The efficiency of injectable biomaterials as minimally invasive therapeutics significantly relies on biomaterial’s characteristics, such as stability, biodegradation rate, and interaction with the host tissue, which requires real-time tracking of the biomaterials. Fluorescence imaging is considered as a noninvasive technique for monitoring biomaterials; however, the commonly used fluorescent agents are often accompanied by photobleaching and toxicity. Herein, graphene quantum dots (GQDs) are introduced as a biocompatible and stable fluorophore for imaging and noninvasive monitoring of a physically cross-linked injectable shear-thinning biomaterial (STB) of gelatin–silicate nanoplatelets. Silicate nanoplatelets and GQDs serve as the physical cross-linkers of gelatin making electrostatic interaction with gelatin chains. Different STB-GQDs formulations are assessed in terms of fluorescence intensity, injectability, thermal stability, and cellular biocompatibility. STB-GQDs with 0.06% GQDs, 6% solid material, and 50% silicate in the solid material show the strongest in vitro fluorescence and the highest thermal stability. In vivo monitoring of STB-GQDs is also achieved through fluorescent imaging where incorporated GQDs exhibit a robust and stable signal, suggesting their promising applications in long-term tracking of gelatin-based STBs.

1. Introduction

Biocompatible and biodegradable hydrogels have been widely used for controlled drug delivery, tissue regeneration, and other biomedical applications. In particular, hydrogel-based shear-thinning biomaterials (STBs) have attracted tremendous attention due to their injectability and self-healing properties, making them useful in delivering therapeutic agents through catheters, syringe needles, and other extrusion-based methods. Shear-thinning hydrogels are often formed by noncovalent cross-linking of hydrogel chains through physical interactions, such as hydrogen bonding, electrostatic interactions, hydrophobic interactions, and host–guest chemistry with nano- or microparticles. The hydrogel’s properties are balanced and controlled based on competition of attraction and repulsion forces. This reversible physical interaction is responsive to the environment, making hydrogels stimuli-responsive materials with shear-thinning behavior. In general, STBs exhibit a considerable decrease in the viscosity while applying shear stress during injection, and a self-healing property after removing the stress. We have introduced a gelatin-based STB through electrostatic interactions between gelatin and silicate nanoplatelets. Synthetic silicate nanoplatelets are disks of 20–30 nm diameter with bioactive properties, which are used as the rheological modifier in STB. We demonstrated promising applications of the injectable STB for the...

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treatment of hemorrhage, endovascular embolization and treatment of aneurysm, and the delivery of cells and growth factors. Monitoring of the STB in the abovementioned applications is of great interest to evaluate the STB’s function and behavior. In particular, biodegradation rate, retention in the target region, and interaction with the host tissues are important parameters and need to be tracked precisely.

Although in vitro models can show hydrogels’ behavior, they do not precisely reveal their in vivo behavior. To better understand the in vivo behavior of hydrogels in a real-time and dynamic manner, it is necessary to sacrifice a large number of animals. Therefore, the development of imaging techniques for real-time and noninvasive monitoring of STBs is an urgent need, which minimizes uncontrollable parameters and reduces the number of animal models in experiments.

Several imaging techniques, including magnetic resonance imaging (MRI) with high spatial resolution, ultrasound imaging, nuclear imaging, fluorescence imaging, and a combination of these techniques, have been suggested for noninvasive monitoring of hydrogels in vivo. For example, Albadawi et al. prepared an injectable embolic hydrogel loaded with tantalum. The tantalum served as an imaging agent to visualize fragmentation or migration of the hydrogel using a combination of MRI, ultrasound, and computed tomography techniques. However, the application of MRI and ultrasound techniques in imaging is limited by their low sensitivity and complicated instruments. Nuclear imaging technique also requires labeling of biomaterials with radio-active contrast agents. Fluorescence imaging is considered as a noninvasive imaging technique with several advantages over other diagnostic methods, including high sensitivity, rich color choice, no risk of radioactivity, and simplicity. In addition, it does not require complicated and expensive instruments for bioimaging and hydrogel functionalization.

Fluorescein, rhodamine B, protoporphyrin, and IRDye 800CW have been suggested for fluorescent labeling of hydrogels for in vivo monitoring of their biodegradation and drug release mechanism. However, these commonly used organic dyes and fluorescent proteins are often expensive and accompanied by photobleaching. Semiconductor metal quantum dots with stable fluorescence properties have extensively been used for bioimaging and targeted delivery. For example, Zhao et al. introduced core/multishell CdSe/ZnS quantum dots functionalized with multifunctional polypeptide ligands for pH-responsive protein delivery and cellular imaging. Also, the integration of semiconductor quantum dots within different hydrogels has been evaluated for fluorescence imaging and biosensing applications. Although semiconductor quantum dots show a stable and considerable fluorescence without photobleaching, their significant toxicity has limited their applications in biomedical imaging. Graphene quantum dots (GQDs) have been introduced as novel fluorescent agents due to their tunable and robust photoluminescence for in vivo and in vitro imaging. Exceptional physicochemical properties of GQDs, owing to their ultrasmall size (<10 nm), including photostability, extreme physiological stability, biocompatibility, low cost, and facile synthesis, distinguish them from other common fluorescent agents. In addition, low in vitro toxicity of GQDs with ≈80% cellular viability at a concentration of 100 μg mL⁻¹ on different cell lines along with their in vivo nontoxicity makes them potentially useful as fluorophores for bioimaging.

