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Arginine-based polyester amide/polysaccharide hydrogels and their biological response

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ABSTRACT

An advanced family of biodegradable cationic hybrid hydrogels was designed and fabricated from two precursors via a UV photocrosslinking in an aqueous medium: unsaturated arginine (Arg)-based functional poly(ester amide) (Arg-UPEA) and glycidyl methacrylate chitosan (GMA-chitosan). These Arg-UPEA/GMA-chitosan hybrid hydrogels were characterized in terms of their chemical structure, equilibrium swelling ratio (Q_{eq}), compressive modulus, interior morphology and biodegradation properties. Lysozyme effectively accelerated the biodegradation of the hybrid hydrogels. The mixture of both precursors in an aqueous solution showed near non-cytotoxicity toward porcine aortic valve smooth muscle cells at total concentrations up to 6 mg ml^{-1}. The live/dead assay data showed that 3T3 fibroblasts were able to attach and grow on the hybrid hydrogel and pure GMA-chitosan hydrogel well. Arg-UPEA/GMA-chitosan hybrid hydrogels activated both TNF-α and NO production by RAW 264.7 macrophages, and the arginase activity was also elevated. The integration of the biodegradable Arg-UPEA into the GMA-chitosan can provide advantages in terms of elevated and balanced NO production and arginase activity that free Arg supplement could not achieve. The hybrid hydrogels may have potential application as a wound healing accelerator.

1. Introduction

Wound healing is closely related to the metabolism of arginine (Arg), particularly in the inflammation and proliferation phases [1,2]. In many experimental wound model studies, activated macrophages avidly consumed or depleted arginine from culture medium via the action of inducible nitric oxide synthase (*i*NOS) and arginase [3,4]. The process of Arg metabolism generates several essential nitrogen-containing compounds, including creatine, polyamines, agmatine and nitric oxide (NO) [5]. Arg is the sole substrate for NO synthesis in biological systems, as NO is synthesized from Arg by the activity of nitric oxide synthase (NOS). In wound healing, NO production has an antibacterial function and is also critical to wound collagen accumulation for restoring mechanical strength [6]. Moreover, through the action of arginase, Arg is converted to urea and ornithine, generating polyamines, including putrescine, spermine and spermidine [2,7]. Ornithine is a precursor for proline, which serves as the substrate for collagen synthesis, whereas polyamines are involved in cell proliferation [8].

Because of the Arg's role in wound healing, free Arg supplement to patients has been suggested and reported. For example, free Arg therapeutic treatment was applied to diabetic ulcer wounds, but the outcomes were not greatly improved [9–15]. Continuous supplemental arginine infusion produced significant and sustained increases in NO production in wound fluid, but no significant difference in concentrations of ornithine, citrulline or proline was found [9]. The lack of significant wound healing improvement may be attributed to such sustained NO production that may inhibit arginase function, thereby limiting polyamines and ornithine synthesis, which are also crucial to wound healing [9–17]. Moreover, free Arg through oral administration is easily dissolved in plasma and distributes systematically, instead of concentrating locally around the wound site.

Wu et al., Song et al. and Pang et al. [18–24] reported the incorporation of Arg into the design and synthesis of a new family of

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synthetic biodegradable amino acid-based poly(ester amide)s (AA-PEA) biomaterials. Functional AA-PEAs have recently been reported to be a viable family of biodegradable biomaterials that have both protein and non-protein characteristics, and hence are referred to as "pseudo-proteins" [25,26]. Wu et al. reported that those Arg-based PEAs have some unique biological properties, such as improving cell attachment and proliferation [18–21], easy penetration through cell membranes, and the capability of capturing and delivering DNA as non-viral gene transfection vectors at a much lower level of cytotoxicity than Lipofectamine 2000® and Superfect® [22,23]. Among the Arg-PEA biomaterials reported, one particular series of cationic Arg-based PEAs has photocrosslinkable double bonds on its polymer backbone (Arg-UPEA) [18,20]. These functional Arg-UPEAs were used as co-precursors to fabricate photocrosslinked hybrid hydrogels with synthetic functional pluronic acid diacrylate (F127) with a significant improvement in cellular adhesion and proliferation [18]. Like polyethylene glycol, F127 co-precursor, however, is a relatively biologically inert biomaterial, and in its hybrid with Arg-UPEA, F127 did not contribute any biological function, and therefore did not have a synergistic effect with Arg-UPEA. In addition, the Arg-UPEA/F127 hybrid hydrogels were relatively mechanically weak, with a compression modulus ranging from 0.77 to 4.35 kPa.

In the present study, a modified but biologically active polysaccharide from chitosan was used as a co-precursor to fabricate an advanced generation of Arg-UPEA-based hybrid hydrogels for improving both the mechanical properties and the potential biological benefits of the resulting hybrids. Chitosan is a linear polymer of *N*-acetyl-D-glucosamine and a deacetylated glucosamine, which could be used as a wound-healing accelerator in clinical and veterinary medicine [27,28]. Shibata et al. [29] observed that the chitin oligosaccharides could activate the macrophage production of TNF-α and interleukin-12. The stimulation relies on the acetylated units. The mechanism of chitosan-induced macrophage activation involves mannose receptor-mediated phagocytosis. During inflammation, the mannose receptor is highly regulated on local macrophages, increasing the possibility to interact with the suitable ligands such as chitosan. Chitosan signaling through the up-regulated mannose and other possible receptors is one possible mechanism for the enhancement of the arginase pathway [28]. Glycidyl methacrylate modified chitosan (GMA-chitosan) was very recently synthesized by a new improved method, which could also achieve a much higher degree of GMA substitution with high yields [30]. GMA-chitosan is water soluble and biocompatible and, owing to the presence of the pendant photo-reactive vinyl group, GMA-chitosan can be photocrosslinked to form biodegradable hydrogels by itself (i.e. without a co-precursor) [30].

