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Programmable Protein-DNA Hybrid Hydrogels for Immobilization and Release of Functional Proteins

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A modular approach for the precise assembly of multi-component hydrogels consisting of protein and DNA building blocks is described for the first time. Multi-arm DNA is designed for crosslinking and stepwise, non-covalent assembly of active proteins inside the hydrogel.

Protein and DNA constitute the two major classes of biomacromolecules in nature which have been recently esteemed by material scientists for designing unique functional materials. Their application as biomedical materials, such as hydrogels, is particularly attractive due to their natural biocompatibility. In comparison to synthetic polymers, the polypeptide backbones of proteins offer many unique features such as precisely defined chain lengths and amino acid sequences, secondary structures as well as many different functional groups along the main chain thereby allowing various chemical modifications. Previously, proteins have been converted into versatile polymeric materials for drug delivery[1] and as biocompatible coatings[2] and they were found to exhibit excellent biocompatibility, biodegradability as well as multifunctionality[3]. In this manuscript, the unique features of protein polymers will be combined with DNA technology to achieve customized hydrogels. The preparation of hydrogels only consisting of DNA has been intensively studied and it offers unique superiority such as biocompatible gelation[4], stimuli responsiveness[5], as well as shape memory effect[6]. In addition, single stranded DNA (ssDNA) served as codons to tag functional protein allowing the immobilization of proteins with high efficiency and specificity[7]. This strategy has been widely applied for decorating proteins onto sensor chips,[8] membranes[9] and inorganic biomaterials.[10] It would be highly attractive to implement DNA codons into hydrogel materials for the programmable immobilization of guest molecules. To date, functional proteins are mainly loaded into hydrogels by either covalent chemical reactions[11] or by non-covalent interactions through physical binding pairs, e.g. barnase–barstar and streptavidin–biotin[12]. Hybridization of ssDNA would allow orthogonal encoding of multiple proteins with easily programmable DNA sequences and precise immobilization of various proteins by using the same reaction scheme under physiological conditions. Therefore, taking the advantages of both protein and DNA materials, herein, we describe the first example of a protein-DNA hybrid hydrogel that uses a protein as polymeric hydrogel backbone and DNA as functional crosslinkers and adaptors for functional protein immobilization.