Graphene-incorporated hybrid hydrogels have been developed for various biomedical and nonbiomedical applications. For instance, 3D graphene hydrogel networks incorporated with nickel-based mesoporous nanosheets, nanoflowers, or CoO quantum dots have demonstrated promising application as electrodes in supercapacitors. In another study, Paul et al. developed an injectable and biocompatible gelatin methacryloyl hydrogel, mixed with functionalized graphene oxide (GO) nanoparticles, which were loaded with DNA vascular endothelial growth factor, for angiogenic gene delivery. In addition to sustained delivery of therapeutic agents, GO nanoparticles can enhance the shear-thinning properties of injectable polymer hydrogels. However, the application of GO nanoparticles in hydrogels for imaging purposes is limited, as they do not exhibit fluorescence property. Surface charge of biocompatible GQDs in addition to their hydrophilic functional groups suggests their promising applications in the fabrication of injectable hydrogels through physical interactions with polymer matrix, while facilitating hydrogel’s real-time monitoring via fluorescence imaging.

For example, Khabibullin et al. prepared an injectable hydrogel composed of cellulose nanocrystals (CNCs) and GQDs as a fluorescent bioink for 3D printing. Although both GQDs and CNCs possess negative charges, robust hydrogen bonding and hydrophobic interactions between them resulted in the formation of the injectable hydrogel for 3D printing applications. Biswas et al. developed a blue-emitting self-healing hydrogel by embedding GQDs in synthesized Amoc (N-anthracenemethyloxycarbonyl) capped aromatic amino acid-based materials. However, these investigations did not study in vivo tracking of injectable hydrogels using fluorescent GQDs. More importantly, our STB formulations will provide regeneration capacity for developed hybrid hydrogels.

Positive charge of gelatin chains enables physical electrostatic interactions with negatively charged silicate nanoplatelets and GQDs, leading to the formation of injectable STB-GQDs. Considering demonstrated medical applications of our developed gelatin-based STB particularly in endovascular hemostasis, real-time monitoring of hydrogel is a critical need to prevent non-target embolization. To the best of our knowledge, there is no report on fluorescence in vivo monitoring of injectable gelatin-based STBs using fluorophore GQDs. In this work, GQDs are integrated within our gelatin-based STB, enabling real-time monitoring and tracking of STBs through noninvasive fluorescence imaging. We evaluated the effect of GQDs on the injectability and shear-thinning behavior of STBs. In addition, considering the possibility of a quench of GQDs’s fluorescence after integration within the STB, the fluorescence property of STB-GQDs was studied. The optimum formulation of STB-GQDs was determined based on its injectability, thermal stability, fluorescence intensity, and cellular biocompatibility. In vivo monitoring of STB-GQDs and STB was finally evaluated at different time intervals using fluorescence imaging to reveal the STB’s stability in animal models.
2. Results and Discussion

2.1. Preparation and Characterization of STB-GQDs

Single-layered GQDs were synthesized by chemical oxidation of carbon black and separation of ultrasmall size nanoparticles using a low-cost, facile, and high-yield method.\(^{[14]}\) The synthesized GQDs were highly stable in water and physiological environments, such as cell culture medium and phosphate-buffered saline (PBS) for several months without any visible precipitation (Figure S1a (inset), Supporting Information). The absorbance of GQDs showed the typical UV absorbance of GQDs at 231 nm (Figure S1a, Supporting Information).\(^{[62]}\) FT-IR spectroscopy of GQDs showed stretching vibration of O=H groups at 3441 cm\(^{-1}\), C=O vibration of aromatic rings at 1616 cm\(^{-1}\), C=O vibration of carboxyl groups at 1691 cm\(^{-1}\), and C–O vibration of carboxyl, hydroxyl, and epoxy groups at 1427, 1263, and 1080 cm\(^{-1}\), respectively (Figure S1b, Supporting Information).\(^{[63]}\) The oxygen functional groups in the structure of GQDs play an essential role in the high stability of GQDs in biological solutions. High-resolution transmission electron microscopy (HRTEM) image of GQDs in Figure 1a shows synthesized GQD nanoparticles with an average diameter of 2.8 ± 0.7 nm, which are uniformly dispersed in solution. It also confirmed the lattice spacing of 0.28 nm in the crystalline structure of GQDs, corresponding to the in-plane lattice spacing of graphitic carbon. In addition, the typical X-ray diffraction (XRD) peak of GQDs at 21.5°, equal to the interlayer spacing of 0.395 nm, was observed in the X-ray diffraction pattern of synthesized GQDs (Figure 1a).\(^{[44]}\) To evaluate the application of GQDs in fluorescence imaging, fluorescence spectroscopy of GQDs was studied at different excitation wavelengths. The GQDs exhibited an excitation-dependent emission similar to other carbon quantum dots,\(^{[64]}\) with the highest emission at 510 nm occurred at the excitation wavelength of 400 nm (Figure 1b). The GQDs solution exhibited a robust green fluorescence under UV light, easily observed by naked eyes (Figure 1b (inset)), suggesting their promising application for fluorescence imaging.

