Acta Biomaterialia 22 (2015) 32–38

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Supermacroporous chemically cross-linked poly(aspartic acid) hydrogels

Benjámin Gyarmati [a], E. Zsuzsanna Mészár [a], Lóránd Kiss [b], Mária A. Deli [b], Krisztina László [a], András Szilágyi [a,*]

[a] Department of Physical Chemistry and Materials Science, Budapest University of Technology and Economics, Budafoki út 8., H-1111 Budapest, Hungary
[b] Institute of Biophysics, Biological Research Centre of the Hungarian Academy of Sciences, Temesvári krt. 62, H-6726 Szeged, Hungary

ARTICLE INFO

Article history:
Received 3 November 2014
Received in revised form 5 April 2015
Accepted 21 April 2015
Available online 25 April 2015

Keywords:
Poly(aspartic acid)
Hydrogel
Macroporous morphology
Responsive character

ABSTRACT

Chemically cross-linked poly(aspartic acid) (PASP) gels were prepared by a solid–liquid phase separation technique, cryogelation, to achieve a supermacroporous interconnected pore structure. The precursor polymer of PASP, polysuccinimide (PSI) was cross-linked below the freezing point of the solvent and the forming crystals acted as templates for the pores. Dimethyl sulfoxide was chosen as solvent instead of the more commonly used water. Thus larger temperatures could be utilized for the preparation and the drawback of increase in specific volume of water upon freezing could be eliminated. The morphology of the hydrogels was characterized by scanning electron microscopy and interconnectivity of the pores was proven by the small flow resistance of the gels. Compression tests also confirmed the interconnected porous structure and the complete re-swelling and shape recovery of the supermacroporous PASP hydrogels. The prepared hydrogels are of interest for several biomedical applications as scaffolding materials because of their cytocompatibility, controllable morphology and pH-responsive character.

1. Introduction

Poly(aspartic acid) (PASP), a synthetic poly(amino acid), has gained remarkable attention recently for multiple reasons. PASP is biocompatible because of its chemical structure [1,2]. It is proved to be biodegradable, but its degradation rate depends on the type and degree of substitution [3]. Its precursor anhydride, polysuccinimide (PSI), reacts with primary amines under mild reaction conditions even without catalyst [1,4]. As PSI derivatives can be easily hydrolyzed to the corresponding PASP, a wide range of PASP derivatives can be synthesized including responsive PASP polymers and hydrogels [5–9]. All these benefits have initiated the fabrication of PASP-based biomedical devices both on macro and nanoscale [7–10]. Including aspartic acid repeating units makes possible the conjugation with *N*-terminated biomolecules *via* the carboxyl groups. The controllable ionic charge of this repeating unit was already utilized in the fabrication of biomimetic materials [11]. Nevertheless, the use of cross-linked PASP hydrogels has not yet been explored in tissue engineering, although several characteristics, *e.g.* large water uptake, externally controlled properties, or soft characteristics, make PASP hydrogels suitable for applications in regenerative medicine.

In tissue engineering, damaged tissues or organs are replaced by implantation of cell cultures seeded on a biomaterial *in vitro*, or by implantation of scaffolds to promote the self-repair of the injured tissues [12]. Scaffolding materials must meet some basic requirements that are independent of the proposed application: they must be biocompatible and possess high porosity with a pore size suitable for the accommodation of the cells [13]. The pore size should be between 5 and 200 μm depending on the type of cell, and the pore structure in this specific size range is also referred to as supermacroporous morphology. A further requirement is the interconnectivity of pores to provide sufficient flow of the nutrient solution between the cells and the environment. The biomaterial must possess functional groups to enable facile modification of the surface with recognition sites, growth factors and ligands for control of the cell-scaffold interactions [12]. In addition, *in vitro* cell seeding and harvesting require easy control of cell adhesion and detachment by external stimulus (e.g. enzymatic treatment or temperature stimulus), while *in vivo* application of scaffolds demands controlled biodegradability of the biomaterial [14]. Finally, proper mechanical strength is also a general requirement towards scaffolding materials [15].

