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Chitosan Hydrogels for Targeted Dye and Protein Adsorption

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ABSTRACT

There is an urgent requirement to devise and develop highly-effective materials for the purification of industrial wastewater. Coloured effluents are particularly problematic due to their toxicity in plants, aquatic organisms and animals, dictating that polymeric adsorbents are highly sought for their capture. Chitosan hydrogels are a cost-effective substrate for the adsorption of dye molecules as they are contain a biopolymeric gelator that can form hydrogels with a polymer content as low as 3% by mass. Such materials are also highly suited for use within a biomedical context as carrier vehicles for the encapsulation and delivery of protein macromolecules, due to their hydrophilic nature. We disclose the capability chitosan-based hydrogels to non-covalently adsorb both reactive and disperse dye molecules, and a model anionic protein from aqueous solution. The materials generated offer both a platform for dye removal from industrial wastewater, and for the encapsulation and pH-mediated release of protein macromolecules.

INTRODUCTION

The presence of organic pollutants in industrial wastewater is an acute environmental concern.[1] Coloured effluents are especially problematic due to their extensive toxicity.[2] In particular, reactive dyes are frequently used as effective colorants due to their capability to covalently bind to cellulosic fibres.[3] However, their extended aromatic structures retard their degradation, ensuring that their environmental release causes extreme ecological damage.[4] C.I. Reactive Black 5 (RB5) is a commonly used reactive dye that is toxic to aquatic life, therefore its environmental accumulation is highly detrimental to wildlife and whole ecosystems.[5] It is resistant to previously implemented dye bath clean-up methods such as biodegradation,[6] and

so alternative methods must be implemented in order to remove RB5 from dye bath wastewater.

Highly effective methods for dye adsorption are urgently sought for application in dye clean-up to eliminate environmental damage.[7] A number of innovative approaches for dye capture have been reported to date, including chemical oxidation,[8] ion exchange,[9] photocatalytic degradation,[10] and membrane filtration.[10] Dye adsorption by polymeric materials offers a particularly effective method of dye retrieval, owing to its ease of application and simplicity.[12-14] The development of economically viable polymeric materials that efficiently adsorb and sequester dyes are therefore highly sought.

Chitosan boasts numerous advantageous features as a dye adsorbent.[15] Firstly, chitosan is produced by the deacetylation of the polysaccharide chitin, the second most abundant biopolymer and a waste material from the food industry, thus ensuring that the economic case for utilising chitosan is highly compelling.[16] Secondly, chitosan exhibits extensive amine functionality that may be exploited for the non-covalent binding of dye molecules.[17] Finally, chitosan lacks toxicity.[18] Chitosan does, however, possess a number of features that render its application as a dye absorbent imperfect, including low mechanical strength and a tendency to deplete over time.[19] Consequently, chitosan has been modified by crosslinking reactions [20] and/or the grafting to nanoparticles [21] and dendrimers [22] in order to yield a more robust material that possess a larger surface area than chitosan in its powder form.[23]

Polymer hydrogels consist of a three-dimensional polymeric network fully dispersed within water. The extended distribution of the polymer within the water ensures that any functionality

present on the polymer is highly accessible for effectual molecular adsorption. Dye transport is permitted throughout the high surface area network, further enhancing hydrogel capability as dye adsorbents.[24] These factors, in addition to the economic and environmental benefits of a material that typically consists of at least 90% water by mass, make polymer hydrogels particularly appealing as substrates for dye adsorption. Synthetic polymers including poly(2-hydroxyethylmethacrylate),[25] poly[2-(methacryloyloxy)ethyl]trimethylammonium chloride [26] and poly(N,N-diethylamino ethyl methacrylate),[27] copolymers including poly(vinyl alcohol)-*block*-poly(ether amine)[28] and poly(acrylic acid-*co*-acrylamide),[29] and clay-modified poly(acrylic acid)[30] and poly(N-isopropylacrylamide)-based hydrogels[31] have independently been employed for targeted dye adsorption.

Polymer hydrogels also have applicability within a biomedical context, for instance as drug delivery vehicles[32] and scaffolds for tissue regeneration.[33] The high water content that hydrogels possess deems them to be highly suited for protein encapsulation, and controlled delivery, as protein denaturation is avoided in the aqueous environment presented.[34] In the absence of a macromolecular carrier, protein drugs are susceptible to low bioavailability and degradation by proteases in the bloodstream.[35] Consequently, a number of polymer-based hydrogels have been proposed for the effective encapsulation and release of protein molecules upon interaction with a targeted stimulus.[36]

Utilising a hydrogel that incorporates a biopolymer as the gelator, in the absence of oil-derived and non-biodegradable polymers, has clear environmental benefits, particularly for use in an application that seeks to be environmentally beneficial, and for employment *in vivo*. There are

numerous examples of modified biopolymers being employed as dye adsorbents; including chitosan-containing materials such as chitosan-montmorillonite,[37] chitosan-polyurethane,[38] and chitosan-palm oil composites.[39] Chitosan hydrogel beads have been prepared in the presence of sodium dodecyl sulfate, which instigates associative phase separation between chitosan and the surfactant in aqueous solution, and results in hydrogel formation.[40] Notably, the hydrogel beads formed were effective for the adsorption of the cationic dye methylene blue dye. [41]

In this proof-of-concept study, we present the creation of physically crosslinked chitosan hydrogels for molecular sequestration. Hydrogel preparation is facile and is performed in the absence of additional crosslinking and/or clay molecules. In particular, gelating agents that interact with the primary amine sites of chitosan, such as sodium dodecyl sulfate, which has associated toxicity,[42] are avoided. Consequently, the materials are highly cost-effective and biodegradable. The continuous structure of the reported materials ensures that hydrogel recovery is simplistic after deployment, compared to the recovery of dispersed hydrogel beads. The capability of the chitosan-based hydrogels to adsorb both anionic molecules, and molecules that sufficiently interact with chitosan *via* hydrogen bonding, offers the materials clear applicability in the fields of wastewater purification and protein encapsulation.