Dynamic light scattering measurement indicated a zeta potential of −40 mV for GQD aqueous solution due to oxygen functional groups’ presence on their surface. The negative surface charge of GQDs facilitates their physical interaction with gelatin, leading to the formation of gelatin-GQDs hydrogel with fluorescence properties originating from GQDs. However, the prepared gelatin-GQDs hydrogel was not stable at 37 °C. Our previous investigations demonstrated that the addition of a specific concentration of silicate nanoplatelets to gelatin results in the thermal stability of hydrogel at 37 °C by increasing sol–gel transition temperature, in addition to the appearance of the shear-thinning property.\(^{[14,18]}\) Thus, silicate nanoplatelets were added to the gelatin-GQDs mixture to prepare a thermally stable and fluorescent hydrogel with shear-thinning properties.

Electrostatic interaction between gelatin and silicate nanoplatelets occurred in the physically cross-linked hydrogel with the shear-thinning property. In addition, GQDs served as a secondary cross-linker by the formation of electrostatic interaction with gelatin chains. To prepare gelatin-based STB-GQDs, heated gelatin solution was mixed with GQDs, followed by vigorous mixing with silicate gel to make a uniform distribution of negatively charged silicate nanoplatelets and GQDs within positively charged gelatin chains (Figure 1c). Different formulations of STB-GQDs (xNCy-zGQDs) were prepared based on the weight percentage of total solid weight (x), the weight percentage of silicate nanoplatelets in the entire solid content (y), and the percentage of GQDs in the mixture (z). The STB-GQDs hydrogel was comfortably injectable through needles and catheters while showing self-healing property after injection (Figure 1d).

Significant change (P < 0.01) of zeta potential from +15 mV in 6NC0 to negative charge of −25 mV in 6NC50 (without GQDs) revealed the formation of electrostatic interaction between silicate nanoplatelets and gelatin chains after the addition of silicate nanoplatelets to gelatin solution. In addition, a significant increase (P < 0.05) in the negative zeta potential of 6NC50 by the addition of 0.06% and 0.12% GQDs was observed, which also demonstrates the formation of electrostatic interaction between GQDs and gelatin, showing the role of the GQDs as the secondary physical cross-linker (Figure 1e). To obtain the best STB-GQDs formulation, the formulation of STB was first optimized without the addition of GQDs based on injectability and thermal stability of hydrogel by changing the amount of gelatin and silicate nanoplatelets. The GQDs concentration was subsequently optimized based on the fluorescence intensity of STB-GQDs hydrogel. We recently showed that the STBs with a lower than 5% solid content easily liquefy at 37 °C, whereas a solid content of more than 8% required a high force for manual injection through a 5F catheter.\(^{[15]}\) Therefore, two formulations of 6NC50 and 6NC75 with 6% solid content were chosen, which showed thermal stability and feasible injectability.

2.2. Fluorescence Property of STB-GQDs

To determine the optimum concentration of GQDs in STB-GQDs, fluorescence spectroscopies of GQDs, 6NC50-GQDs, and 6NC75-GQDs were measured at different concentrations of GQDs and the excitation wavelength of 400 nm. As shown in the fluorescence spectrum of GQDs in Figure 2a, the highest fluorescence intensity occurred at 0.015% GQD, and quench of fluorescence was observed at the concentrations of higher than 0.015%. This behavior is due to the proximity of GQDs at high concentrations, which enhances electron transfer between GQD layers. This results in an efficient Förster resonance energy transfer before emission of energy and, thereby, the quench of emitted fluorescence.\(^{[65,66]}\) As a result, adding a high concentration of GQDs to STBs does not necessarily enhance the hydrogel’s fluorescence intensity.

