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Investigation of 2D WS_2 nanosheet-reinforced tough DNA hydrogel as a biomedical scaffold: preparation and *in vitro* characterization

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1. Materials and Methods

1.1 Preparation and characterization of WS_2-Alg nanosheets

2D WS_2-Alg nanosheets were prepared based on a standard TMD exfoliation protocol with minor modifications [1]. A 1% w/v sodium alginate (PROTANAL LF20/40, FMC BioPolymer, Philadelphia, PA) solution was prepared through vigorous agitation in MilliQ water. Three different concentrations of tungsten disulfide (Sigma-Aldrich, powder, $2\ \mu m$, 99%) at 0.5%, 1%, and 2% w/v respectively, were dispersed in the alginate solution through vortexing for 30 seconds. This WS_2 dispersion was subsequently sonicated in an ice bath by an ultrasonic liquid processor with a probe tip (SONICATOR XL2020, Misonix Incorporated, Farmingdale, NY) at an amplitude setting of five for 5 hours to allow for exfoliation of suspended WS_2 into 2D-nanosheets. The resulting 2D WS_2-Alg nanosheets were characterized using Transmission Electron Microscopy (Phillips) and UV-Vis Spectroscopy (Beckman Coulter).

1.2 Fabrication of single and double network DNA-based hydrogels

The covalently crosslinked DNA network was generated following a previously published protocol [2]. DNA sodium salt from salmon testes (Sigma-Aldrich, $M_n \approx 1.3 \times 10^6\ Da$, ca. 2000 bp) was solubilized overnight in MilliQ water at $37\ ^{\circ}C$. To form the single network (SN) nanocomposite hydrogels, exfoliated WS_2 was added into the DNA solution and stirred for 5 minutes, followed by the addition of the cross-linker, poly ethylene glycol diepoxide (PEGDE - Sigma-Aldrich, $M_n = 500$) and TEMED (044% w/v) (tetramethylethylenediamine, Bio-Rad Laboratories Inc.) to initiate the gelation. TEMED induces denaturation of DNA to expose the amine groups on the

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nucleobases for further reaction with the epoxide end groups of PEGDE [2–4]. The final concentration of DNA was fixed at 5% w/v and a DNA/PEGDE mass ratio of 2 was used for all the hydrogels. Thereafter, the reaction mixture was placed in a bead bath at $55\ ^{\circ}C$ for 4 hours to form the cross-linked network and the cross-linked gels were allowed to cool overnight. The final concentration of WS_2 in the resultant hydrogels were 0.1875%, 0.375% and 0.75% w/v. These hydrogels with three different WS_2 concentrations were named as "Low", "Med", and "High" respectively. To form the double-network (DN) hydrogels, the gels were soaked in a 0.025(M) $CaCl_2$ (96%, anhydrous, ACROS Organics, NJ, USA) solution and placed for 24 hours in an incubator shaker at 37°C.

1.3 Mechanical Characterization

Rheological studies were conducted using an AR2000 rheometer (TA Instruments, New Castle, DE) with a 20 mm rough parallel plate and plate geometry. An established protocol was used to collect and analyze data, [2].The SN gels were soaked in PBS for 24 h prior to testing, while the DN gels were soaked in the $CaCl_2$ solution for 24 h to form the second network and subsequently washed with PBS prior to analysis. All the experiments were performed at $37\ ^{\circ}C$. Strain sweeps were first carried out within strain values from 0.001 to 100 at a fixed frequency of 1 Hz for the detection of the linear viscoelastic region. Having found a constant strain of 0.01 to be within the linear region, oscillatory frequency sweeps at this fixed strain within a frequency range of 0.01 – 10 Hz were conducted. Furthermore, stress sweeps at 1 Hz were carried out by varying the shear stress from 0.1 – 10,000 Pa. Finally, uniaxial compression tests were conducted with a TA ARES RSA3 rheometer (TA Instruments, New Castle, DE), where 5 mm biopsy punches were utilized to obtain cylindrical samples. The compressive moduli of the hydrogel constructs were obtained from the linear regimes of the stress-strain plots in the range of 10 to 20% strain.

1.4 Physical and morphological characterization

The hydration capacity of the hydrogel constructs was determined via swelling studies [2], whereby the samples were freeze-dried to obtain their dry mass (M_0) and subsequently soaked in PBS. The increase in the mass of the hydrogels was measured in 5-minute intervals for the first 15 minutes, in 15-minute intervals up to the 2-hour timepoint, and every hour thereafter for a total of 8 hours. The swelling ratio was calculated as the ratio of the mass gain to the initial dry mass as represented by the following equation,

$$swelling\ ratio = \frac{M_t - M_0}{M_0}$$

where, M_t is the mass evaluated at the aforementioned time points and M_0 is the mass of the dry hydrogel.

The morphology of the single and double network hydrogels was visualized by performing scanning electron microscopy with the freeze-dried hydrogels after sputter coating the samples with gold[22]. An immersion-lens detector (FEI Tecnai F20 XT) with an acceleration voltage of 1 - 10 kV was used for acquiring the images.

1.5 Evaluation of *in vitro* biocompatibility

Human adipose-derived stem cells (hASCs, RoosterBio, USA) were grown with α-minimum essential medium (Invitrogen), 10% fetal bovine serum (Thermo-Fischer Scientific, USA), and 1% penicilin/streptomycin, following manufacturer's protocol. The *in vitro* biocompatibility assays were performed following a previously established protocol [2].The hASCs were grown in 24 well plates. The DNA hydrogels were positioned inside cell culture inserts (Corning Incorporated-Life Sciences, 3 µm diameter of the pore) and placed on top of the wells. With this setup, the cells were exposed to diffused hydrogel debris. This non-contact method of testing cytotoxicity of hydrogels avoids cell apoptosis in adherent cell cultures arising from weight-induced mechanical stress [5]. In direct-contact methods, an overestimation of cytotoxicity may happen because of apoptosis from the gels smothering the adherent cells. Furthermore, cell-culture inserts are frequently used to evaluate drug delivery-based scaffolds, where the gels release the entrapped therapeutic materials as a function of time . Next, MTS assays were performed, following manufacturer's protocol, after 24h and 72h of incubation with the hydrogels. Finally, live/dead assays were conducted, following previously established protocol [6], to image the cells after 24h and 72h.

1.6 Statistical Analysis

Statistical significance was obtained by performing one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. All statistical analyses were performed using Microsoft Excel. Statistically relevant p value was dispayed as *= $p<0.05$, **= $p<0.01$, and ***= $p<0.001$.

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