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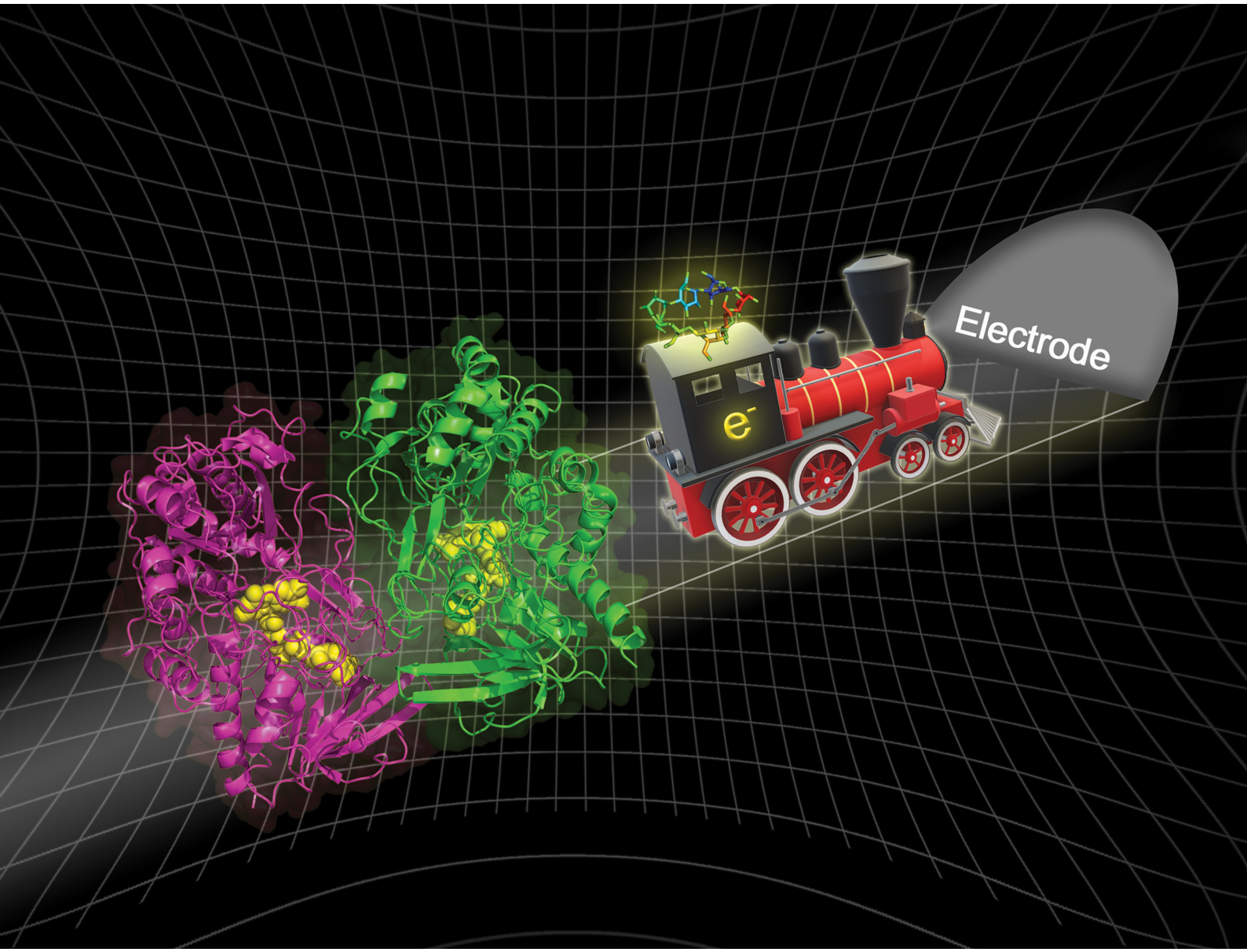

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UV-cured cyclodextrin modified hydrogels for the immobilization of electron transfer mediators and enzymes on electrode surfaces†

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Electron transfer mediator ferrocene is immobilized on a UV-cured cyclodextrin modified hydrogel *via* the formation of a host–guest inclusion complex. This surface allows enzyme immobilization and bioelectrochemical response, as well as cell adhesion resistance to fibroblast cells.