Fluorescence spectra of STB-GQDs for formulations of 6NC50 and 6NC75 at different GQD concentrations (Figure 2b,c) showed that the STB itself exhibits a weak emission peak at 450 nm originated from gelatin. The addition of GQDs to the STBs resulted in a considerable increase in the fluorescence intensity of STB-GQDs. In addition, a shift of maximum emission wavelength was observed from gelatin’s characteristic peak at 450 nm to GQD’s distinct peak at 510 nm in both 6NC50 and 6NC75. As shown in Figure 2d, a sharp quench of fluorescence for GQDs was revealed at the concentrations of higher than 0.015%. Similarly, a gradual reduction of fluorescence intensity was observed for STB-GQDs at GQD concentrations of higher
than 0.06% for 6NC50-GQDs and higher than 0.12% for 6NC75-GQDs. Significantly higher fluorescence intensity was observed for STB-GQDs compared with GQDs at the GQD concentrations of higher than 0.03%. This can be explained by the presence of gelatin chains between GQDs, preventing the proximity of GQD layers to each other. This phenomenon resulted in a gradual quench of fluorescence by increasing GQD concentration in STB-GQDs, whereas a sharp quench of fluorescence was observed in GQDs only. The highest fluorescence intensity of STB-GQDs occurred at the GQD concentration of 0.06% for 6NC50-GQDs, indicating 6NC50-0.06GQDs as the optimum formulation of STB-GQDs. Also, slightly higher fluorescence intensity was observed for 6NC50-GQDs than 6NC75-GQDs (Figure 2d,e). This can be explained by the higher content of gelatin in 6NC50 compared with 6NC75, resulting in higher fluorescence intensity of 6NC50, which originated from gelatin.

A robust green fluorescence from GQDs, 6NC50-GQDs, and 6NC75-GQDs after exposure to UV light was easily observed by
naked eyes for different concentrations of GQDs (Figure 2e). STBs alone did not show significant fluorescence, whereas the addition of GQDs resulted in the appearance of green fluorescence emitted from STB-GQDs. Figure 2e also confirms the quench of fluorescence at high concentrations of GQDs and the appearance of the most robust fluorescence at the GQD concentrations of 0.06% and 0.12% for 6NC50-GQDs and 6NC75-GQDs, respectively.

Figure 2. Fluorescence spectrum of a) GQDs, b) 6NC50-GQDs, and c) 6NC75-GQDs at GQD concentrations of 0, 0.0035, 0.0075, 0.015, 0.03, 0.06, 0.12, and 0.24% (w/v) and an excitation wavelength of 400 nm. d) Fluorescence intensity of 6NC50-GQDs, 6NC75-GQDs, and GQDs at different concentrations of GQDs and excitation and emission wavelengths of 400 and 510 nm, respectively. e) Photographs of GQD aqueous solution and 6NC50-GQDs and 6NC75-GQDs to varying concentrations of GQDs under visible light (left) and UV light (right). Note that the images of 0% GQDs were taken separately from the rest.
2.3. Injectability and Rheological Property of STB-GQDs

The injectability of STB-GQDs was evaluated to examine its facile injection through catheters or syringe needles for imaging purposes. STB-GQDs was easily injectable through 18G and 23G syringe needles, and also 5F catheter at the optimum GQD concentration of 0.06% without using any equipment. The injectability of STB-GQDs through catheters and needles was further studied by measuring the required injection force and evaluating the effect of different parameters on it. An injection force was applied to loaded samples in a syringe using a compressive platen in an Instron mechanical tester. Applying the injection force resulted in an initial linear increase in the force within STB’s network, which was required for disturbance of the network structure of the STB and its extrusion from 5F catheter (Figure 3a,b and Figure S2, Supporting Information) and 18G and 23G needles (Figure 3c,d). Subsequently, a slight decrease in the injection force was observed due to a reduction of STB viscosity. The injection force finally reached a plateau once the STB was extruded from the syringe, measured as the required injection force. Similar behavior was observed for STB-GQDs in 18G and 23G needles and 5F catheters, with a higher required injection force compared with 6NC50 and 6NC75. The injection force in the 5F catheter reached a plateau after a longer time than the syringe needles due to its longer length. In addition, no significant difference was observed in the maximum injection force, whether the catheter was prefilled with hydrogel or not, showing that the presence of remaining STB-GQDs in catheters does not affect the required injection force (Figure 3a,b).

To assess the effect of injection parameters (i.e., needle size and flow rate), required injection force was measured for STB-GQDs at GQD concentration of 0.06% using 5F catheter, 18G, and 23G needles at the injection rates of 1, 2, and 3 mL min⁻¹ (Figure 3e,f). The results showed a significant increase (P < 0.01) in the required injection force through the 5F catheter compared with the needles for all STB and STB-GQDs formulations (Figure 3e). However, no significant difference was observed between 18G and 23G needles for 6NC50 and 6NC50-GQDs. However, the 6NC75-GQDs showed a significant increase in the required injection force for the 23G needle compared with the 18G needle. Furthermore, an increase in the flow rate from 1 to 3 mL min⁻¹ through the 5F catheter did not significantly affect the STB samples’ injection force. In contrast, a significant increase in the injection force was observed for the STB-GQDs samples (Figure 3f). In addition, the STB composition (6NC75 or 6NC50) did not significantly affect the required injection force through the needles. At the same time, the 5F catheter showed a considerably higher force of injection needed for 6NC75 formulation compared with 6NC50 in both STB and STB-GQDs at different flow rates (Figure 3e,f). Moreover, the addition of GQDs to STB did not significantly affect the injection force through the needles. However, a significant increase was observed in the required injection force through the 5F catheter at different flow rates after the addition of GQDs.

