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RGD-mimetic poly(amidoamine) hydrogel for the fabrication of complex cell-laden micro constructs

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ABSTRACT

The potential of the 3D cell culture approach for creating *in vitro* models for drug screening and cellular studies, has led to the development of hydrogels that are able to mimic the *in vivo* 3D cellular milieu. To this aim, synthetic polymer-based hydrogels, with which it is possible to fine-tune the chemical and biophysical properties of the cell microenvironment, are becoming more and more acclaimed. Of all synthetic materials, poly(amidoamine)s (PAAs) hydrogels are known to have promising properties. In particular, PAAs hydrogels containing the 2,2-bisacrylamidoacetic acid-agmatine monomeric unit are capable of enhancing cellular adhesion by interacting with the RGD-binding αVβ3 integrin. The synthesis of a new photocrosslinkable, biomimetic PAA-Jeffamine®-PAA triblock copolymer (PJP) hydrogel is reported in this paper with the aim of improving the optical, biocompatibility and cell-adhesion properties of previously studied PAA hydrogels and providing an inexpensive alternative to the RGD peptide based hydrogels. The physicochemical properties of PJP hydrogels are extensively discussed and the behavior of 2D and 3D cell cultures was analyzed in depth with different cell types. Moreover, cell-laden PJP hydrogels were patterned with perfusable microchannels and seeded with endothelial cells, in order to investigate the possibility of using PJP hydrogels for fabricating cell laden tissue-like micro constructs and microfluidic devices. Overall the data obtained suggest that PJP could ultimately become a useful tool for fabricating improved *in vitro* models in order to potentially enhance the effectiveness of drug screening and clinical treatments.

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1. Introduction

In recent years much attention has been paid to three-dimensional (3D) cell culture as a tool for *in vitro* biological experimentation [1]. In fact, researchers have come to appreciate the different biological response between cells cultured in monolayers and in 3D, which is more consistent with the environment in which cells physiologically operate [2,3]. In particular, as the complex relationships between cells and the extracellular matrix (ECM) *in vivo* play critical roles in controlling their behavior and functioning [4], much effort has been made to design synthetic 3D ECMs that are capable of mimicking the properties of natural cellular milieu, thus ensuring that the cells cultured *in vitro* behave similarly to their analog *in vivo* [5]. The potential of the 3D cell culture approach to create *in vitro* models for toxicity testing, drug screening and disease or cellular studies [6,7], has led to the development of materials with controllable biological, chemical and mechanical properties [8,9] that are capable of recapitulating natural ECM microstructures, with the ultimate goal of controlling cell and tissue functions [5,10–13]. To this aim hydrogels have proved to be desirable biomaterial systems for guiding 3D cell behavior to specific functions [14]. There are various advantages in using hydrogels such as their high water content and biocompatibility. Naturally derived molecules are commonly used to create cell-laden hydrogels owing to their intrinsic bioactivity, biocompatibility and biodegradability [15–22]. In particular, cell-laden hydrogels have been created from a wide range of native ECM molecules, including collagen [15], fibrin [16,17], hyaluronic acid [18,19], Matrigel [20,21], and gelatin [22]. These biomolecules naturally contain cell-signaling attributes that enable high cellular viability and

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proliferation. Nevertheless, the cues that enable cells to prosper in these materials are so abundant that it is not easy to accurately control and assess their effects on cellular behavior [5]. In addition, their intrinsic batch-to-batch variability results in hydrogels with physico-chemical properties that are difficult to reproduce and engineer [5]. In general, this leads to the inability to reliably control the hydrogels' mechanical properties thus limiting their versatility for creating 3D cellular constructs. In an attempt to solve some of the above issues, synthetic polymer-based hydrogels have been increasingly studied as 3D culture platforms, due to simple synthetic process, low costs and ease of functionalization, resulting in high tunability of the chemical and biophysical properties [8,9,12–14]. On the other hand, hydrogels from synthetic molecules generally lack a mechanical behavior comparable to natural origin materials such as, for instance, strain stiffening as observed for collagen and fibrin [23,24]. In addition, synthetic hydrogels are commonly characterized by small mesh size values hampering the cellular motility if compared to natural hydrogels [25,26].

