthermore, the RGD peptide market is projected to break US $1.5 billion in 2025 with a compound annual growth rate (CAGR) of 12%. [9-10] For all intents and purposes, the design basis being evaluated assumes 1 kg/year of cyclo-RGDfK in order to illustrate the potential economic upside of a greener synthesis process. Currently, cyclo-RGDfK is sold at ~$2990/gram, or roughly $2,990,000 in total revenue annually.

These peptides function as high-affinity ligands for the αvβ3 and α5β1 integrins, which are receptors that regulate cell adhesion, migration, angiogenesis, and extracellular matrix interactions. [4-6] The cyclic backbone, illustrated by Figure 1 on the title page, allows for chemically unique characteristics such as stability, specificity, and binding affinity when compared to linear motifs. As a result, cyclo-RGD conjugates have become a popular and requisite molecule amongst a broad range of applications. Within that range, conjugates are widely used in targeted drug delivery, biomaterial functionalization, and studies of attachment and migration. More specifically, they are prevalent in regenerative medicine, tumour biology, and research on human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC CMs), such as the work being done here at the University of Michigan's Baker Laboratory. [6-8] Clinically, radiolabeled cyclo-RGD derivatives, done so by conjugating a radioisotope to a chelator attached to the peptide, function as tracers for positron emission tomography (PET)

and single photon emission computed tomography (SPECT) to visualize integrin-rich tissue associated with cancer progression and cardiovascular diseases. [7] The range and depth of these applications demonstrate the scientific and commercial relevance of cyclo-RGD peptides and molecules like them, and further support their specific synthesis selection for a greener process design.

Classical Synthesis of Cyclo-RGDfK:

As cyclo-RGDfK is a specialized peptide produced on a laboratory or pilot scale, batch sizes typically range from 1 to 100 milligrams. From a chemical engineering standpoint, the microscale production of high-value biomolecules is an often-overlooked field in the synthesis optimization conversation. Despite the scaling problem, cyclo-RGDfK synthesis faces the same constraints as large-scale chemical operations, including mass transfer limitations, energy-intensive operations, solvent cycling, and hazardous reagent management.

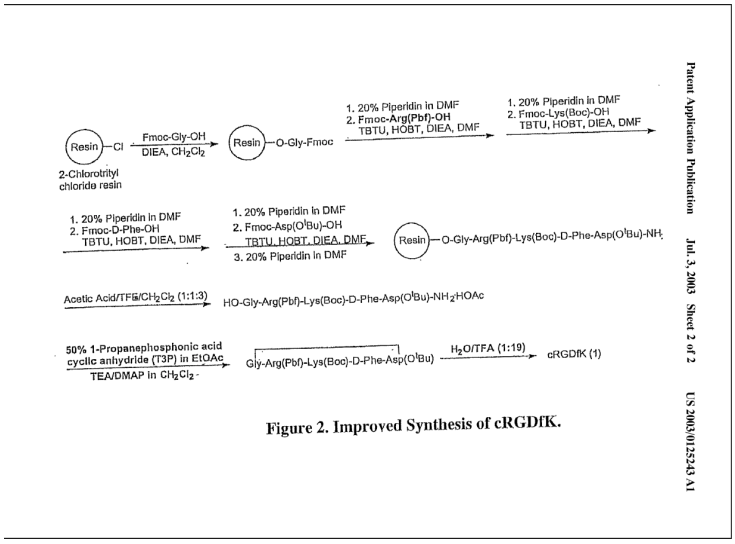


Figure 2. Improved Synthesis of cRGDfK.

Figure 4: Figure 2 from US Patent US20030125243A1, *Synthesis of Cyclic Peptides*

A widely utilized route to synthesis, and a patented process assigned to the Massachusetts Institute of Technology, US patent US20030125243A1, issued in 2003, describes an older but classical "*Synthesis of Cyclic Peptides*". Jun Liu, Xuedong Dai, and Zhuang Su, the inventors of the synthesis, start with: (1), 2-Chlorotrityl chloride resin (Resin-Cl) and then proceed to react the resin with Fmoc-Glycine-OH in a CH2Cl2 solution utilizing DIEA as a base. This first reaction forms a Resin-Oxygen-Glycine-Fmoc motif, anchoring glycine to the solid support. Following this initial reaction: (2), (i), the resin is treated with 20% piperidine in DMF, removing the protecting group, Fmoc, on glycine, then, (ii), the coupling of Fmoc-Arginine(Pbf)-OH using TBTU/HOBt/DIEA in DMF solution is utilized to add Arginine to the amino acid motif. This is repeated with Lysine, D-Phenylalanine, and finally Asparagine in this order, and then the final rinse with 20% piperidine in DMF removes the Fmoc protecting group. At this stage the linear motif is Resin–O–Gly–Arg(Pbf)–Lys(Boc)–D-Phe–Asp(OtBu), where the amino acids with reactive side chains, Arg, Lys, Asp, still maintain their protecting groups to prevent prevent potential side reactions, like aspartimide formation, and to prevent premature cyclization, the formation of a cyclic structure.