In general, the injection force results showed that flow rate, STB formulation, and the addition of GQDs to STB did not considerably affect the injection force through needles, whereas the injection force through 5F catheters is significantly affected by these parameters. Considering the higher fluorescence intensity and lower injection force of 6NC50-GQDs than 6NC75-GQDs, 6NC50-GQDs with the GQD concentration of 0.06% were chosen for further experiments. Although strong electrostatic interaction between GQDs and gelatin increased the injection force needed for STB-GQDs, it was still easily injectable through 5F catheters.

In addition to thermal stability, the addition of silicate nanoparticles to gelatin can lead to the appearance of yield stress and shear-thinning behavior in STB. The oscillatory shear rate sweep was conducted on 6NC75, 6NC50, 6NC75-0.06GQDs, and 6NC50-0.06GQDs to study the effect of GQDs on the shear-thinning behavior of STBs. The yielding behavior and yield stress (the break in the linearity of shear rate–shear stress at 37 °C) are shown in Figure 4a. The appearance of yield stress in STB-GQDs samples suggests the maintenance of the shear-thinning property of STBs after the addition of GQDs. The yield stress increased by increasing silicate nanoparticles in 6NC75 compared with 6NC50. In addition, the addition of GQDs to STBs in 6NC75 and 6NC50 increased the yield stress and viscosity, along with a slight decrease in the linear viscoelastic range (Figure 4a,b). The oscillatory strain was performed to evaluate the recovery of STB and STB-GQDs after applying a high strain amplitude (100%) above the linear viscoelastic region, followed by the removal of strain (1%) (Figure 4c,d). The break of the network at 100% strain amplitude resulted in liquid-like behavior and the higher amount of loss modules than storage modules (G″ > G′), whereas a solid-like behavior with G″ < G′ was observed in 1% strain amplitude (Figure 4c). The 6NC50 and 6NC50-GQDs at the GQD concentrations of 0.06% and 0.12% exhibited a rapid mechanical recovery in a few seconds after removing the strain. This behavior indicates a rapid self-healing of the network, preventing the flow of STB-GQDs in the targeted region after injection (Figure 4d). Although the addition of GQDs increased the storage and loss modulus of STBs, it did not impair the recovery of STBs.

2.4. Biocompatibility and In Vivo Bioimaging

In vitro biocompatibility of STB-GQDs formulations was evaluated using live–dead, MTT, and PrestoBlue assays by incubating 3T3 mouse fibroblast cells with GQDs, STB, and STB-GQDs. The MTT assay demonstrated the biocompatibility of GQDs, STB, and STB-GQDs with no significant difference in cellular metabolic activity (Figure 5a). Biocompatibility of STB-GQDs (6NC50 at GQD concentration of 0.06%) was further confirmed using live/dead assay. The live–dead assay revealed a large number of live cells (green) compared with dead cells (red) with a cellular viability of ≈100% after 1, 3, and 5 days of incubation with 6NC50-GQDs, 6NC50, and GQDs (Figure 5b,c). In addition, the cells incubated with GQDs, 6NC50, and 6NC50-GQDs exhibited a significant increase in metabolic activity from day 1 to day 3, further demonstrating the biocompatibility of 6NC50-GQDs (Figure 5d).

The potential ability of GQDs as a fluorescent agent in the in vivo monitoring and tracking of STB was evaluated by subcutaneous injection of 6NC50 and 6NC50-GQDs at the GQD concentration of 0.06%. The samples were injected into mouse
models with subsequent fluorescence imaging after 1 and 3 days using the in vivo imaging system. The in vivo monitoring of 6NC50-GQDs showed a high fluorescence signal emitted from the injection site, whereas 6NC50 did not show any signal (Figure 5e). This result confirms the promising application of GQDs in STB composition as a potent fluorescent agent for tracking STBs in biomedical applications. In addition, no significant decrease was observed in the in vivo fluorescence intensity of 6NC50-0.06GQDs after 3 days, showing no considerable degradation of hydrogels during this time. This confirms the results