Mediated electron transfer (MET) is an important mechanism used in enzymatic bioelectrodes for a range of bioelectrochemical applications, *e.g.*, biosensors and biofuel cells.[1–3] MET relies on the usage of an electron-transfer mediator with a suitable redox potential,[4] typically a synthetic compound with a reversible redox behaviour, to mediate the electron transfer (ET) between the redox-active enzyme and the electrode surface.[5] Mediators are preferably immobilized onto the electrode with the enzyme to prevent leakage of mediators to the working solution, making redox mediator modified polymers of great interest.[6,7] Covalent grafting is frequently used to graft redox mediators to hydrogel polymers, but requires multiple-step functionalization and labour-intensive purification.[8–10]

β-cyclodextrin (CD) is a cyclic oligosaccharide composed of seven D-glucose subunits connected *via* α-1,4-glucosidic bonds.[11] The conical shape of CD and arrangement of the hydroxyl groups create a hydrophobic cavity (0.6–0.65 nm diameter) and a hydrophilic outer surface. The hydrophobic cavity of CD has been used to load many redox mediators (such as ferrocene (Fc),[12,13] tetrazine-naphthalimide,[14] bis-pyrene-2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid),[15] 9,10-phenanthrenequinone,[16] and anthraquinone-2-sulfonate[17]) with low water solubility by forming a supramolecular inclusion complex for enzymatic bioelectrodes. Previous efforts employed CD inclusion complexes directly,[18] a self-assembled monolayer of thiol-linked CD,[19] or glyconanoparticles of CD linked block copolymers,[15,16] necessitating the development of a more universal strategy to immobilize the CD inclusion on a solid electrode surface. Accordingly, CD polymers offer a promising strategy.

In this contribution, we propose to use a UV cured hydrogel with light-induced cross-linking of benzophenone (Bzp) modified dextrans (Bzp-dex) and CD modified dextrans (CD-dex) for the co-immobilization of Fc and enzyme relying on host–guest inclusion with CD and film entrapment, respectively (Scheme 1). Specifically, β-CD and Bzp are first separately appended on dextran, a linear polysaccharide featuring high biocompatibility and water solubility (see the ESI†). Fc is then loaded into CD-dex, leading to the formation of inclusion complexes (Fc–CD-dex). Fc–CD-dex and Bzp-dex, with the addition of enzyme, are finally mixed and UV cured, through the formation of a robust covalent bond between Bzp and CD,[20] on the surface of a glassy carbon electrode (GCE). According to the literature, nuclear magnetic resonance (NMR) showed that the Bzp ketyl radical binds at C3 and/or C5 inside the hydrophobic cavity of CD and/or at C6 at the narrower rim[21] (Scheme 1 only shows the binding to C5). This so-called "dock'n'flash" method, comprising (i) the docking of the benzophenone group with CD *via* inclusion complex formation and (2) the subsequent UV flash triggered photo-crosslinking between them,[22,23] is quick and versatile, with the potential to include a variety of redox mediators for MET based bioelectrodes. The mechanical properties of the hydrogel resulting from the combination of mediator-CD-dex and Bzp-dec furthermore offer a possibility for 3D-printing, thus the ease of scale-up and mass production. Furthermore, the antibiofouling performance of the hydrogel is investigated, holding the potential for implantable bioelectrodes.