EXPERIMENTAL

Instrumentation and Materials

All reagents and products were weighed using an Ohaus Pioneer balance. UV-visible (UV-Vis) spectroscopy readings were carried out on an Agilent Technologies Cary 100 UV-Vis Spectrophotometer. Pixel intensity analysis was performed using ImageJ software. pH measurements were recorded using a Checker portable pH meter by Hanna Instruments. A Buchi R-210 rotary evaporator and a FiStream vacuum oven were used to remove solvent and dry samples. Samples were separated using a MSE Mistral 3000i centrifuge. Thermogravimetric analysis (TGA) was performed using a TA Instruments Q50 instrument to determine the water content of the hydrogels produced. Heating was maintained at 100 °C under a nitrogen purge gas for 40 min whilst the bound water evaporated. The weight loss over time was attributed to the loss of water. A Nikon SMZ 1500 optical microscope was used to image dye adsorption. Brightfield (illumination from fluorescent tube light box in transmission mode) image sequences were obtained using NIS-Advanced software-controlled operation of Nikon DS-Fi2 full colour CCD camera/DS-U3 controller system. Fluorescence microscopy for time-drive studies of FITC-tagged albumin (FITC-albumin) sequestration in chitosan hydrogels was conducted on Zeiss Observer Z1 with Zeiss LD A Pln 5x/0.15 Ph1 objective. Brightfield (full spectral illumination in transmission mode, intensity attenuated to avoid saturation) and fluorescence (filtered excitation light “GFP” centred at 488 nm, unfiltered emitted light detected in greyscale) image sequences were obtained using Zeiss Zen software-controlled operation of Zeiss Axiocam ICm1. A Malvern Instruments ZetaSizer Nano ZSP was used to determine the zeta potential of

chitosan. Five zeta runs were obtained at 25 °C, using a measurement position of 2.00 mm. An Elmi intelli-mixer RM-2L was used to invert the samples, using mode C3, and 50 rpm. To mix the hydrogel, a Scientific Industries vortex genie 2 at level 5 was used. The hydrogel was sonicated using a Bransonic B1510 sonicator at 40 kHz.

Low molecular weight chitosan powder (M_v = 50 – 190 kDa, 75-85% average deacetylation), RB5, C.I. disperse orange 3 (DO3), FITC-Albumin, benzoylated dialysis tubing (Mw cut off 2 kDa), hexane, chloroform, diethyl ether and tetrahydrofuran (THF) were all supplied by Sigma-Aldrich. C.I. disperse blue 3 (DB3) was supplied by ChemCruzBiochemicals. VWR International supplied ethyl acetate, reagent grade acetone and ethanol. Hydrochloric acid (HCl) (approx. 37%) and sodium hydroxide (NaOH) pellets were supplied by Fisher Scientific. All chemicals were used without further purification. Reagent quality dyes were used throughout this research. Though these are not indicative of industrial dyes nor real dye effluents, since this research project was intended to act as a model study, this approach was considered to be valid.

Formation of Chitosan Hydrogels

Varying amounts of chitosan powder (Table 1) were added to deionised water (15 mL) with stirring. Concentrated HCl (0.3 mL, 37.2% w/w%) was then added, followed by ethanol (15 mL). This formed an off white, viscous liquid. The solution was then stirred for 24 h and then added to aqueous NaOH solution (1 mol dm^{-3}, 75 mL), producing a white hydrogel that was left for 24 h in the NaOH solution. The hydrogels formed were then dialysed against deionised water for 48 h, with frequent water changes, to decrease the solution pH to pH 7. The pH of the

surrounding medium was monitored to ensure the removal of NaOH, which was confirmed when it matched the pH of the deionised water employed (Figure S1)

Rheological Evaluation of the Chitosan Hydrogels

Rheological tests were carried out at 25 °C using a stress-controlled AR 1500ex rheometer (TA instruments). The instrument was equipped with a steel-parallel plate geometry (40 mm in diameter) with the geometry gap distance maintained at 500 μm. The storage modulus (G') and the loss modulus (G") were determined by performing a frequency sweep test (5 rad s^{-1} to 65 rad s^{-1}) at a constant stress (5 Pa).

Dye Uptake Studies by UV-Vis Spectroscopy

Each hydrogel (0.50 g) was weighed into 6 separate vials and an aqueous solution of the dye under investigation (0.1 $mg.mL^{-1}$, 4 mL for RB5, 0.05 $mg.mL^{-1}$, 2 mL for DO3 and DB3) was added to each vial. A sample of the external dye solution (1 mL) was taken at regular time intervals and immediately measured by UV-Visible (UV-Vis) spectroscopy and then replaced into the vial. This procedure was repeated at regular time intervals for 300 min.

Dye Uptake Studies by Optical Microscopy

An optical microscope was utilised to visually monitor dye uptake by the chitosan hydrogels. A sample of Hydrogel **2** (10 mg) was placed between a microscope slide and a cover slip. This was then sealed around three of the sides with epoxy adhesive. Aqueous dye solution (0.1 $mg.mL^{-1}$, 100 μL) was added and the final edge was sealed. This was immediately placed under an optical microscope and set to take an image every five min for 12 h.

Dye Release from Chitosan Hydrogels

Following hydrogel incubation with the various dye solutions tested, the capability of the hydrogels to release the dye molecules upon washing with various organic and aqueous solvents was assessed. In each case, the hydrogels (0.5 g) were added to the selected solvent (1 mL), before the solvent was removed after 30 seconds, and replaced with fresh solvent (1 mL). This was repeated six times. The solutions were analysed by UV-Vis spectroscopy and the cumulative absorbance determined.

FITC-Albumin Uptake Studies by UV-Vis Spectroscopy

Each hydrogel (0.5 g) was added to FITC-albumin aqueous solution (0.5 mg mL^{-1}, 2 mL). At regular time intervals the absorbance of the external FITC-albumin solution was measured by UV-Vis spectroscopy. The decreasing absorbance with time indicated the uptake of FITC-albumin by the hydrogel.

FITC-Albumin Uptake Studies by Fluorescence Microscopy

A sample of hydrogel (10 mg) was placed between a microscope slide and a cover slip, and aqueous FITC-albumin solution (0.05 mg mL^{-1}, 100 µL) was added. This was then imaged by fluorescence microscopy at the initial incubation time and then over a period of 24 h.

FITC-Albumin Release Studies by Fluorescence Microscopy

A sample of hydrogel (10 mg) loaded with FITC-albumin was placed between a microscope slide and a cover slip, and aqueous solution of pH 4.2 (0.05 mg mL^{-1}, 100 µL) was added. The hydrogel and the surrounding solution were then imaged by fluorescence microscopy over a

period of 540 min. ImageJ software was then used to measure the pixel intensity of the hydrogel and act as a guide that reveals the diffusion of FITC-albumin from within the hydrogel to the surrounding, acidic, solution.

RESULTS AND DISCUSSION

Chitosan Hydrogel Formation

Chitosan-based physical hydrogels were produced following a method disclosed by Ladet *et al.* [43] Three different hydrogels were produced that contained comparable water contents as assessed by thermogravimetric analysis (Table 1). The low polymer content of the hydrogels ensures they are highly cost-effective materials, that can feasibly be produced on a large-scale, and so are deemed suitable for the intended application as dye adsorbents.