Figure 3. a–d) Dynamics of injection force for 6NC50, 6NC75, 6NC50-0.06GQDs, and 6NC75-0.06GQDs samples at a flow rate of 2 mL min$^{-1}$ through a) 5F catheter, pre-filled with samples, b) 5F catheter, not pre-filled with samples, c) 18G needle, and d) 23G needle. The average value of the plateau was used to quantify the injection force. e,f) Measured force for injection of 6NC50, 6NC75, 6NC50-0.06GQDs, and 6NC75-0.06GQDs samples e) through 5F catheter, 18G, and 23G needles at a flow rate of 2 mL min$^{-1}$ (*) vs the same sample at 18G, # vs the same sample at 23G and f) at the flow rates of 1, 2, and 3 mL min$^{-1}$ through 5F catheters († vs 6NC50 at the same flow rate, ‡ vs 6NC75-0.06GQDs at the same flow rate, and § vs the same sample at the flow rate of 1 mL min$^{-1}$). Data are mean ± SD (n = 3). The double and single symbols indicate P < 0.01 and P < 0.05, respectively, as calculated by two-way ANOVA analysis to show a significant difference between groups.
of our previous studies, which revealed the in vitro degradation of less than 20% for 6NC50 after 48 h of incubation at 37 °C and no considerable in vivo degradation of 6NC50 after 3 and 7 days of subcutaneous injection. The significant in vivo fluorescence of 6NC50-0.06GQDs after 72 h of injection, along with its stable in vitro fluorescence after incubation in PBS at 37 °C for 72 h (Figure S3, Supporting Information), demonstrates the stability of GQDs in STBs due to their robust ionic interaction with the STBs and their long-term photostability. These findings suggest the potential application of STB-GQDs in long-term and localized imaging. The injected STB and STB-GQDs did not cause any adverse effect or significant inflammation in animals. Also, our previous studies demonstrated in vivo biocompatibility of different STB formulations including 6NC50 and 6NC75 after 28 days of injection or implantation in mice. 

3. Conclusion

We successfully synthesized an injectable and fluorescently labeled gelatin-based hydrogel prepared by the physical cross-linking of gelatin chains with silicate nanoplatelets and GQDs through electrostatic interactions. Silicate nanoplatelets played a crucial role in inducing shear-thinning properties in the biomaterial, resulting in its feasible injectability through catheters and syringe needles. In addition to the secondary physical cross-linker, GQDs served as the fluorescent agent providing fluorescence property for STB, facilitating in vitro and in vivo monitoring of STBs in various biomedical applications. The GQD concentration of 0.06% with STB formulation of 6NC50 was determined as the optimum STB-GQDs formulation with the highest fluorescence intensity, feasible injectability, and shear-thinning properties. The 6NC50-0.06GQDs could be easily monitored in the mice models without any significant decrease in the fluorescence intensity after 3 days, showing the photostability of GQDs and their application in long-time tracking of hydrogels. Our results demonstrated facile in vivo monitoring of hydrogels by integrating GQDs as a universal and robust fluorophore within hydrogels, which helps to better understand their in vitro and in vivo behavior during therapeutic applications.

4. Experimental Section

Materials: Vulcan CX-72 carbon black, synthetic silicate nanoplatelets (Laponite XLG), and porcine skin gelatin (type-A) were purchased from Cabot Corporation (Boston, USA), Southern Clay Products Inc. (Louisville, USA), and Sigma-Aldrich (Milwaukee, WI, USA), respectively; 10 kDa Amicon ultracentrifugal filters were purchased from Merck (Darmstadt, Germany). Nitric acid and dimethylsulfoxide (DMSO) were obtained from Merck Chemical Co. Dulbecco’s modified eagle media (DMEM), PBS, PrestoBlue cell viability reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin-streptomycin (Pen Strep), live/dead viability/cytotoxicity kit containing calcein and ethidium homodimer, fetal bovine serum (FBS), and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) were obtained from Thermo Fisher Scientific. 3T3 mouse fibroblast cells were obtained from American Type Culture Collection (ATCC). A Milli-Q gradient water purification system (Millipore, USA) was used for water purification in all experiments.

GQDs Preparation and Characterization: Chemical oxidation of CX-72 carbon black using nitric acid was performed to prepare GQDs. In brief,
0.22 g CX-72 carbon black was added to 50 mL nitric acid of 6 M and was refluxed for 24 h at 135 °C. The mixture was filtered through qualitative filter papers to separate unreacted components and was heated to evaporate nitric acid. The obtained reddish-brown solid was dispersed in water, and the dispersion was centrifuged for 30 min (10 000 rpm), using a 10 kDa Amicon ultracentrifugal filter. Ultrasmall GQDs in the filtrate were collected and freeze-dried for subsequent characterization experiments.

Morphology, size, and surface properties of prepared GQDs were characterized using HRTEM (Philips CM300, Netherlands), UV–visible (UV–vis) spectroscopy (Shimadzu UV 2450, Japan), XRD (Bruker D8 Advance, Germany), and Fourier transform IR (FT-IR; Bruker Vector22, Germany). The droplets of GQDs dispersion were placed on HRTEM copper grids and dried at room temperature to be imaged by HRTEM.