The successful inclusion of Fc within CD-dex is verified by comparing the electrochemical behaviours of free Fc (Fig. 1A) and the inclusion complex (Fig. 1B). It's clearly seen that free Fc in 0.1 M pH 7 phosphate buffer solution (PBS) displays a

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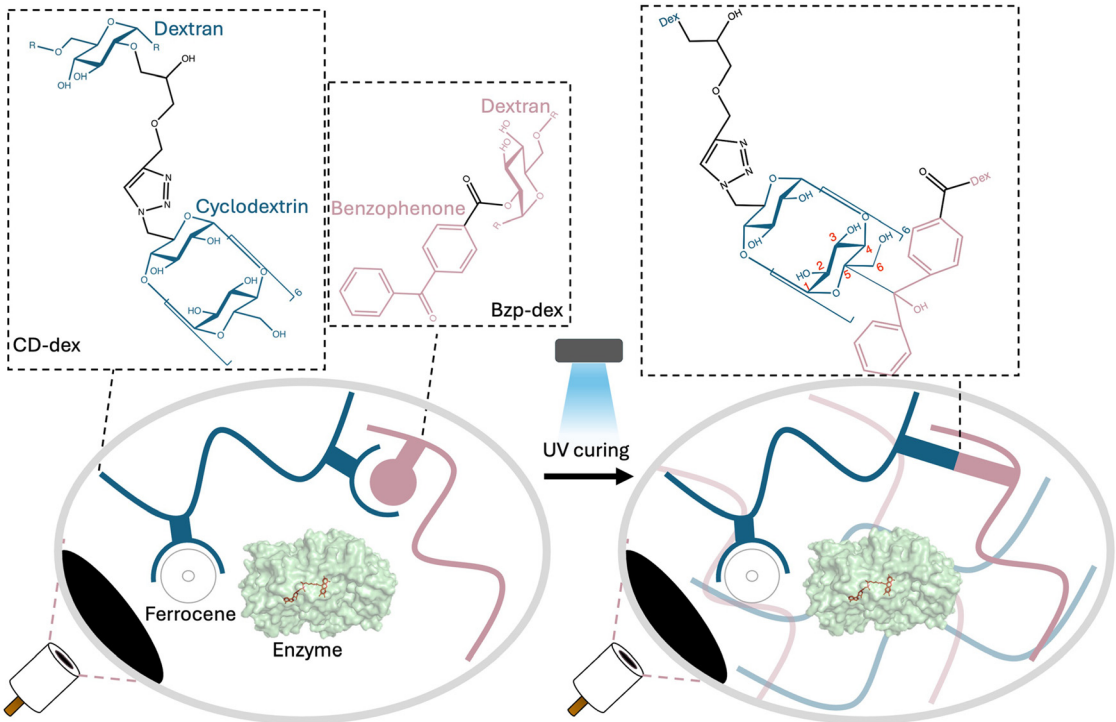

Scheme 1 Schematic drawing of the process of immobilizing and entrapping ferrocene and enzyme on an electrode surface within a UV cured hydrogel matrix. Note: there are likely several reaction routes between CD and Bzp, and Bzp may also bind to dex and/or the enzyme. However, due to the high tendency for Bzp to form inclusion complexes with CD and the stabilization effect of the hydrophobic cavity towards the Bzp ketyl radical, the former interaction is more favored. Here is a simplified illustration only showing the binding to the C5 position.

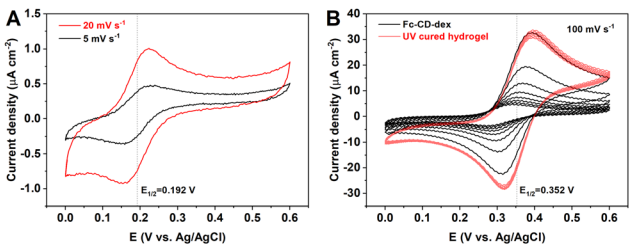

Fig. 1 Cyclic voltammograms (CVs) of (A) a bare GCE in 0.1 M pH 7 PBS containing free Fc with a saturated concentration and (B) modified GCEs with Fc–CD-dex and the UV cured hydrogel.