Once the desired linear motif is acquired (3), the resin with added linear RGDfK motifs is put into an acetic acid/TFE/CH2Cl2 (1:1:3) solution in order to cleave the motif from the solid resin,

producing: HO-Gly-Arg(Pbf)-Lys(Boc)-D-Phe-Asp(O'Bu)-NH2HOAc. Next, (4), macrocyclization takes place using 50% 1-Propanephosphonic acid cyclic anhydride (T3P) in EtOAc, with TEA/DMAP in CH2Cl2, forming the macrocyclic intermediate shown in Figure 4, labelled “Figure 2” in the patent image. This consists of initiating an intramolecular lactam formation, an amide bond, between the ε-amino terminus of the Asparagine, and the β-carboxylate of the Glycine. In the final stage (5), H2O/TFA (1:19) is used to treat the macrocyclic intermediate and remove the Pbf, Boc, and OtBu side-chain protecting groups, leaving the final product cyclic-RGDfK, although not quite concentrated.

The patent further notes that, following cyclization, the crude product is “easily” purified by silica gel chromatography, thereby further reducing the need for diphenyl phosphoryl azide (DPPA). Silica gel chromatography, also known as flash chromatography, uses a silica gel polar stationary phase and a nonpolar mobile phase, typically an organic solvent such as hexane, EtOAc, or CH2Cl2, to separate molecules based on polarity and adsorption. This process is iterated multiple times, requiring an additional solvent-intensive step to isolate the desired cyclo-RGDfK isomer.

All synthetic details in this section are adapted from: (12) Liu, J.; Dai, X.; Su, Z. Synthesis of cyclic peptides. U.S. Patent Application US 2003/0125243 A1, July 3, 2003.

Sustainability and Health Concerns of US Patent US20030125243A1 *Synthesis of Cyclic Peptides:*

The solid-phase and solution-phase synthesis of cyclo-RGDfK, outlined in *Synthesis of Cyclic Peptides,* exhibits several safety and environmental drawbacks when evaluated through the modern chemical engineering lens. First, the route relies heavily on large volumes of hazardous polar aprotic solvents, including DMF, a regulated reproductive toxin used in every deprotection and coupling cycle of the amino acid polymerization. Each iteration requires a DMF wash, substantially increasing the solvent footprint as rinsing solvents are generally used in excess to maximize deprotection and coupling efficiency. [12,13] The macrocyclization step employs propanephosphonic acid cyclic anhydride (T3P) in EtOAc/CH2Cl2 solution. T3P’s chemical classifications detail high flammability, acute inhalation toxicity, and reproductive toxicity. T3P also generates phosphorus-constraining byproducts as waste from the macrocyclization step, so the batch reaction must be conducted under high-dilution conditions to suppress oligomerization. [14] Concerning the batch reactor itself, because reactions continue to run without product and byproduct removal, the synthesis runs the risk of temperature and/or acidic buildup that may degrade desired peptides. Silica gel chromatography, a normal-phase process that consumes large quantities of organic solvents alike (EtOAc, CH2Cl2, and hexane), must be repeated multiple times to isolate the product. [12] Flash chromatography, synonymous with silica gel chromatography, is often an unsustainable purification method due to low solvent recovery, high adsorbent waste, and low flow rates. Collectively, although the patented process has demonstrated advancements with the exclusion of DPPA, TFA, and Mtr, three hazardous reagents, continuing to refine peptide synthesis to reduce the manufacturing footprint is a crucial step as the specialized peptide industry continues to grow.

Description of Thiol-Ene Separation Units:

Following the same SPPS resin process to acquire the Resin–O–Gly–Arg(Pbf)–Lys(Boc)–D-Phe–Asp(OtBu) motif, the improved synthesis employs a streamlined separation train enabled by the cleaner impurity profile of thiol–ene macrocyclization [13,14]. First, membrane filtration removes resin particulates and enables an

early transition to fully aqueous media, minimizing exposure to toxic organic solvents. Continuous flow thiol–ene macrocyclization, in which linear thiolated peptides and an alkene bearing partner are pumped through a UV-irradiated microreactor. It is in the microreactor that a controlled flux of radicals drives intramolecular thioether bond formation without dilute conditions. Following the reactor, the product undergoes its first separation, an inline liquid-liquid phase separation. This allows for rapid removal of photoinitiators, molecules that absorb light and initiate chemical reactions, into a recyclable organic phase. Ultrafiltration using membrane filters removes low-molecular-weight impurities, including radical initiators, buffer salts, and unreacted thiols. This allows the concentrated cRGDfK to pass through more concentrated. A single preparative RP-HPLC run is performed to achieve final purification to >95% purity, using ethanol-water or low-acetonitrile gradients to reduce environmental impact. Vacuum solvent removal purifies cRGDfK at freezing temperatures prior to lyophilization, a process that removes water from a frozen product by sublimation, yielding a stable, dry peptide solid. Finally, milling and packaging produce a flowable powder suitable for distribution. [15,16]

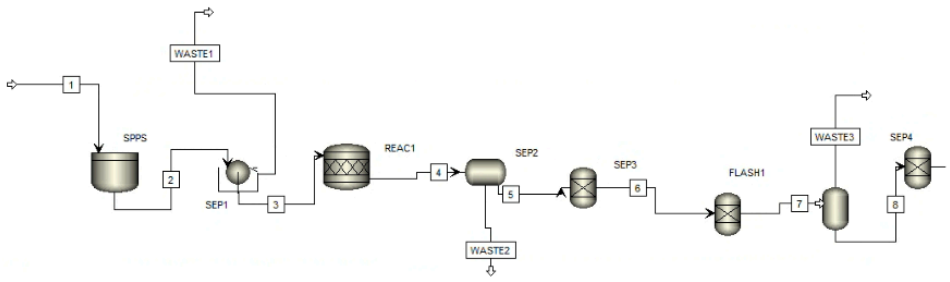
Thiol-Ene Separation Units Selection Reasoning:

Both the chemical properties of cRGDfK and the unique benefits of thio-ene macrocyclization are realized in the specific sequence of separation operations. Filtration and washing remove the resin from the linearized RGDfK motifs and prepare the incomplete peptides for thiol-ene chemistry using an aqueous rinsing solution. [15] Continuous-flow macrocyclization, rather than the standardized batch reaction used in this synthesis, is selected because thiol-ene chemistry yields high product under mild conditions. In turn, it's best to select a reactor/reaction type that avoids both thermal and acidic conditions that can lead to byproduct buildup in a batch reactor. Avoiding buildup additionally protects the peptides from potential degradation [16]. An inline liquid-liquid separator takes the continuous flow from the reactor and prevents reactive reagents from traversing downstream, furthermore reducing the burden on other separation features. Ultrafiltration is then placed directly before the chromatography because thiol-ene-mediated macrocyclization reduces isomeric impurities. As a result, flash chromatography is replaced by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) due to reduced purification requirements. Essentially, the new cyclization type takes the load off the front end of the chromatography. Frozen drying and purification via sublimation are then used because they are the most optimal methods to prevent peptide degradation. [16]

Expected Yield, Performance of Steps and Overall Process:

Thiol-ene macrocyclization and typical SPPS performance, improved synthesis is expected to achieve an overall isolated cRGDfK yield of approximately 75%-80%. Nearly double the previously expected ~40~50% of the US20030125243A1 route. [12, 15,16] Individual steps such as SPPS cleavage (~95%), thiol–ene macrocyclization (~90–95%), UF/DF membrane purification (~95–97%), and the reduced-load single-pass RP-HPLC (~90–93%) each contribute to the overall efficiency. The burden on chromatography from previous processes is nearly eliminated by the thiol-ene reaction, resulting in higher purity and lower overall solvent use and operating costs. Economically, reducing DMF, DCM, EtOAc, silica gel, and the high-dilution bath macrocyclization yields a greener process more suitable for small-scale cyclo-RGDfK manufacturing.

Thiol-Ene Separation PFD:



Appendix:

1 - Reagents for SPPS Linear cRGDfK motif
2 - Resin–O–Gly–Arg(Pbf)–Lys(Boc)–D-Phe–Asp(OtBu)
SEP 1 - SPPS cleavage and filtration
3 - HO-Gly-Arg(Pbf)-Lys(Boc)-D-Phe-Asp(O'Bu)-NH2HOAc
REAC1 - Continuous flow thiol-ene macrocyclization
4 - cRGDfK an various byproducts
SEP2 - Ultrafiltration/Diafiltration Unit
5 - Partially filtered product
SEP 3 - RP-HPLC Unit
6 - >95% purity cRGDfK
FLASH1 - Vaccum solvent removal
7 - Frozen (low temperature) product steam
SEP4 - Lyphilization

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