Among synthetic hydrogels, poly(ethylene glycol) (PEG) is considered to be the gold standard thanks to its high hydrophilicity and its inert network resistant to protein adsorption [4,11]. However, pure PEG hydrogel lacks the cell-responsive features able to promote cell adhesion, viability and migration, that are crucial features for the creation of organized 3D cellular constructs. In order to overcome these limitations, modifications with binding motif Arg-Gly-Asp (RGD) [27–29], incorporation of proteolytically degradable peptide backbone [30,31] or physical mixing with ECM components [19] have been presented. In particular, PEG hydrogels containing both cell binding RGD motifs and matrix metalloproteinase (MMP)-sensitive degradation regions, have been shown to promote cell elongation, migration and interconnection *in vitro* with different cell lines [32–39]. Their main drawback lies in the use of peptide sequences, which contribute to increase the cost and complexity of the synthesis and may weaken their micropatterning ability in comparison to unmodified photopolymerizable materials [22]. For these reasons, the use of PEG hydrogels is mainly restricted to the creation of *in vitro* cellular tissues and models. A promising class of synthetic polymers for cell culture applications are poly(amidoamine)s (PAAs). They are obtained by means of Michael-type addition of PAA to Jeffamine® to primary amines and/or secondary diamines, under mild conditions [40]. Their simple synthetic process allows for the incorporation of various bioactive molecules in the PAA's backbone through covalent attachment [41–43]. In general, PAA-based hydrogels have good biocompatibility and are easily modifiable by introducing different co-monomers such as carboxylic acids, thiols and amino groups [44–49] that provide additional chemical functions. In order to improve the cell adhesion properties of PAA hydrogels, Ferruti and co-workers developed an RGD-mimetic PAA with a repeating unit composed of 2,2-bisacrylamidoacetic acid (BAC) and 4-aminobutylguanidine (ABG, Agmatine) [50]. Introducing ABG units in a cross-linked amphoteric PAA hydrogel caused an increase in fibroblast cell adhesion [50]. Moreover, it has been demonstrated that the ABG residue in PAA hydrogels maintains cell adhesion properties even when copolymerized with other monomers [48]. Notably, despite their interesting biological properties, various limitations have been observed when using PAA hydrogels for cell encapsulation such as limited transparency, poor mechanical properties and the need of several washing cycles to eliminate unreacted monomers [50]. These issues have prevented them from being used for developing *in vitro* models for 3D cell culture.

In this study, we report the synthesis of a new photocrosslinkable, biomimetic PAA-Jeffamine®-PAA triblock copolymer (PJP) hydrogel in order to provide an inexpensive alternative to using RGD peptide-based hydrogels. In particular, we presumed that by copolymerizing the PAA-based ABG with Jeffamine® it would be possible to obtain a hybrid RGD-mimetic PAA macromer to be used in the synthesis of hydrogels with improved biocompatibility, cell-adhesion, optical and mechanical properties. The physico-chemical properties of PJP hydrogels are extensively discussed. An in-depth analysis was carried out on 2D cell culture and 3D cell encapsulation behavior with different cell types. Moreover, cell-laden PJP hydrogels were patterned with perfusale microchannels and seeded with endothelial cells in order to determine if it is possible to use PJP hydrogels for the fabrication of complex cell-laden micro constructs.

2. Materials and methods

2.1. Materials

4-Aminobutylguanidine sulfate (ABG, agmatine, Biosynth/Sigma Aldrich), ammonium persulfate (APS), *O,O*-Bis(2-aminopropyl) poly(propylene glycol)-*block*-poly(ethylene glycol)-*block*-poly (propylene glycol) with molecular weight of 600, 900 and 1900 (Jeffamine® 600, 900 and 1900), Poly(ethylene glycol) diacrylate 4000 (PEGDA), 2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone (Irgacure 2959, CIBA Chemicals), low-melting point agarose, dimethyl sulfoxide (DMSO), lithium hydroxide monohydrate (LiOH), hydrochloric acid 37% (HCl), trichlorododecylsilane (TCS), and tetramethylethylenediamine (TEMED), sodium chloride (NaCl), sodium phosphate dibasic (Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4) were purchased from Sigma–Aldrich at the highest degree of purity available and used as received. Milli-Q grade water was used for all the experiments. The phosphate buffered saline (PBS) used in the experiments contained 2.69 mM KCl, 136.89 mM NaCl, 3.21 mM Na_2HPO_4, 1.47 mM KH_2PO_4. Lastly, 2,2-bis(acrylamido)acetic acid (BAC) was prepared as previously reported and its purity (99.9%) was determined both by acid–base titration and by NMR spectroscopy [51]. The materials were all purchased from Sigma Aldrich and used as supplied, unless otherwise stated.

2.2. Glass slides silanization and mold fabrication

Glass slides were silanized for fabricating cylindrical molds. In short, the glass slides were washed with acetone, activated by plasma oxygen (70 W, 70 s) and immersed in a TCS solution (5% v/v in petroleum ether) for 10 min. The slides were then repeatedly washed with petroleum, ether, ethanol and water, and dried with a gentle air flow and stored at room temperature. Different-sized molds were prepared by interposing preformed silicone spacers (0.3, 1, 3 and 6 mm thick) between two silanized glass slides. It is important to note that void circles (Ø of 6, 10 and 12 mm, respectively) had previously been cut into the preformed silicone spacers. The mold for microfluidic experiment was prepared in a similar way using two preformed 1-mm-thick silicone spacers, cut in order to obtain a 15 × 5 mm rectangular void area. A poly(lactic acid) (PLA) filament of ~200 μm was then fabricated with a commercial 3D printer (CREATR, Leapfrog). The PLA filament was placed between the two silicone spacers and suspended in the middle of the cylindrical chamber created by the void parts of the spacers.

2.3. PAA oligomer synthesis

In a 2 mL glass tube, BAC (200 mg, 1.0 mM), water (333 μL), lithium hydroxide (48.8 mg, 1.14 mM) and ABG (for OPAA-2: 168.8 mg, 0.717 mM; for OPAA-4: 199.4 mg; for OPAA-6: 210.7 mg) were added in this order and the resulting pH 9 mixture was stirred at 35 °C for 7 days in the dark and under inert atmosphere. The mixture was then diluted 1:50 with bi-distilled water

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