midpoint redox potential ($E_{1/2}$) of 0.192 V *vs.* Ag/AgCl, while a positive shift is observed for the inclusion complex by 160 mV ($E_{1/2}$: 0.352 V *vs.* Ag/AgCl). Such a positive shift of $E_{1/2}$, governed by the association constant for the equilibrium between free host CD and free guest Fc and the resulting inclusion complex, is consistent with the previous report.[24] The positive shift of $E_{1/2}$ can be regarded as direct evidence for complex formation, as it reflects the changes in solvation and electrostatic interactions in the nanoconfinement. The formation of the inclusion complex is further verified by ^{1}H NMR, which shows a chemical shift from 4.255 to 4.227 ppm for the free and complexed Fc protons, respectively (Fig. S1, ESI†). From Fig. 1B, it can be further observed that uncured Fc–CD-dex cannot form a stable functional surface due to the high water solubility of dextran, demonstrated by the decrease in current over multiple scans of potential. In contrast, the UV cured hydrogel displayed a much better preservation of current response, due to the formation of a stable cross-linked hydrogel.

Prior to enzyme immobilization, the UV curing parameters such as the drying/mixing time of Fc–CD-dex and Bzp-dex before UV curing (Fig. S2, ESI†), UV light intensity (Fig. S3, ESI†) and UV curing duration (Fig. S4, ESI†) are examined. It can be concluded that a proper mixing of the two modified dextran polymers is necessary. Insufficient mixing leads to the formation of non-uniform hydrogel layers, leading to CVs that are not reversible, where the ratio between the anodic and cathodic peak current density (j_a/j_c) is far larger than 1 (Fig. S2A, ESI†). Instead of a simple E mechanism for the redox process of free Fc with a reversible CV (Fig. 1A), an EC-like mechanism is suggested for the redox of Fc in the inclusion complex. One possible explanation by the fact is that the oxidized Fc (Fc^+ cation) is more water soluble than Fc itself,[24] so that the formation of Fc^+ will lead to partial decomplexation from CD and the formation of a new equilibrium with a lower association constant. The decomplexation is a supramolecular interaction, instead of a physical desorption process. Thus, the EC mechanism can be summarized as:

$$\text{E step: Fc–CD-dex} \rightleftarrows \text{Fc}^+\text{–CD-dex} + e^-$$

$$\text{C step: Fc}^+\text{–CD-dex} \rightarrow \text{Fc}^+ + \text{CD-dex}$$

By applying sufficient mixing, it is possible to obtain thin and uniform electrode surface films displaying more reversible CV curves with a j_a/j_c closer to unity (Fig. S2C, ESI†). The

observed CV response is the direct observation of the self-exchange of electrons, *via* collisions between mobile redox active centers,[8] of included Fc tethered to the dextran backbone. It has been revealed that the electron diffusion redox hydrogels are highly sensitive to the mobility of chain segments.[8,25] UV light intensity and duration (Fig. S3 and S4, ESI†) have certain effects on the initial CV behaviours of Fc in the hydrogel, as they affect the rigidity of the hydrogel film and thus self-exchange of electrons within the hydrogel. In particular, 20–60 s UV curing is found to make a reasonably stable hydrogel (Fig. S4B–D, ESI†). A duration of 20 s is selected primarily based on considerations of process efficiency.

The composition of the hydrogel is further tuned by varying the molecular weight (MW) of Bzp-dex, degree of substitution (DS) of CD-dex and molecular ratios of introduced Fc to CD-dex for inclusion formation (Fig. 2). Decreasing the MW of Bzp-dex and increasing the DS of CD-dex are expected to enhance the extent of cross-linking of the two modified dextran polymers, thus hindering electron diffusion and lowering the redox current of Fc (Fig. 2A and B). This is further validated *via* a rheological test on the UV cured hydrogel, showing that the storage modulus, loss modulus, and complex viscosity of the hydrogel with a higher DS are all greater than those of the hydrogel with a lower DS in a frequency range from 0.1 to 100 rad s^{-1}. (Fig. S5, ESI†). Similar behaviours were observed with an electrochemical addressable osmium complex modified polymer with the increasing ratio of cross-linker.[26] The improvement of current density with a higher concentration of Fc/Fc–CD inclusion complexes is shown in Fig. 2C.