**STB-GQDs Preparation:** STB was prepared based on a previously described method, followed by the incorporation of GQDs. Gelatin stock solution (18% (w/v)) was prepared by dissolving gelatin (1.8 g) in the water while keeping it in an incubator at 80 °C for 30 min. Silicate nanoplatelets (0.9 g) were dispersed at 4 °C cold water, while vortexing to provide a uniform gel of nanoplatelets in water (9% (w/w)). To prepare 900 mg of 6NC50-GQDs (6% (w/w) solid material and 50% gelatin and 50% silicate in the solid material)-GQDs, different amounts of GQD dispersion were added to 150 μL of gelatin stock solution. The mixture was subsequently added to 300 mg silicate stock gel, followed by the addition of water to reach the solid content of 6% and vigorous vortexing to prepare a uniform gel. The same experiment was conducted to prepare 6NC75-GQDs (900 mg) in which 450 mg and 75 μL of silicate and gelatin stock solutions were used, respectively. GQDs were taken from 0.6% (w/v) stock aqueous solution, to reach to the final GQDs percentage (w/v) of 0.24%, 0.12%, 0.06%, 0.03%, 0.015%, 0.0075%, and 0.00375%.

**STB-GQDs Characterization:** Zeta potential of STB-GQDs at different concentrations of GQDs was measured using Zetasizer (Malvern, UK). Fluorescence spectra of GQDs, STB, and STB-GQDs were recorded for two STB formulations of 6NC75 and 6NC50 at different GQD concentrations, using a microplate reader at the excitation wavelength of 400 nm (Tecan Infinite F200 plate reader, Switzerland).

**STB-GQDs Rheological Analysis:** Rheological properties of STB and STB-GQDs were analyzed using a rheometer (MCR 301, Anton Paar, USA) with a 25 mm diameter parallel plate and a gap height of 500 μm at 37 °C. Mineral oil was cast around the plate to avoid the evaporation of water in the sample. Shear rate sweeps were performed from 0.001 to 10 s⁻¹. Gel recovery was monitored by applying 100% strain above the linear viscoelastic range for 5 min, followed by applying 1% strain at

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Figure 5. a) Relative viability of 3T3 cells incubated with 6NC50-GQDs, 6NC75-GQDs, and GQDs at the GQD concentrations of 0%, 0.6%, and 1.2% using MTT assay after 48 h of incubation. b) Live–dead staining images of 3T3 cells after 1, 3, and 5 days of incubation with 6NC50-0.06GQDs, 6NC50, and GQDs. Scale bars are 100 μm. c) Cellular viability measured from live–dead images. d) Metabolic activity of 3T3 cells after 1 and 3 days of incubation with 6NC50-0.06GQDs, 6NC50, and GQDs (**p < 0.01 shows statistically significant difference calculated by two-way ANOVA analysis). e) In vivo fluorescence images of mice subcutaneously injected with 6NC50 and 6NC50-0.06GQDs immediately and after 72 h of injection.
1 Hz for 5 min. Samples were equilibrated for 10 min and were under 10 S \(^{-1}\) steady shear for 2 min before conducting experiments.

**STB-GQDs Injection Test:** Injectable ability of STB and STB-GQDs was analyzed by measuring the required injection force using a mechanical tester (Instron Model 5542). Samples were loaded inside 3 mL syringes (BD Biosciences) and injected through 18 G needle (ID = 0.84 mm, L = 38 mm, BD Biosciences), 23 G needle (ID = 0.34 mm, L = 38 mm, BD Biosciences), and 5-French (SF) catheter (ID = 0.97 mm, L = 1000 mm, Cook Inc.). The syringe was fixed between a compressive platen compressing the top of the syringe plunger, and a tensile grip keeping the lower part of the syringe. The compression rate of compressive platen regulated the injection flow, and experiments were performed at the injection rates of 1, 2, and 3 mL min \(^{-1}\). The time-dependent injection force measured on the syringe plunger was recorded and plotted on the software.

**Cell Culture:** The 3T3 mouse fibroblast cell line was used to study the biocompatibility of STB-GQDs. The cells were cultured in culture flasks (T-175) containing DMEM medium supplemented with 1% (v/v) Pen Strep and 10% (v/v) FBS while incubating at 37 °C in a humidified atmosphere containing 5% CO\(_2\). The media were changed every 2 days, and cells with 3–5 passage numbers were used for experiments.

**In Vitro Cellular Biocompatibility Assays:** Viability and metabolism of cells were studied by performing live–dead and PrestoBlue assays by incubating cells with STB-GQDs at 0.06% (w/v) GQDs. Cells were seeded in 24 well transwell plates (20,000 cells mL \(^{-1}\), 1 mL cell-contained medium in each well) for 24 h, followed by the addition of gels to the inserts immersed in the cell medium and incubated for 1, 3, and 5 days. To perform live–dead staining, the cell media were removed, and the cells were washed with PBS three times, followed by incubation with 500 μL live–dead solution for 20 min. The live–dead solution contained calcein AM (0.05% (v/v)) for detecting live cells (green color) and ethidium homodimer-1 (0.2% (v/v)) for detecting dead cells (red color). The cells were washed with PBS after the removal of the solution and were monitored using a fluorescence microscope (Zeiss Axio Observer D1, USA). The PrestoBlue staining was performed by removing the cell culture medium and subsequent incubation of the cells with 500 μL PrestoBlue solution (10%, v/v) in fresh DMEM for 3 h. Each well's absorbance was recorded at fluorescence excitation and emission wavelengths of 530 and 590 nm using a microplate reader (Tecan Infinite F200 plate reader, Switzerland). Biocompatibility and possible cytotoxicity of samples were further studied by adding 100 μL of 5 mg mL \(^{-1}\) MTT stock solution to each well and incubating for 3 h. Subsequently, the cell media were removed, and DMSO (500 μL) was added to each well. To determine relative cell viability, each well's UV absorbance was recorded using a microplate reader at the wavelength of 570 nm. Each live–dead, PrestoBlue, and MTT assay was repeated and after 72 h of injection. The mice were kept under anesthesia using 2% isoflurane gas during the imaging process.