Table 1. The chitosan-based hydrogels produced

Name	Chitosan Mass (g)	Mass of Gel Produced (g)	Water Content (wt %)
Hydrogel **1**	0.30	13.70	97.0
Hydrogel **2**	0.50	14.18	94.6
Hydrogel **3**	0.75	15.10	96.4

Ideal gels behave as viscous flowing liquids over long time scales and so the storage modulus (*G*') must dominate the loss modulus (*G*'') in order for the system to be qualified as a gel. Rheological assessment of the hydrogels revealed that the storage modulus (G') dominated the

loss modulus (G'') over the frequency range tested, confirming the materials as hydrogels (Figure 1).

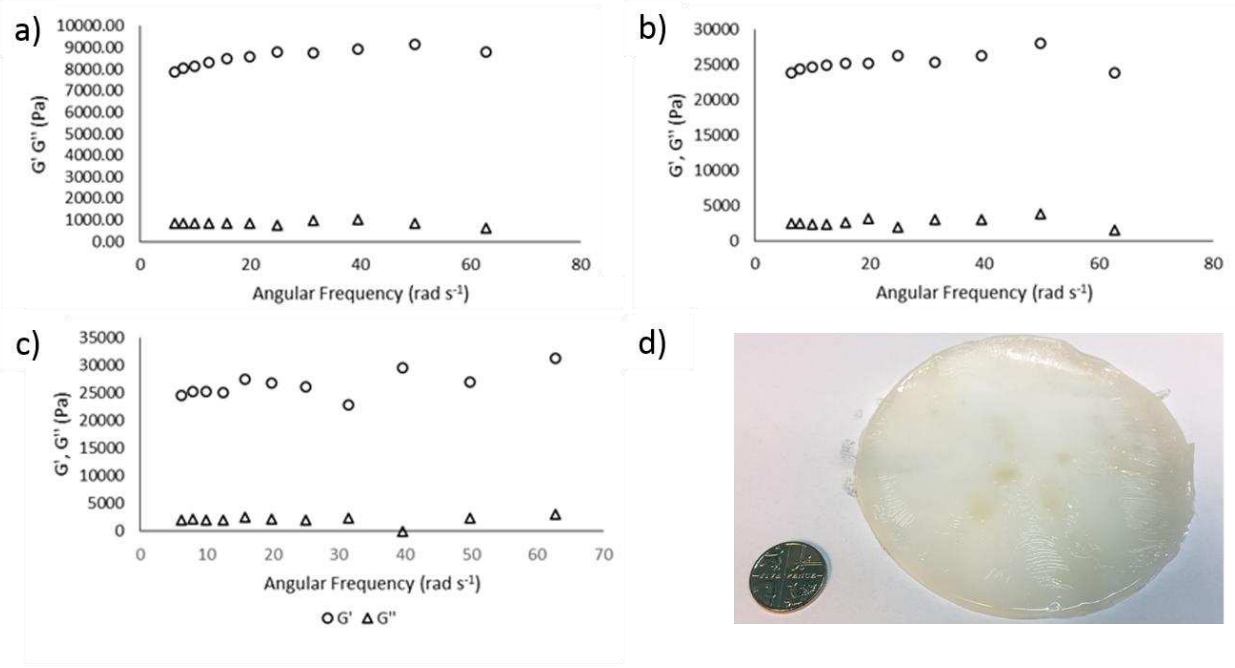


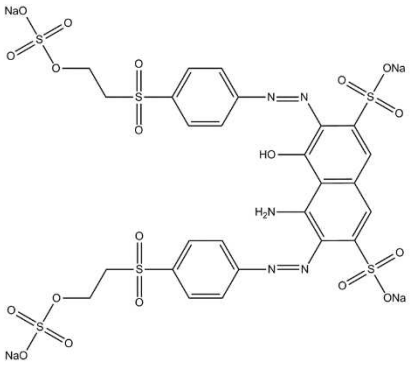
Figure 1. Rheological assessment of a) Hydrogel **1**, b) Hydrogel **2**, and c) Hydrogel **3.** The hydrogels can readily be manipulated to form discs, or other forms, that may be applied to filter columns, d). A U. K. five pence coin (diameter = 18 mm) is included in the image as a comparison of scale.

The zeta potential of the chitosan used at pH 7 was measured to be 12.4 ± 4.45 mV. This is comparable to other reported values,[44] and suggests that chitosan-dye interactions may form by electrostatic and/or hydrogen bonding interactions, depending on the nature of the target molecule.

Dye Encapsulation by Chitosan-Based Hydrogels

Three dyes were evaluated for their ability to adsorb to the chitosan hydrogels: RB5, DO3 and DB3, the structures of which are given in Figure 2. DO3 is commonly used for the colouration of polyester, nylon and acrylic fibres, whilst DB3 is more frequently employed for the dyeing of polyamides.

a)



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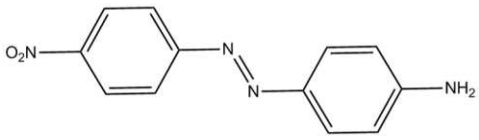

c)

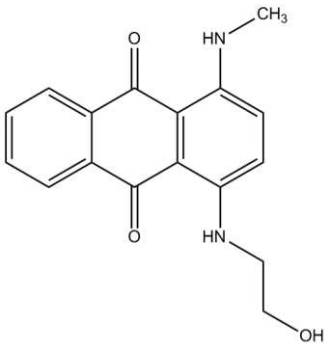

Figure 2. The structures of a) C.I. Reactive Black 5, b) C.I. Disperse Orange 3, and c) C.I. Disperse Blue 3.

The capability of the three hydrogels (0.5 g) to adsorb RB5 from solution (0.1 mg mL^{-1}, 4 mL) without agitation was assessed by time-driven UV-Vis spectroscopy on bulk RB5 solution (Figure 3). The RB5 content within the surrounding solution decreased over time; in each instance more than 70% of RB5 present in aqueous solution was adsorbed and retained by the hydrogels produced. This corresponds to a minimum of 0.56 mg of RB5 being adsorbed per gram of hydrogel in the absence of agitation, alterations to the solution pH, and/or heating. The maximum adsorption by Hydrogel **2** was 83%, corresponding to 0.66 mg of RB5 adsorption per gram of hydrogel used and 12.2 mg of RB5 adsorption per gram of chitosan used.