The present paper reports on the feasibility of integrating this newly developed GMA-chitosan polysaccharide with Arg-UPEA, their characterization and some preliminary in vitro biological properties with a view to pursuing future in vivo study. The resulting hybrids could have the merits of both biologically active polysaccharides (i.e. chitosan) and pseudo-proteins (i.e. Arg-based PEAs) biomaterials. Such an integration could lead to an advanced family of biodegradable and biologically active biomaterials for a variety of biomedical applications, such as a potential treatment option to accelerate wound healing, which is difficult using a traditional free Arg supplement (dietary or infusion). Arg-UPEA/GMA-chitosan-based hybrid hydrogels may have potential as model biomaterials in the study of wound healing, because they are able to provide an Arg-rich environment in situ. In addition, the hydrophilicity and soft, flexible nature of Arg-UPEA-based hybrid hydrogels may make this family of advanced biomaterials suitable for the treatment of wound healing.

2. Materials and methods

2.1. Materials

L-Arginine (L-Arg), fumaryl chloride, ethylene glycol, 1,4-butanediol were purchased from Alfa Aesar (Ward Hill, MA). *p*-Toluene sulfonic acid monohydrate ($TsOH{\cdot}H_2O$), *p*-nitrophenol (J.T. Baker, Philipsburg, NJ) and triethylamine (Avantor Performance Materials, Center Valley, PA) were used without further purification. Solvents including toluene, isopropyl alcohol, *N*,*N*-dimethylacetamide and DMSO were purchased from VWR Scientific (West Chester, PA); ethyl acetate and acetone were purchased from Mallinckrodt (St. Louis, MO). All solvents were ACS grade and used without further purification. Chitosan (77% deacetylated) of molecular weight (MW) 150 kg mol^{-1} and bovine serum albumin (BSA) of molecular weight ~66 kg mol^{-1}, polyethylene glycol diacrylate (PEGDA, $M_n \approx 750$) and α-isonitrosopropiophenone were purchased from Sigma Chemical Company (St. Louis, MO). GMA (97%), 4-(*N*,*N*-dimethylamino) pyridine (DMAP, 99%), 0.05 M buffer solutions (pH 3, pH 7.4, pH 10), thiazolyl blue tetrazolium bromide and $MnCl_2{\cdot}4H_2O$ and lysozyme (from chicken egg) were purchased from VWR Scientific (West Chester, PA). Irgacure 2959 was donated by Ciba Specialty Chemicals Corp.

2.2. Synthesis of Arg-UPEA

Two Arg-UPEA were synthesized by the same procedures reported before [18,20]. Briefly, the preparation steps could be divided into three major steps: the preparation of TsOH salts of Arg alkylene diester monomer (I); the preparation of di-*p*-nitrophenyl ester of dicarboxylic acid monomer (II), and the polymer synthesis of Arg-UPEA via solution polycondensation of the two monomers from (I) and (II) above. The detailed procedures are given elsewhere [18,20]. The M_n and M_w of 2-UArg-2-S synthesized by this method are 12.93 and 14.01 kg mol^{-1}, and those of 2-UArg-4-S are 15.71 and 17.49 kg mol^{-1} [18].

2.3. Synthesis of GMA-chitosan

The GMA-chitosan was synthesized by the procedure described in a prior study [30]. The degree of substitution (DS; the amount of methacrylate (MA) groups per 100 chitosan repeat unit) of GMA-chitosan was 37, which was determined by ^{1}H NMR spectroscopy.

2.4. Fabrication of Arg-UPEA/GMA-chitosan hybrid hydrogel

Although almost all Arg-UPEA have water solubility at room temperature, the methylene chain length (x) in the diol part between the two adjacent ester groups is one crucial material parameter of Arg-UPEA that can largely influence the polymer solubility in water [18]. Only Arg-UPEA with $x = 2$ and 4 were used in the fabrication of Arg-UPEA/GMA-chitosan hybrid hydrogel, because the water solubility of Arg-UPEA with $x > 4$ is too low to form a water-soluble hydrogel precursor solution with some other water-soluble polymer precursors, such as the GMA-chitosan. In a typical Arg-UPEA/GMA-chitosan hybrid hydrogel fabrication process, 0.3 g GMA-chitosan (DS 37) and desired amounts of Arg-UPEA (2-UArg-4-S or 2-UArg-2-S, ~43–150 mg) were added to a glass vial and dissolved in deionized water (6.0 ml) to form a clear homogeneous solution with light yellow color. Then, 10 mg Irgacure 2959 photo-initiator was added to the precursors' solution and dissolved completely. In order to keep the structure integrity of the hybrid hydrogels, the weight feed ratio of Arg-UPEA to GMA-chitosan could not be higher than 33/67.