Thermally induced solid–liquid phase-separation during polymerization and/or cross-linking – also called cryogelation – is used to prepare porous structures. In the case of hydrogels, it

* Corresponding author. Tel.: +36 1 463 3518; fax: +36 1 463 3767.
E-mail addresses: bgyarmati@mail.bme.hu (B. Gyarmati), meszar.ezsuzsa@gmail.com (E.Zs. Mészár), lorand.kiss.work@gmail.com (L. Kiss), deli@brc.hu (M.A. Deli), klaszlo@mail.bme.hu (K. László), aszilagyi@mail.bme.hu (A. Szilágyi).

http://dx.doi.org/10.1016/j.actbio.2015.04.033
1742-7061/

is a well-established technique to create supermacroporous hydrogels below the melting point of the solvent [13]. The major part of the solvent is frozen during cryogelation, while the reaction occurs in the liquid phase, which is a concentrated solution of the polymer [16,17]. The growing crystals of the solvent act as templates for the interconnected pores forming after melting. Here we report the synthesis of supermacroporous PASP hydrogels by cryogelation in DMSO. Additionally the pore morphology, pore interconnectivity and mechanical properties of prepared hydrogels were investigated.

2. Experimental section

2.1. Materials

1,4-diaminobutane (DAB, 99%), imidazole (puriss) and 2,4,6-trinitrobenzenesulfonic acid (picrylsulfonic acid, TNBS, 1 M aqueous solution, analytical reagent) were obtained from Sigma–Aldrich. Dimethyl sulfoxide (DMSO, dried, max. 0.025% H_2O), L-aspartic acid (99%), methanol (MeOH, 99.9%), potassium chloride (99.5%), sodium tetraborate decahydrate (a.r.), mesitylene (for synthesis) and sulfolane (for synthesis) were acquired from Merck. Phosphoric acid (cc. 85%) and hydrochloric acid (HCl, 35%) were purchased from Lach Ner. Citric acid monohydrate (99%) and sodium hydroxide (NaOH, a.r.) were bought from Reanal (Hungary). For cytotoxicity tests Eagle's minimum essential medium (MEM; Gibco), fetal bovine serum (Lonza), Na-piruvate (Gibco), XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt, Roche) and cytotoxicity detection kit measuring LDH (lactate dehydrogenase) release (Roche), 96-well and 24-well cell culture plates (Orange, UK) were used. All reagents and solvents were used without further purification.

The aqueous buffer solutions used were prepared from citric acid (c = 0.033 M, pH = 2 to 6), imidazole (c = 0.1 M, pH = 6 to 8) and sodium tetraborate (c = 0.025 M, pH = 8 to 12). pH was adjusted by the addition of 1 M HCl or 1 M NaOH. Ionic strength of the solutions was adjusted to 0.15 M by the addition of KCl. The pH of the buffer solutions was checked with a pH/ion analyzer (Radelkis OP-271/1).