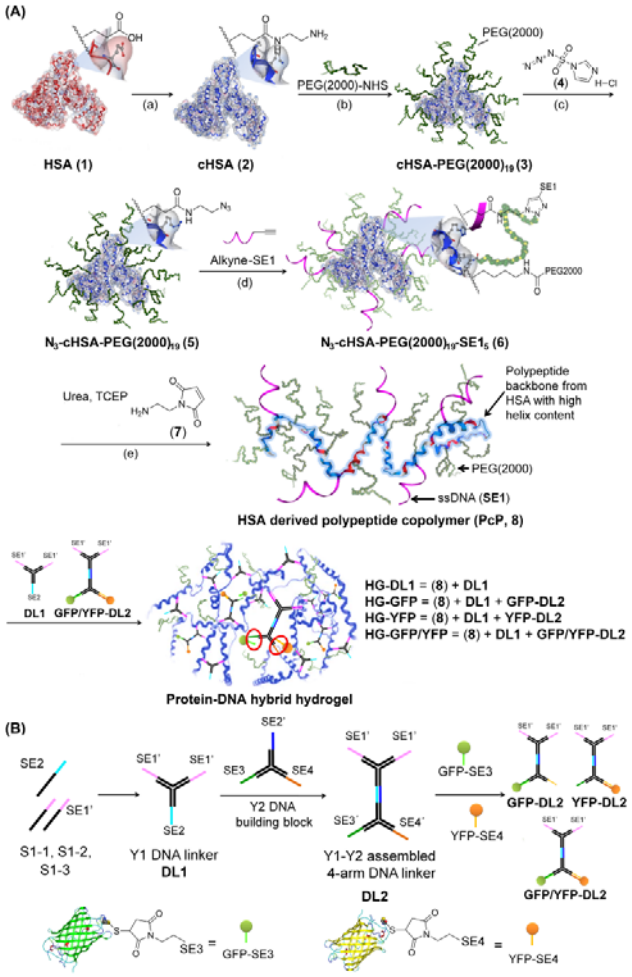

Fig. 1 (A) Synthesis of a protein-DNA hybrid hydrogel. Reaction conditions: (a) Ethylenediamine 50 mM, EDC 267 mM, pH 4.75, yield 98%; (b) PEG(2000)-NHS 30 eq, pH 8.0, yield 84%; (c) K_2CO_3 177 eq, $CuSO_4$ 0.87 eq, (**4**) 100 eq, yield 80%; (d) Alkyne-SE1 8 eq, $CuSO_4$ 60 eq, sodium ascorbate 90 eq, pH 7.4, yield 93%; (e) 5M Urea, TCEP 100 eq, (**7**) 100 eq, pH 7.4, yield 92%. (B) Preparation of the 3-arm DNA crosslinker (**DL1**) and the 4-arm DNA crosslinker (**DL2**) and controlled attachment of GFP and YFP onto **DL2**.

The naturally abundant plasma protein human serum albumin (HSA) is selected as polypeptide backbone of the hydrogel due to

its high plasma stability, enzymatic degradability and less side-effects.[13] The HSA-derived hydrogel backbone is prepared by sequential conjugation of polyethyleneglycol (PEG) and ssDNA sticky ends to the polypeptide chains (Fig. 1A). Briefly, HSA is firstly converted to cHSA (**2**) containing ~159 primary amino groups[14] (Fig. S2). Thereafter, ~19 PEG chains (Mw = 2000) are conjugated to the amino groups of cHSA (**2**) to afford cHSA-PEG(2000)$_{19}$ (**3**) (Fig. S2). Introducing PEG components into hydrogels is a common strategy to significantly increase the water content and to reduce nonspecific protein absorption and immunogenicity.[15] Thereafter, for bioorthogonal conjugation of ssDNA without side reactions on the nucleobases, ~50 amino groups of (**3**) are converted to azido groups by applying diazo transfer reagent (**4**) (Table S1).[16] The alkyne functionalized ssDNA sequence (alkyne-SE1, Scheme S1, Fig. S1) is then conjugated to the azido-HSA derivative (**5**) *via* Huisgen cycloaddition and serves as the interaction point for gelation with DNA corsslinker. On average five SE1 per polymer chain are found according to gel electrophoresis and UV-Vis spectra (Fig. S4, S6). Apply DNA conjugation after PEGylation is critical to ensure both entities with sufficient yields. Finally, the resulting HSA derivative (**6**) is denatured in concentrated urea buffer, followed by reduction of the disulfide bridges by TCEP (tris(2-carboxyethyl)phosphine) and capping of the free sulfurhydryl groups with (**7**) as reported[3] (Fig. 1A). The stable polypeptide copolymer (PcP, **8**) is thus obtained carrying PEG chains contributing to water-solubility and ssDNA (SE1) for interacting with DNA crosslinkers and further functionalization.

Gelation is achieved by crosslinking PcP (**8**) with 3-arm DNA crosslinker **DL1** (Fig. 1A). The **DL1** is assembled from three ssDNA (S1-1, S1-2, S1-3) as shown in Fig. 1B and 2A. It consists of two sticky ends (SE1') complementary to SE1 on PcP (**8**) to crosslink the polymer chains[17] and one third sticky end (SE2) available for further extension (Fig. 1B). The hydrogel (**HG-DL1**) is prepared by simply mixing PcP (**8**) and **DL1** in various physiological buffers (e.g. TBE buffer, PBS buffer and DMEM medium). Gelation occurs within one minute at room temperature. Since the gelation procedure is rapid and biocompatible, it potentially allows *in situ* gelation at target tissue by simply injecting both protein and DNA components. Injectable hydrogels are of great interest since drugs, proteins and cells can be easily added to the solutions before administration, and no surgical procedures are required for the insertion of such gels into the body.[18]