The optimized recipe is then adopted to immobilize a model enzyme glucose oxidase (GOx), with a relatively short UV irradiation period (100% intensity) of 20 s. GOx is frequently used for the bioelectrochemical oxidation of glucose[1] and can use Fc as a mediator due to their suitable redox potentials (E°),[27] *i.e.*, the E° of the redox mediator Fc is higher than that of GOx to favour the electrochemical oxidation.[28] As shown in Fig. 3, the CVs towards 6 mM glucose of the hydrogel with GOx immobilized display a sigmoidal shape without cathodic peaks. It indicates quick ET mediation between the electrode and GOx by Fc–CD-dex.[29,30] The bioelectrode exhibits a good storage stability over a time course of 48 h as the electrochemical response is largely preserved by 64% of the initial value after soaking in PBS. An observed partial loss of electrochemical

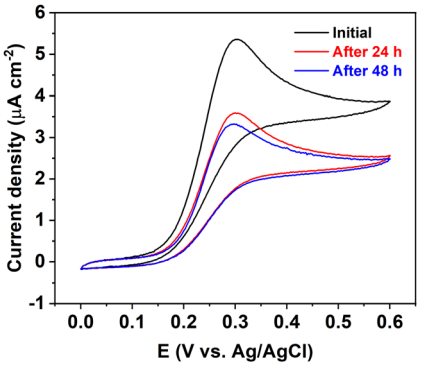

Fig. 3 CVs of the electrode modified with GOx immobilized in the hydrogel towards 6 mM glucose in 0.1 M pH 7 PBS. Scan rate: 5 mV s^{-1}. Storage condition: 4 °C.

response to glucose is likely due to (1) enzyme and mediator leakage from the electrode as the hydrogel considerably swells in the water (Fig. S6, ESI†) and (2) enzyme inactivation.[31] Blank CVs of the bioelectrode in the absence of glucose show a decrease in the surface coverage of Fc from *ca.* 125.4 to 41.1 μC cm^{-2} over 48 h (Fig. S7, ESI†), corresponding to a loss of 67%. It's thus reasonable to assume that the mediator leakage constitutes the major factor of the declined storage activity, which can be overcome in the future by covalently grafting Fc to Bzp-dex. This bioelectrode holds the promise to be used in biofuel cells and biosensors, which will be investigated in a follow-up study. The general nature of this methodology to immobilize other mediators and enzymes is also under study.

Antibiofouling properties of electrode surfaces are of high importance in relation to implantable bioelectrodes as a biofouling layer consisting of a protein and cells will inevitably hinder the diffusion of substrates to the bioelectrode.[32] The antifouling property of CD-dex has previously been revealed to reduce the nonspecific adsorption of albumin,[33] due to the high hydrophilic nature of the polymer. Herein, we further investigate the ability of the hydrogel to suppress cell adhesion. Specifically, we use both standard optical microscopy and fluorescence microscopy to evaluate fibroblast cell attachment on the hydrogel modified surface. A Nafion coating, which is often used for enzyme immobilization on microelectrodes, promotes the cell attachment to the surface, as evidenced by the clearly identified cell