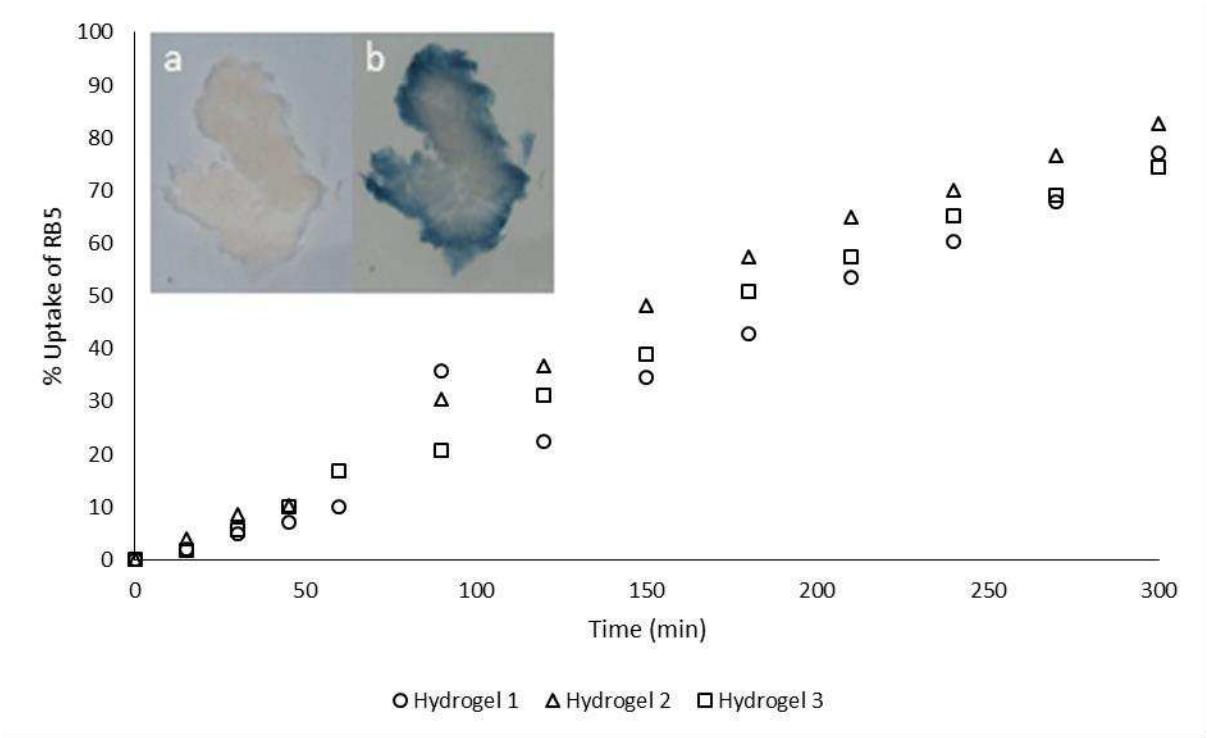

Figure 3. The absorbance of the RB5 external solution over 300 min for the hydrogels. Insert: Chitosan Hydrogel **2**: a) at time 0 h and b) after 900 min of RB5 adsorption.

Optical microscopy was employed to visually observe RB5 uptake from aqueous solution by Hydrogel **2** over a period of 22 h. The inset of Figure 3 reveals the chitosan hydrogel at 0 min (Figure 3a) and the same hydrogel following 15 h of static immersion in RB5 solution (Figure 3b). After 15 h the surrounding solution is colourless to the eye, suggesting complete dye uptake by the hydrogel. The rapid decrease in mean pixel intensity values of the optical micrographs obtained in the first 300 min of incubation signified RB5 adsorption, and correlated well to the UV-Vis spectroscopy data produced (Figure 4).

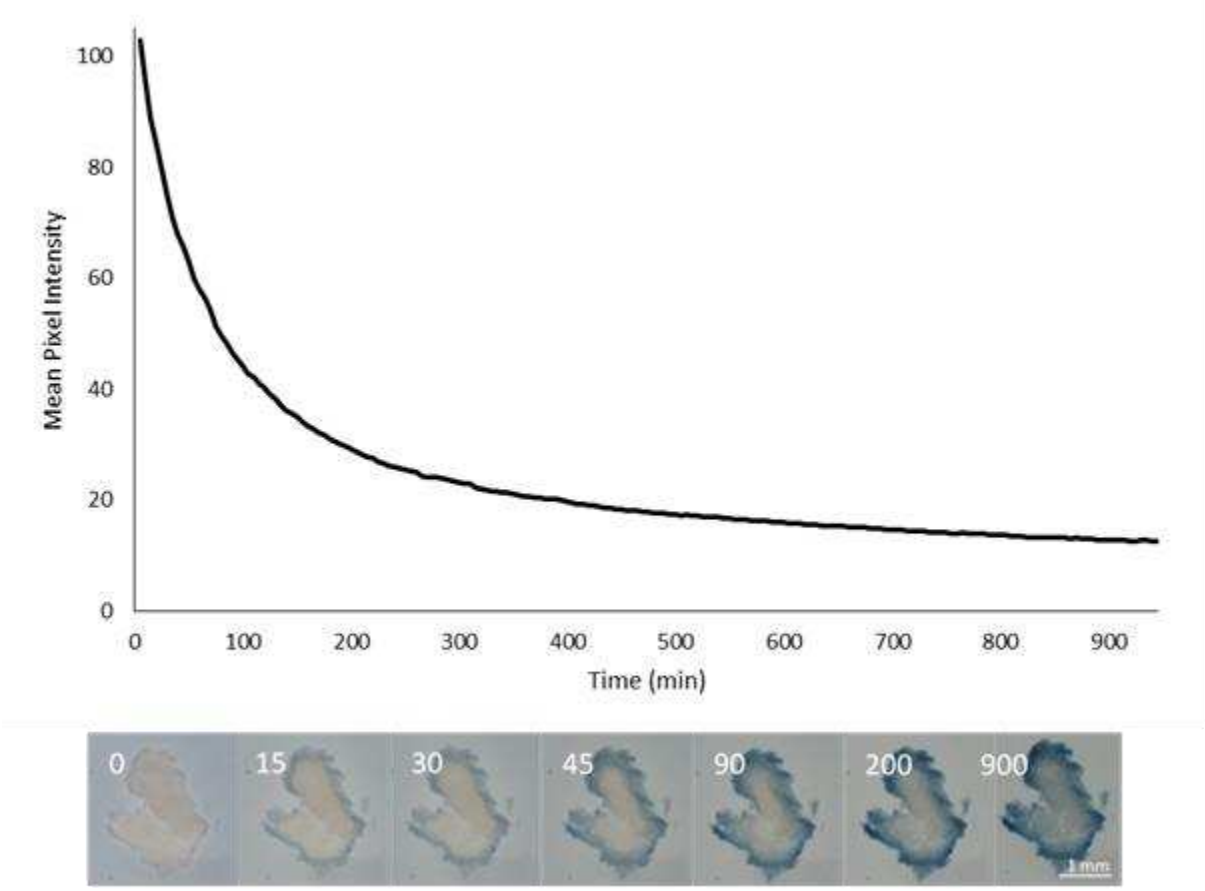

Figure 4. *Top*: A plot of mean pixel intensity from within the hydrogel area as determined by ImageJ against time. *Bottom*: Optical microscopy images of Hydrogel **2** with aqueous RB5 solution (the top left hand corner of each image indicates the number of min passed).