**In Vivo Imaging:** MF1 female mice with the weights of 20–25 g were purchased from Charles River Laboratories (USA) and maintained at the animal center at the University of California-Los Angeles, Center for the Health Sciences with the Animal Welfare Assurance Number of A3330-01. The in vivo experiments were carried out based on the approved protocol number of ARC-2010-045 and institutional animal use and care regulations. To evaluate the fluorescence imaging of STB-GQDs in vivo, 200 μL STB-GQDs and 200 μL STB with the optimum STB formulation of 6NC50 at 0.06% GQD were subcutaneously injected on the back of mice using 23G syringe needles. The mice were anesthetized before injection, and the noninjected mice were considered as the negative control. The in vivo imaging system (IVIS Lumina XR, Caliper) was used for the fluorescence imaging of mice, and the imaging was taken immediately and after 72 h of injection. The mice were kept under anesthesia using 2.5% isoflurane gas during the imaging process.

**Statistical Analysis:** Data are presented based on three independent experiments as the mean ± standard deviation. One-way and two-way ANOVA analyses with the Tukey post hoc method were used to determine P values for each pair of groups using the Graphpad Prism software. One-way ANOVA is used to analyze the data in Figure 1e, and two-way ANOVA is used to analyze the data in Figure 3e,f and 5a,c,d. The statistically significant difference was considered for the P values of less than 0.05.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

Research data are not shared.

**Keywords**

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[1] J. Li, D. J. Mooney, *Nat. Rev. Mater.* 2016, 1, 16071.
[2] S. Mantha, S. Pillai, P. Khayambashi, A. Upadhyay, Y. Zhang, O. Tao, H. M. Pham, S. D. Tran, *Materials (Basel)* 2019, 12, 3323.
[3] S. Ahadian, R. B. Sadeghian, S. Salehi, S. Ostrovudov, H. Bae, M. Ramalingam, A. Khademhosseini, *Bioconjugate Chem.* 2015, 26, 1894.
[4] M. Tavafoghi, A. Sheikh, R. Tutar, J. Jahangiry, A. Baidya, R. Haghniaz, A. Khademhosseini, *Adv. Healthcare Mater.* 2020, 9, 1901722.
[5] A. Sivashanmugam, R. Arun Kumar, M. Vishnu Priya, S. V. Nair, R. Jayakumar, *Eur. Polym. J.* 2015, 72, 543.
[6] M. Guvendiren, H. D. Lu, J. A. Burdick, *Soft Matter* 2018, 8, 260.
[7] A. P. Mathew, S. Uthaman, K.-H. Cho, C.-S. Cho, I.-K. Park, *Int. J. Biol. Macromol.* 2018, 110, 17.
[8] J.-A. Yang, J. Yeom, B. W. Hwang, A. S. Hoffman, S. K. Hahn, *Progr. Polymer Sci.* 2014, 39, 1973.
[9] A. Thakur, M. K. Jaiswal, C. W. Peak, J. K. Carrow, J. Gentry, A. Dolatshahi-Pirouz, A. K. Gaharwar, *Nanoscale* 2016, 8, 12362.
[10] G. Song, Z. Zhao, X. Peng, C. He, R. A. Weiss, H. Wang, *Macromolecules* 2016, 49, 8265.
[11] C. B. Rodell, J. W. MacArthur, S. M. Dorsey, R. J. Wade, L. L. Wang, Y. J. Woo, J. A. Burdick, *Adv. Funct. Mater.* 2015, 25, 636.
[12] A. Khabibullin, M. Alizadehgiashi, N. Khuu, E. Prince, M. Tebbe, E. Kumacheva, *Langmuir* 2017, 33, 12344.
[13] A. Sheikh, S. Afewerki, R. Oklu, A. K. Gaharwar, A. Khademhosseini, *Biomater. Sci.* 2018, 6, 2073.
[14] A. K. Gaharwar, R. K. Avery, A. Assmann, A. Paul, G. H. McKinley, A. Khademhosseini, B. D. Olsen, *ACS Nano* 2018, 8, 9833.