Depending on the concentration of PcP (**8**) and **DL1** used for gelation, the hydrogel stiffness varied in a broad range. By adjusting the solid contents from 1% to 5%, the storage modulus G´ increased from ~1 pa to ~4200 pa (Fig. 2B). Notably, in our previous studies on hydrogels composed solely of crosslinked DNA, only G´ moduli up to only ~1000 pa were obtained.[5] Hydrogels consisting solely of polypeptides such as the elastin-like peptide (ELP) also reveal G´ of less than 1000 pa.[19] In this aspect, the protein-DNA hybrid hydrogel offers improved stiffness that can be tuned over a broad range most likely based on the synergistic interplay of the two biopolymers. This observation is likely due to (a) the ultra-long polypeptide backbone of HSA, offering sufficient flexibility for effective crosslinking and (b) high contents of rigid helical structures (60 % helices according to circular dichroism, Fig. S13)[3] comparable with native HSA contributing to enhanced stiffness of the high solid content hydrogel. Since hydrogel stiffness is a critical parameter for tissue engineering,[20] its fine-tuning over a broad range is of great interest to customize materials with the most favourable cell interactions.

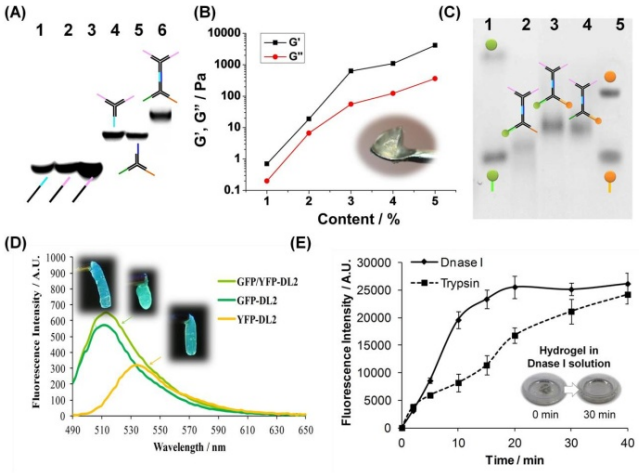

Fig. 2 Characterization of the hydrogels. (A) Gel electrophoresis shows the assembly of DNA crosslinkers. Line 1 to 3: ssDNA S1-1, S1-2 and S1-3; line 4: **DL1**; line 5: Y2; line 6: **DL2**. (B) Rheological properties of **HG-DL1** with different solid contents presented by storage modulus G' and loss modulus G". Inset shows a representative picture of the gel. (C) Gel electrophoresis of protein-DNA conjugates (direct fluorescence image without staining). Line 1: GFP and GFP-SE3; line 2: **GFP-DL2**; line 3: **GFP/YFP-DL2**; line 4: **YFP-DL2**; line 5: YFP and YFP-SE4. (D) Normalized emission spectrum of **GFP-DL2**, **YFP-DL2** and **GFP/YFP-DL2** (λex = 460 nm). Inset shows the representative fluorescence pictures of the gels (excited at 365 nm). (E) Time course of GFP release from **HG-GFP** after treatment with DNase I (2.5 U/μL) or trypsin (1mg/mL).