RB5-loaded hydrogels were then extensively washed with a range of solvents (Table 2), but in each instance the dye remained entrapped by the hydrogel. In a further attempt to liberate the dye molecules from the hydrogels, the dye-containing hydrogels were subjected to ultrasonic agitation for 30 min in the presence of the same solvents. Again, the dye could not be liberated from the hydrogels, confirming the substantial chitosan-RB5 interactions.

Table 2. The range of solvents were independently employed to remove the encapsulated RB5 from the chitosan-based hydrogels.

Solvent Tested
Ethanol
Methanol
Water
Phosphate Buffered Saline Solution (pH 7.4)
Tetrahydrofuran
Ethyl Acetate
Acetone
Diethyl Ether
Hexane
Chloroform

The stability of the hydrogels was then assessed by disrupting samples of Hydrogel **2** by subjecting the hydrogel to sonication (40 kHz for 5 and 10 min), inversion (10 min) and vortex mixing (level 5 for 1 min). The rheological profiles of the hydrogels following attempted disruption are provided in the Figures S2-S4, and demonstrate that in each instance, the hydrogel maintained its structural integrity following attempted destabilisation. The capability of Hydrogel **2** (0.5 g) to adsorb and retain RB5 from aqueous solution (0.1 mg mL^{-1}, 4 mL) was then examined by assessing the extent of RB5 uptake by the hydrogels that had been subject to the methods of destabilisation detailed above. Figure S5 reveals that all the hydrogels that had been subject to destabilisation performed better than the untreated hydrogel, with extents of

adsorption ranging from 95.2% to 100.0% for the agitated hydrogels compared to an adsorption extent of 87.7% for the untreated hydrogel.

The capability of the chitosan hydrogels to adsorb the disperse dye, DO3 was then assessed by UV-Vis spectroscopy to determine whether the hydrogels produced may be effectively applied to adsorb a second class of dye molecule. DO3 lacks the anionic sulfate groups present in RB5, but contains polar nitro and amine moieties that may interact with the hydroxyl and amine groups of chitosan *via* hydrogen bonding (Figure 2b). Dye uptake (0.05 mg mL^{-1}, 2 mL) by 0.5 g of the hydrogels was found to be 59.1%, after 26 h of incubation, which corresponds to 0.12 mg of DO3 being adsorbed per gram of hydrogel (Figure 5a). In the case of Hydrogel **1**, this corresponds to 4 mg of DO3 being adsorbed per gram of chitosan. The reduced dye uptake by the hydrogels suggests that their interaction with DO3 was weaker than the interaction between RB5 and the hydrogels. The extensive release of DO3 (≥ 96% in all cases) upon treatment of the dyes with ethanol confirmed this (Figure 5b).

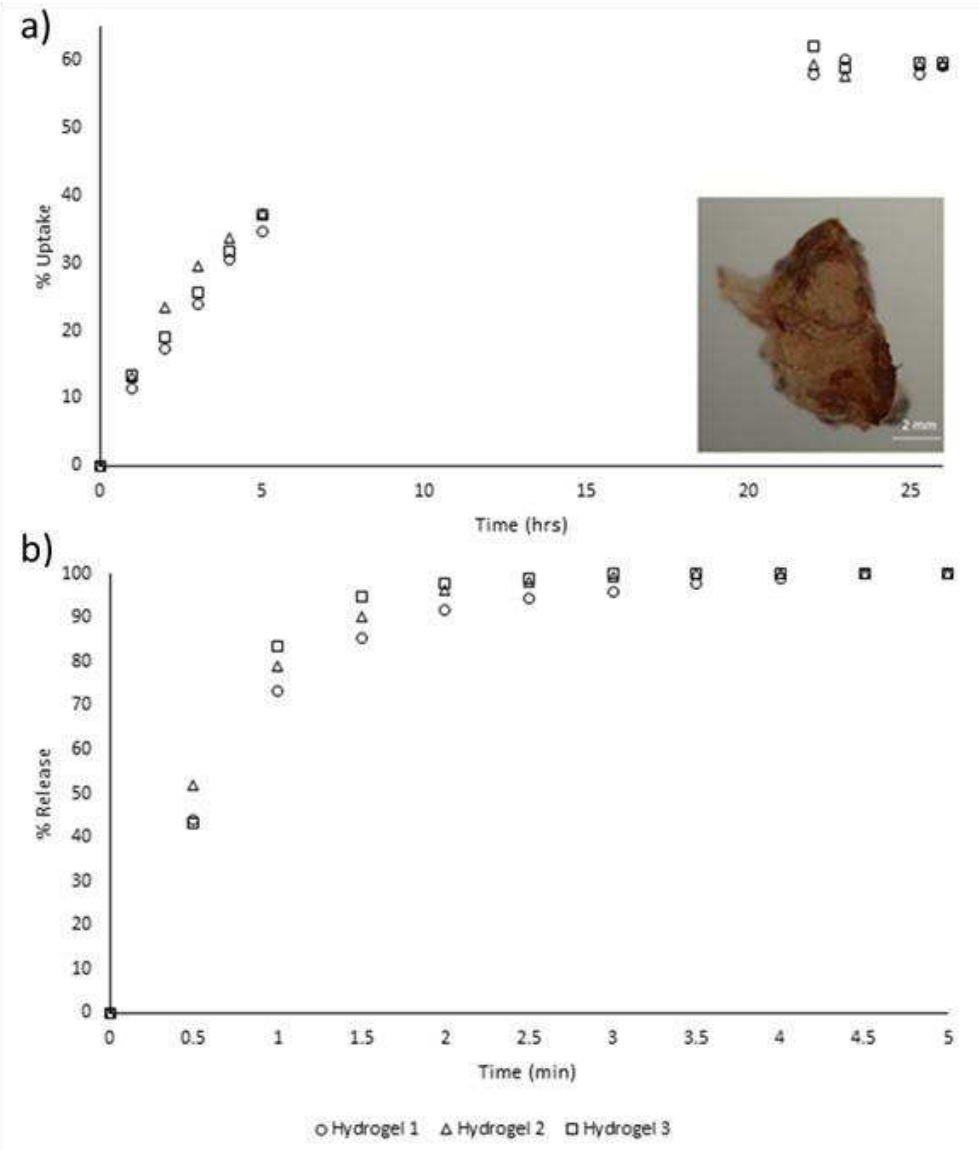

Figure 5. a) UV-Vis spectroscopy data revealing the extent of DB3 uptake by the chitosan hydrogels over time. b) UV-Vis spectroscopy data revealing the rapid release of DB3 from the chitosan hydrogels upon washing with ethanol.