2.2. Methods

2.2.1. Synthesis

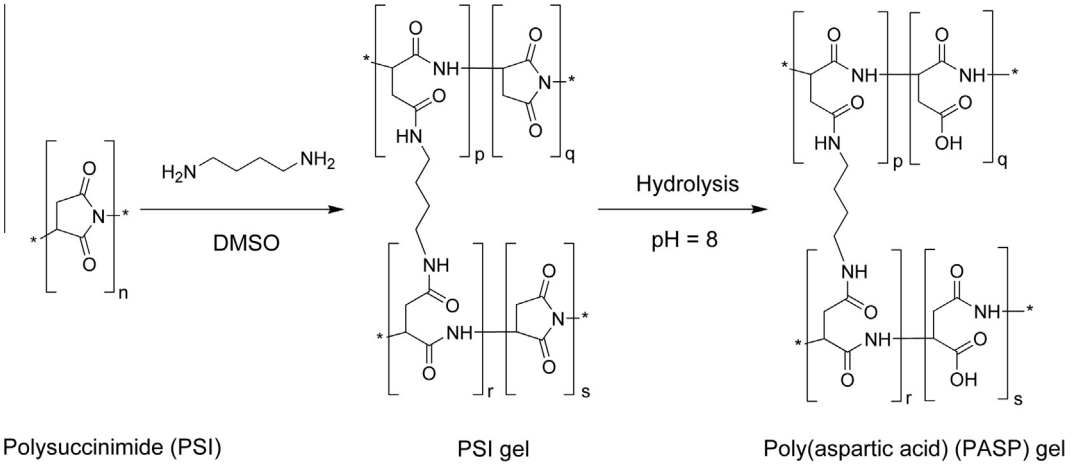
The PSI polymer was synthesized by the acid-catalysed thermal polycondenzation of aspartic acid in a mixture of mesitylene and sulfolane at 160 °C (7 h) [8]. PSI was purified by precipitation in methanol and dried in vacuum at 25 °C. Its chemical structure was confirmed by ^{1}H NMR (300 MHz, DMSO-d_6, δ): 5.10 (d, 1H, CH); 3.20 and 2.75 (s,s, 2H, CH_2). After hydrolysis the average molecular weight of the resultant PASP was determined by HPLC size exclusion chromatography (SEC). A Nucleogel GFC-300 (Macherey–Nagel) column was used (molecular mass range 1–100 kDa) with phosphate buffered saline (PBS, pH = 7.6) eluent. The average molecular mass of PASP was 56.1 kDa with polydispersity index of 1.07.

PSI was cross-linked with 1,4-diaminobutane under cryogenic conditions in DMSO significantly below the melting temperature (−10 to −40 °C) to induce phase separation (Scheme 1). In a typical procedure of gel synthesis, the solution of the cross-linker (8.8 wt.% DAB in DMSO) was added to the solution of PSI (final concentration of 9.70 wt.% in DMSO). The cross-linking ratio (X_{DAB} defined as the molar ratio of cross-linker molecules to the repeat units) in the precursor solution varied between 4.0% and 9.0%. After 30 s vigorous stirring at 25 °C the precursor solution was filled into the sample holders and quickly placed in a freezer. For the swelling experiments and SEM measurements gel sheets with thickness of 2 mm were prepared, while for the mechanical characterization and flow experiments samples were prepared in cylindrical molds (diameter ≈ height ≈ 1.5 cm). The temperature was continuously controlled (deviation less than ±2 °C). After 7 days gelation time, the sample holders containing PSI-DAB gels were quickly immersed into room temperature aqueous buffer solution (pH = 8) to yield the PASP-DAB gels.

2.2.2. Characterization

The conversion of the cross-linking reaction was characterized by the number of unreacted cross-linker molecules, which is determined by a method developed earlier [18]. After cryogelation, the swelling solution of PSI-DAB gels were reacted with TNBS at pH = 9. The concentration of unreacted cross-linker molecules, and hence the conversion, was determined by the UV–Vis spectrophotometric assay of the TNBS adduct of DAB (details in Supplementary Data).

Cytotoxicity and viability tests were made on human intestinal epithelial Caco-2 cells [19]. The PBS solutions in which the PASP hydrogels were swollen during sterilization were used in the biological tests. The negative control group received culture medium diluted with PBS while the positive control group was treated with 1% Triton X-100 detergent. Viability was determined by measuring the cellular conversion of XTT dye, whereas cytotoxicity was



Scheme 1. Synthesis of PSI and PASP gels. Additional cross-linking in water is prohibited after cryogelation of PSI.

ID	Title	Pages
239	Supermacroporous chemically cross-linked poly(aspartic acid) hydrogels	7