



Full length article

Evaluation of the hemocompatibility and rapid hemostasis of (RADA)₄ peptide-based hydrogels

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ABSTRACT

(RADA)₄ peptides are promising biomaterials due to their high degree of hydration (<99.5% (w/v)), programmability at the molecular level, and their subsequent potential to respond to external stimuli. Interestingly, these peptides have also demonstrated the ability to cause rapid (~15 s) hemostasis when applied directly to wounds. General hemocompatibility of (RADA)₄ nanofibers was investigated systematically using clot formation kinetics, C3a generation, and platelet activation (morphology and CD62P) studies. (RADA)₄ nanofibers caused a rapid clot formation, but yielded a low platelet activation and low C3a activation. The study suggests that the rapid hemostasis observed when these materials are employed results principally from humoral coagulation, despite these materials having a net neutral charge and high hydration at physiological conditions. The observed rapid hemostasis may be induced due to the available nanofiber surface area within the hydrogel construct. In conclusion, our experiments strongly support further development of (RADA)₄ peptide based biomaterials.

Statement of Significance

Biomedicine based applications of (RADA)₄ peptides are being extensively studied for the purpose of improving drug carriers, and 3D peptide nanofiber scaffolds. However, this peptide's biocompatibility has not been investigated till now. One particular study has reported a revolutionary and very desirable ability of (RADA)₄ peptide to achieve complete and rapid hemostasis, nevertheless, the literature remains inconclusive on the underlying molecular mechanism. In this manuscript we bridge these two main knowledge gaps by providing the much needed systematic biocompatibility analysis (morphology analysis, platelet and C3a activation) of the (RADA)₄ based hydrogels, and also investigate the underlying hemostatic mechanism of this peptide-induced hemostasis. Our work not only provides the much-needed biocompatibility of the peptide for applicative research, but also explores the molecular mechanism of hemostasis, which will help us design novel biomaterials to achieve hemostasis.

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1. Introduction

Self-assembling peptides have various tissue-engineering applications such as drug delivery [1], membrane protein stabilization [2], 3D peptide nanofiber scaffolds, etc., [3,4]. Particularly, (RADA)₄ peptide based hydrogels have traits that make them amenable for soft-tissue applications, in physiologically relevant solutions [5,6]. These traits include, net neutral charge, applicable to minimally

invasive therapies (i.e. injectable), able to respond to external stimuli under physiological conditions, facilitate 3-D cellular activities, while maintaining an internal hydration of up to 99.5% (w/v) water [7–9]. Also, (RADA)₄ based hydrogels have been observed to achieve complete hemostasis in ~15 s, without employing any of the traditional hemostatic therapies, such as cauterization, vasoconstriction, coagulation, pressure application or the use of cross-linked adhesives [10]. Many protein (such as, collagen and fibrin) based biomaterials have been studied extensively in the past for the purpose of achieving hemostasis, but they all resulted in limited success due to the lack of severe hemorrhage control [11,12]. However, (RADA)₄ based hydrogels have been reported to not only have achieved complete, but rapid hemostasis under

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severe hemorrhagic conditions [10]. Despite the seemingly revolutionary hemostatic capabilities of (RADA)₄ hydrogels, the correlations between underlying material properties with the general hemocompatibility of these materials have not been thoroughly investigated [10,13]. Elucidation of the mechanisms that underlie this somewhat surprising hemostatic ability may hold a key that will facilitate a revolutionary approach to the design of blood-contacting biomaterials.

The primary physiological response to any biomaterial involves the accumulation of a layer of host proteins derived from plasma or interstitial fluids and that this protein–surface interaction may dictate subsequent host responses [14,15]. Previous work has shown that material properties can have a profound effect on how biomaterials interact with protein rich solutions like blood. In fact, fundamental studies have shown that, for surfaces, the key for limiting protein–surface interactions seems to depend upon having hydrophilic, electrically neutral, and hydrogen-bond acceptor moieties [16]; also present on the (RADA)₄ systems employed in this study. Hence here, we assess the hemocompatibility of the (RADA)₄ peptide by investigating biomaterial induced immunological and inflammatory responses including the activation of coagulation and complement systems.