Next, the capability of the dyes to absorb a second disperse dye was assessed. DB3 contains a terminal hydroxyl group that may form hydrogen bonds with the amine groups of chitosan (Figure 2c). The capability of the hydrogels to absorb and withhold DB3 was assessed by UV-Vis

spectroscopy and optical microscopy (Figure 6). UV-vis spectroscopy demonstrated that the three hydrogels produced absorbed, and withheld, between 66% and 89% of DB3 that was present initially in surrounding solution, after 72 h (Figure 6a). This corresponds to 0.13 mg and 0.18 mg of dye molecules being absorbed per gram of hydrogel, respectively. Hydrogel **1** was able to adsorb 6 mg of DB3 per gram of chitosan used. Optical microscopy demonstrated the capability of hydrogels to withhold the dye molecules in water, the inset of Figure 6 reveals DB3-loaded hydrogel after 24 h incubation. Rinsing the hydrogels with ethanol resulted in the complete release of the loaded DO3 (Figure 6b). Greater DB3 uptake compared to DO3 uptake may be attributed to a greater extent of hydrogen bonding between the alkyl hydroxy group of DB3 and chitosan compared to the degree of hydrogen bonding between the aromatic amine/nitro groups of DO3 and chitosan.

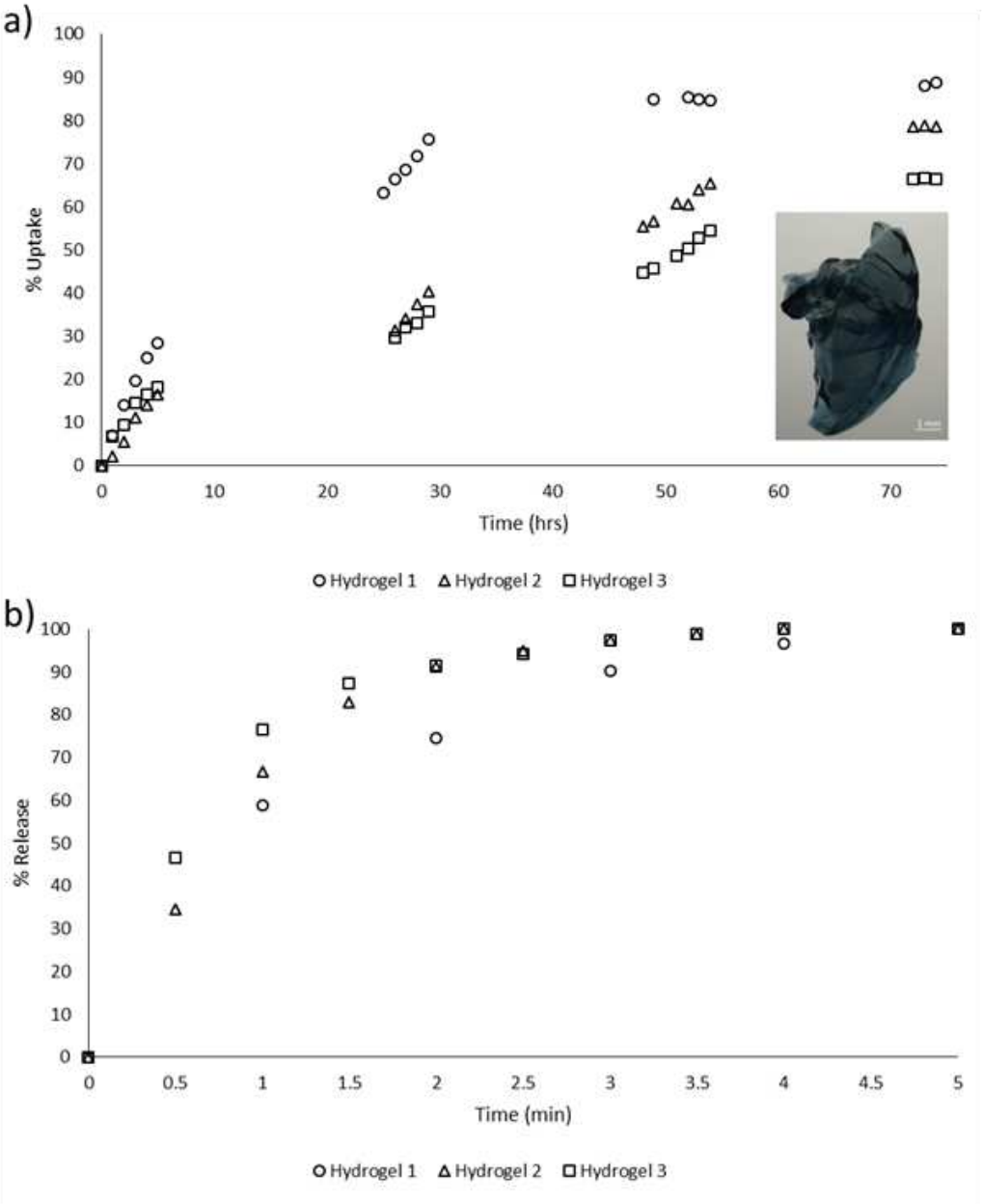

Figure 6. a) UV-Vis spectroscopy data revealing the extent of DB3 uptake by the chitosan hydrogels over time. b) UV-Vis spectroscopy data revealing the rapid release of DB3 from the chitosan hydrogels upon washing with ethanol.