The sticky end SE2 of **DL1** is further extended with DNA building block Y2 (Fig. 1B and 2A) to offer simultaneous conjugation of up to two different proteins. The green fluorescent protein (GFP) and yellow fluorescent protein (YFP) are chosen as model proteins for conjugation into hydrogel, since the structural integrity of fluorescent proteins could be assessed easily by measuring their photoluminescence. The maleimide-modified DNA tags SE3 and SE4 are site-specifically conjugated to GFP and YFP at their single-mutated cysteine residue respectively (see supporting information for details). The assembly of SE3-GFP and SE4-YFP with **DL2** is demonstrated by gel electrophoresis and the emission spectra (Fig. 2C and 2D). Compared with the emission spectra of the native GFP and YFP, no spectral shift or emission intensity changes are observed indicating the retention of structural integrity of both immobilized proteins (Fig. S9). The hydrogel containing immobilized GFP and YFP (**HG-GFP/YFP**) is prepared by mixing 5% of **GFP/YFP-DL2** with **DL1** and subsequently mixing with PcP (**8**) as described above (Fig. 2D). Compared to conventional hydrogels where conjugation of proteins is mainly achieved by covalent reactions, such as EDC couplings,[11] self-assembly of proteins by DNA hybridization proceeds entirely under physiological condition without adding chemical reagents (e.g. catalysts) and with high efficiency.

Protein immobilized inside hydrogel can be enzymatically released as demonstrated with DNase I and trypsin. As shown in Fig. 2E, the complete digestion of **HG-GFP** by DNase I proceed

within 15 min with a concomitant release of GFP. In the presence of trypsin, the complete release of GFP is achieved within 30 min (Fig. 2E) (GFP is resistant to trypsin digestion). In addition, it has been verified that all components for the preparation of these protein-DNA hybrid hydrogels, e.g. PcP (**8**) and **DL1** and even the degradation fragments of **HG-DL1** after treatment with DNase I are non-toxic to different cell lines (Fig. 3C), substantiating the biocompatibility of the hydrogel. The potential of adopting these protein-DNA hybrid hydrogels as extracellular matrices for 3D cell culture is tested with human lung adenocarcinoma cell line A549 and human foreskin fibroblast cells. Cells are seeded inside the hydrogel by mixing the cells in culture medium with PcP (**8**) and **DL1**. After culturing for 48 hrs, the hydrogel is stable and cell viability inside the gel is tested using fluorescence based live/dead staining. As shown in Fig. 3B and 3C, cells are growing in the 3D gel matrix with >95% cell viability for both cell types.

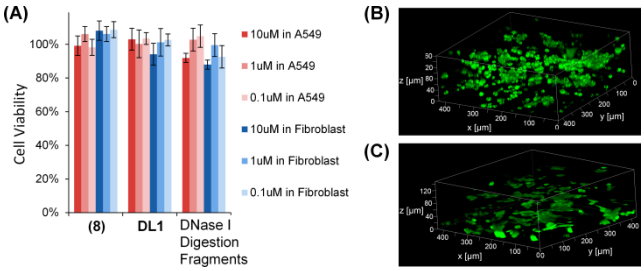

Fig. 3 (A) Cytotoxicity of all components forming the hydrogel and of hydrogel (**HG-DL1**) fragments after DNase I digestion. Confocal imaging of (A) A549 cells and (B) primary fibroblast cells growing in the 3D hydrogel matrix (**HG-DL1**) after 48 hrs. Green: Calcein-AM staining of living cells; red: Propidium iodide (PI) staining of dead cells.

Conclusions

In summary, we have presented a modular approach for the convenient immobilization of proteins into a hydrogel *via* ssDNA hybridization and their controlled release by enzyme-mediated degradation of the hydrogel. The hydrogels are prepared from HSA polypeptides and crosslinked by multi-arm DNAs yielding versatile hybrid materials combining the unique feature of each macromolecular building block. The preparation of these hydrogels proceeds completely under physiological, environmental friendly conditions and no toxic monomers, catalysts, polymerization initiators or organic solvents are required. Tunable stiffness of the hydrogels over a broad range, high biocompatibility and controlled degradation under enzymatic conditions make them attractive candidates as extracellular matrices for 3D cell culture. One could envision that sensitive protein cofactors, such as growth factors, could be efficiently immobilized inside the hydrogel in physiological media and released under controlled, mild conditions which would be of great interest for stem cell culture.

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Notes and references

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