A few studies have demonstrated the first applications of (RADA)₄ in nanotechnology to achieve complete and rapid hemostasis [10,13]. One of these studies investigated (RADA)₄ as an extracellular matrix to facilitate neuronal regeneration, but instead discovered immediate hemostasis when applied directly to open wounds [10]. Interestingly, the mechanism was not tissue specific, as complete hemostasis was observed in various organs and tissues: brain, spinal cord, high pressure femoral artery wounds, highly vascularized liver wounds and skin punches [10]. In an attempt to understand the underlying mechanism responsible for this rapid onset of hemostasis various concentrations of the (RADA)₄ peptide were studied (1.0%, 2.0%, 3.0% and 4.0% (w/v)), and the transmission electron microscopy (TEM) of the tissues were also conducted, however, the resulting observations were somewhat inconclusive [10]. Further work [13] focused on this topic also concluded that the longer the (RADA)₄ nanofibers, the higher was the storage modulus (*G'*), resulting in more control of bleeding. However, even though these studies had similar theories about the rapid hemostasis *via* (RADA)₄ nanofibers, they lack the experimental evidence relating molecular assembly of these fibers with their hemostatic capabilities, as well as any biocompatibility analysis. Therefore, in this work we report the *in-vitro* evaluation of (RADA)₄ based hydrogels hemocompatibility involving: complement activation, platelet morphology and activation, as well as clot formation kinetics.

Complement is a key component of humoral immunity, and its activation leads to the enzymatic cleavage of C3 into C3a and C3b; where C3a mediates an array of inflammatory responses such as smooth muscle contraction, histamine release from mast cells, vasodilation, increased vascular permeability, and chemotaxis [17]. Whereas platelets serve in hemostasis to preserve the integrity of the vascular wall through the formation of a platelet plug [18]. Material-induced platelet activation is associated with undesirable thrombotic complications of the devices, and is rapid and occurs *via* platelet attachment, membrane alteration, spreading, the release of granule contents and aggregation [19–21]. Herein, platelet activation was determined *via* monitoring the expression of CD62P protein on the activated platelets (for CD42 positive platelets) [22,23]. A detailed morphological analysis of platelets upon incubation with (RADA)₄ nanofibers was also conducted and analyzed using a morphology score. Clotting kinetics for these hydrogels were evaluated using a turbidimetric assay using recalcified, platelet-poor human plasma. The lack of platelets in this assay allows for the analysis of the clot formation purely as a function

of protein–surface interactions: surface activation leading to clot formation. Hence, the rapid hemostatic ability of the (RADA)₄ peptide nanofibers was evaluated not only as a function of platelet activation but also *via* cell free clot kinetics. This work provides an in-depth hemocompatibility analysis of hydrogels composed of (RADA)₄ nanofibers for the first time.

2. Materials and methods

Briefly, whole blood was collected from three healthy unmedicated donors, who provided informed consent in accordance with the current CBS and National Ethics Board standards. Blood collected in this manner was used for all subsequent plasma related assays, as described below.

2.1. Peptide synthesis

Self-assembling peptide, (RADA)₄ ([Ac]-RADARADARADARADA-[NH₂]) was commercially synthesized and purified by SynBioSci (Livermore, CA) using Fmoc amino acid derivatives. High performance liquid chromatography and mass spectrometry were used to determine peptide purity and molecular weight (MW). Peptide purity was ~98%, with a calculated MW of 1713.2 that was very similar to the expected MW of 1713.8. These peptides were used without further purification.

2.2. Hydrogel preparation

Peptide solution was prepared by dissolving (RADA)₄ peptide powder in syringe-filtered (0.2 μm) MilliQ water. Hydrogels were prepared by sonicating the aqueous peptide stock solution (2510 Branson sonicator, Crystal Electronics, Newmarket, ON) for 15 min at 25 °C. Aqueous peptide solutions were diluted with syringe filtered, 10× phosphate buffer saline (Sigma–Aldrich P7059, pH 7.4) such that the final working peptide concentrations were 0.5, 1.0, 2.0 or 3.0% (w/v) and so that a final 1× PBS (150 mM) solution was obtained. Solution pH was adjusted to 7.4 through drop wise addition of concentrated NaOH and/or HCl. Peptides were allowed to self-assemble at 37 °C for 30 min, and stored overnight at 4 °C. Prior to use, peptide solutions were sonicated for 30 min at 37 °C, pipetted as needed, and rested for at least 30 min to allow time for complete self-assembly [24].

2.3. Transmission electron microscopy

Samples were loaded onto perforated formvar carbon coated copper grids (Ted Pella, Inc.). A 4% uranyl acetate stain was applied to the peptide samples. All TEM was performed on an FEI Morgagni electron microscope.

2.4. Plasma clot analysis

The plasma clot assay was conducted as previously described [25]. Various concentrations of the (RADA)₄ hydrogels (0.5% (w/v) to 3.0% (w/v)) were thoroughly mixed and incubated with an equal volume of platelet-poor human plasma for 30 min at 37 °C in a 96 well microtiter plate. An equal volume of 0.025 M CaCl₂ was injected into the wells and the optical density at 405 nm was measured with a BioTek ELx808 plate reader, at 1-minute intervals over a period of 60 min. All experiments were repeated four times.

2.5. Complement C3a activation

Complement C3a studies were performed using platelet-poor human plasma with a commercially available kit (Quidel, San Diego, CA) as per the manufacturer's protocol. Briefly, plasma

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