Protein Encapsulation by Chitosan-Based Hydrogels

Finally, the chitosan-based hydrogels were incubated in the presence of negatively charged FITC-albumin, at pH 7.4, to assess whether chitosan hydrogels may be employed for the encapsulation of model protein molecules. Protein encapsulation offers the opportunity to isolate and manipulate the function and structure of proteins.[45] Encapsulation within hydrogels presents materials that may be readily applied to the fields of tissue engineering, [46] protein therapeutics,[47] and nutrient delivery.[48] Consequently, biodegradable, non-cytotoxic hydrogel materials that may be produced, and further modified, in a facile manner are highly sought. The hydrogels reported fit each criterion, deeming them highly promising candidates for use within the contexts outlined.

UV-Vis spectroscopy and fluorescent microscopy analysis revealed complete protein uptake (2 mg per gram of hydrogel, 66.7 mg of FITC-albumin per gram of chitosan used, in the case of hydrogel **2**) by the hydrogels within 5 h (Figure 7). After 50 h of incubation, FITC-albumin adsorption was comparable for the three hydrogels tested, irrespective of their chitosan content. Analogous to RB5 uptake, the protein could not be removed by washing with a range of solvents; release from the hydrogels only occurred upon washing with pH 4 buffer solution, a pH value that is below the isoelectric point of albumin and so further suggests that chitosan-target molecule electrostatic interactions promote the uptake of anionic molecules by the hydrogels, and impede their subsequent release.

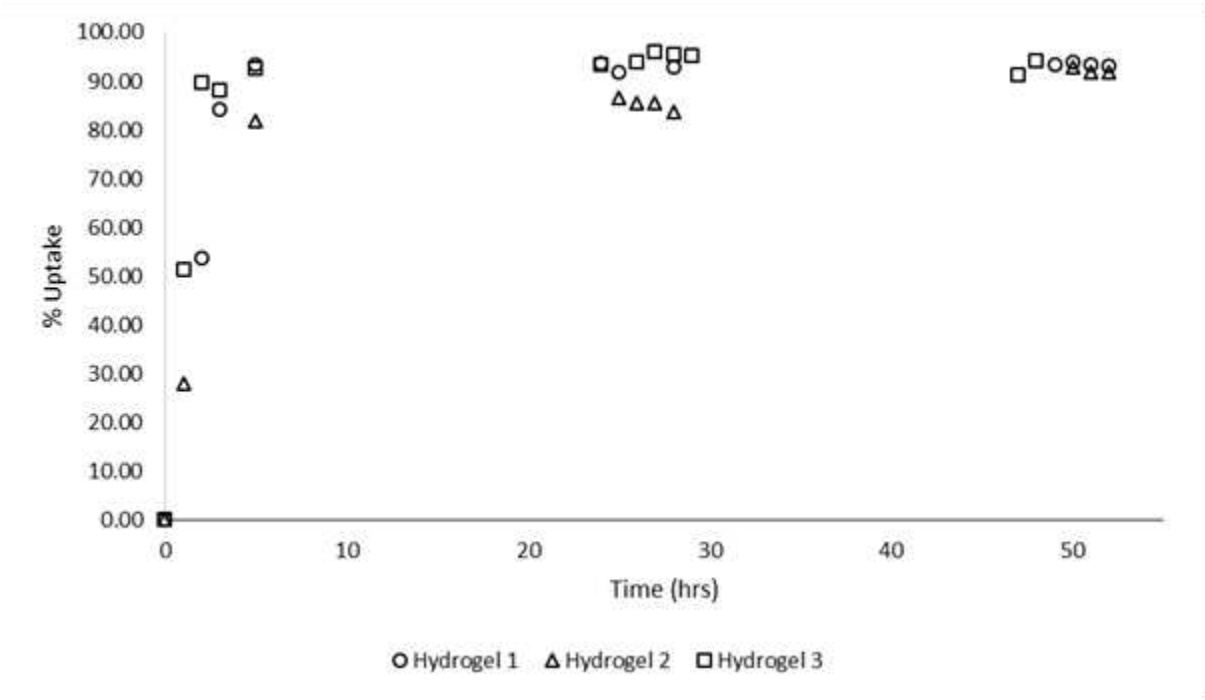

Figure 7. The extent of FITC-albumin uptake by the chitosan-based hydrogels as assessed by UV-Vis spectroscopy.

Figure 8 illustrates the uptake of FITC-albumin by the hydrogel, highlighting the preferential interaction between the protein and the chitosan, causing protein diffusion into, and retention within, the hydrogel.

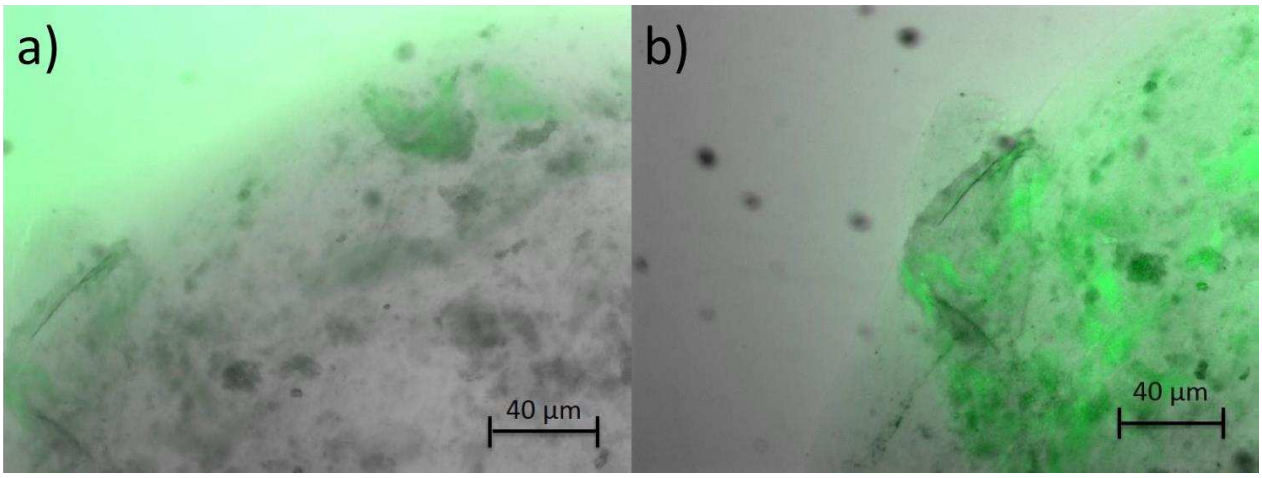

Figure 8. a) Fluorescence microscopy image of Hydrogel **1** (grey area) surrounded FITC-albumin solution (green) at the initial incubation point. b) An image of the same sample after 24 h reveals FITC-albumin uptake by the hydrogel (now green), leaving a colourless solution surrounding the hydrogel.