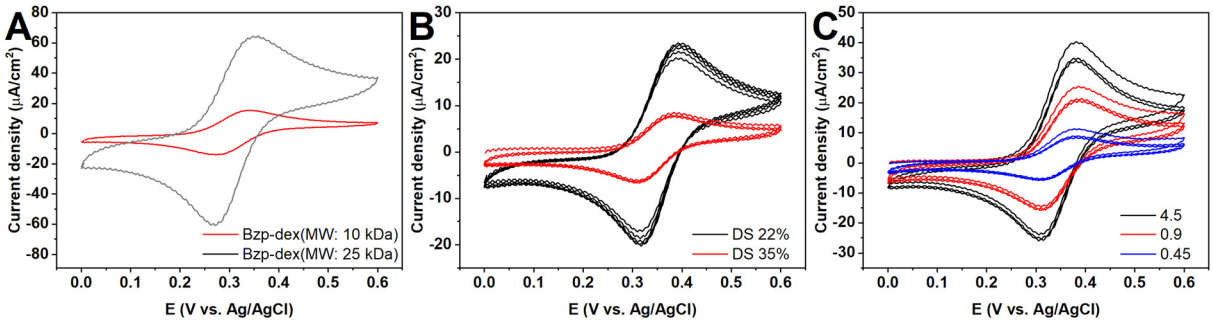

Fig. 2 CVs of the UV cured hydrogel using (A) Bzp-dex with two different MWs, (B) CD-dex with two different DSs and (C) different molecular ratios of introduced Fc to CD-dex. Solution: 0.1 M pH 7 PBS. Scan rate: 100 mV s^{-1}.

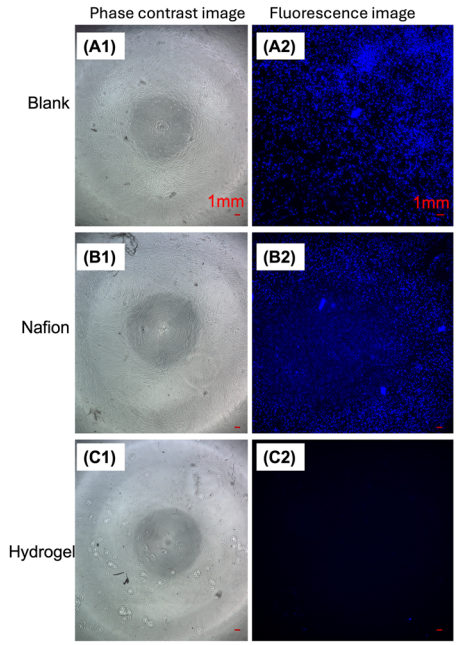

Fig. 4 Cell attachment results. Normal phase contrast and fluorescence images of fibroblasts attached onto the bottom of a 96-well plate without modification (blank, A1–A2) and modified with Nafion (B1–B2) and hydrogel (C1–C2), after 24 hours of culture. Scale bar: 1 mm.

layer (Fig. 4B). This is confirmed by nuclear staining, showing a high number of cells present, similar to a previous work.[32] Similarly, good cell attachment is observed for the blank control group (Fig. 4A). In sharp contrast, cell attachment is inhibited, evidenced by the lack of discernible cells, when the surface is coated with the dextran-based hydrogel (Fig. 4C). For implantable biopower or biosensing applications, the service timeframe of enzyme electrodes is application-dependent, requiring operational stability ranging from hours to years. For example, an ingestible sensing device may only need several hours,[34] while the powering unit for a pacemaker requires a lifetime of at least 10 years.[35] Further optimization and investigation are needed to assess the operational stability of the bioelectrode under biofouling conditions or *in vivo*.

Conclusions

In conclusion, CD and Bzp have been grafted to dextran polymers, respectively. CD-dex is used to immobilize Fc by the formation of inclusion complexes and is photocross-linked with Bzp-dex leading to the formation of a hydrogel allowing for the entrapment of GOx. The immobilized GOx is electrochemically active to oxidize glucose for further applications, such as biosensing or biopowering. Our results show that the electrochemical behaviour of the redox active species in the hydrogel is highly dependent on the UV curing conditions and hydrogel composition. The hydrophilic hydrogel furthermore demonstrates cell adhesion resistance to fibroblast cells. All the merits of the CD based hydrogel as confirmed in this study imply its great potential for use in bioelectrochemical applications. The versatile nature of the modular hydrogel system allows for the inclusion and subsequent immobilization of other low-redox potential and low-water soluble redox mediators such as tetrathiafulvalene, 1,4-naphthoquinone and tetracyanoquinodimethane, as well as the immobilization of a large variety of enzymes, paving the way for a wide spectrum of MET based applications, especially as implantable bioelectrodes.

Conflicts of interest

There are no conflicts to declare.

Data availability

Data are available upon request from the authors.

Acknowledgements

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