The loaded hydrogel was then exposed to pH 4 buffered aqueous solution and imaged by fluorescence microscopy on a time lapse, with an image taken every five min for 540 min. The pixel intensity within the hydrogel area was then analysed by imageJ software (Figure 9).

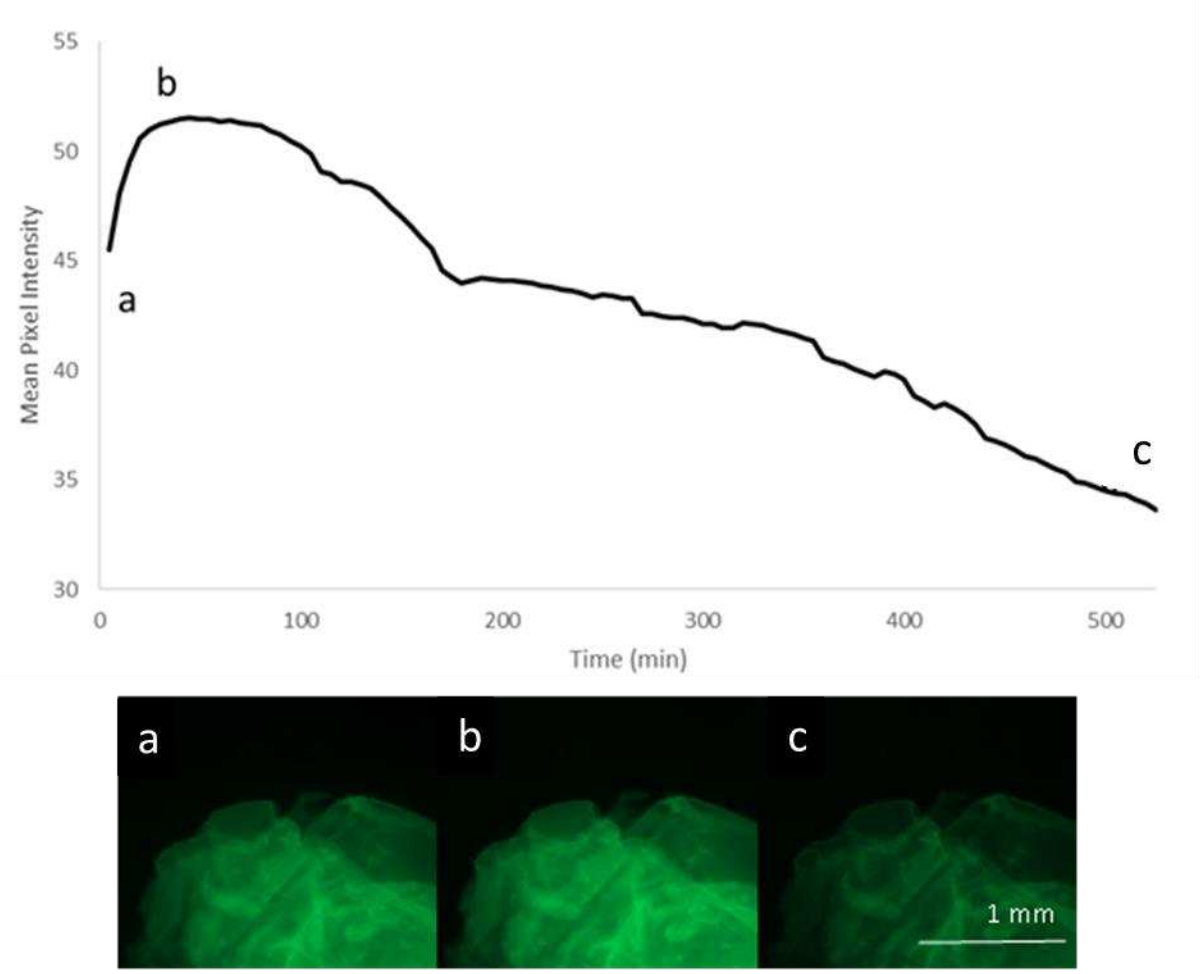

Figure 9. *Top:* ImageJ pixel intensity profile showing a decreasing pixel intensity within the hydrogel area with time. *Bottom:* Microscope images of the loaded hydrogel as it releases the FITC-albumin.

From Figure 8 it can be seen that the hydrogel is able to release the albumin on exposure to a solution of low pH. The increase in pixel intensity between a) and b) is due to the inital diffusion of FITC-albumin from the hydrogel core to the hydrogel perimeter. The pixel intensity then decreases between b) and c) to a value that suggests approximately 24% FITC-albumin release has occurred over the assessed period. Black *et al.* reported that FITC-albumin maintains an α-

helical structure following release from hydrogel carriers upon interaction with aqueous solution of pH 4.[49] Therefore, the chitosan-based hydrogels reported here may be applied for the delivery of protein molecules to acidic environments, such as the endosomes or lysosomes of cells. No release occurred from the hydrogels upon incubation in aqueous solution of pH 5, demonstrating the robustness that the hydrogels possess to withhold a FITC-albumin payload prior to pH-mediated release.

CONCLUSIONS

Chitosan hydrogels capable of RB5 absorption were produced and analysed. Dye adsorption was almost complete, with up to 83% dye adsorption achieved after 300 min of static incubation. RB5 retention by the hydrogel occurred, even upon washing the hydrogel with a range of organic solvents. This suggests that the hydrogels may be of use as dye transfer inhibitors in advanced laundry detergents, in which instance dye release must be prevented. In addition, the hydrogels produced had an affinity to adsorb both DO3 and DB3, demonstrating versatility across a range of dyes with contrasting chemical functionalities. The reduced affinity of DO3 and DB3 for the hydrogels resulted in diminished dye uptake, compared to anionic RB5 uptake, but allowed for complete dye release upon washing with ethanol. This feature may enable the recycling of such disperse dyes that are recovered from wastewater following treatment with the chitosan hydrogels presented. A final application of the hydrogels is as release vehicles for the encapsulation and pH-mediated release of protein molecules. The hydrogels demonstrated the capability to extensively encapsulate FITC-albumin (2 mg of FITC-albumin per gram of hydrogel, 66.7 mg of FITC-albumin per gram of chitosan as minimum

loading concentrations), prior to the partial release of the protein (24% after 540 min static incubation) upon immersing the protein-loaded hydrogel with an acidic solution. The hydrogels produced therefore also have clear potential to be used as a medium for the delivery of proteins to acidic environments.

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GRAPHICAL ABSTRACT