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Versatile Biocompatible Polymer Hydrogels: Scaffolds for Cell Growth**

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cells; DNA; hydrogels; polymers; scaffolds; chitosan-graft-polyethylenimine; nanocomposite hydrogels; delivery-systems; gene carrier; microarrays; fabrication; copolymers; microgels; gels; poly(ethylenimine)

Abstract

A three-dimensional, biocompatible hydrogel (see picture) was generated by combining two cationic polymers, chitosan and poly(ethylenimine). The hydrogels were stable under cell-culture conditions and facilitated cell proliferation, yet prevented dedifferentiation of primary human skeletal cells into fibroblasts. A variety of materials such as DNA, proteins, and peptides can be stably incorporated into the gel network.

Main text

Hydrogels have attracted considerable attention as so-called "smart materials" because of the various and often intriguing physical and chemical phenomena that they can display when subjected to a variety of external stimuli, such as changes in pH, temperature, light, and electric fields.[1–5] As a result, hydrogels have been applied as fundamental components in a range of applications such as controlled drug delivery,[6] soft linear actuators, sensors, and energy-transducing devices.[2–5, 7] Many hydrogels exist, as well as methods of their synthesis, which include the cross-linking of linear poly(*N*-isopropylacrylamide) (PNIPAM),[7] poly(ethylene glycol),[8] polyacrylamide,[9] and poly(acrylic acid) based polymers[10] and their copolymers[11, 12] to name but a few.

Chitosan and poly(ethylenimine) are two widely used polymers (Figure 1 a.). Chitosan is a polysaccharide derived from chitin, and is an attractive material for use in the biomedical field[13] because of its controlled biodegradability[14] and biocompatibility.[15] Chitosan forms so-called "hydrogels" by the neutralization of acidic solutions of chitosan, although the resulting materials are opaque, with a granular crystalline morphology.[16] Poly(ethylenimine) (PEI) is a linear-branch polymer which has been extensively used in the gene delivery field[17] and as a coating material in biosensor applications.[18] Chitosan and PEI have been chemically grafted to give materials with enhanced gene-carrier abilities.[19] Herein we report the preparation of quite remarkable hydrogels that support 3D cell growth by the simple expedient of mixing solutions of these two cationic polymers.

Polymer blends were generated by mixing chitosan (partially hydrolyzed, *M*w=250 kDa, 1 % aqueous acetic acid, pH≈4.0) and poly(ethylenimine) (*M*w=300 kDa, 10 % in water, pH≈11) in various molar ratios (90:10 to 10:90). The resulting solutions (pH≈7.5) became, over a period of 5 minutes, gels that were stable to inversion and manipulation. All compositions showed gelation, but these varied from clear (chitosan/PEI 10:90) to more opaque gels (chitosan/PEI 40:60; Figure 1 b.).

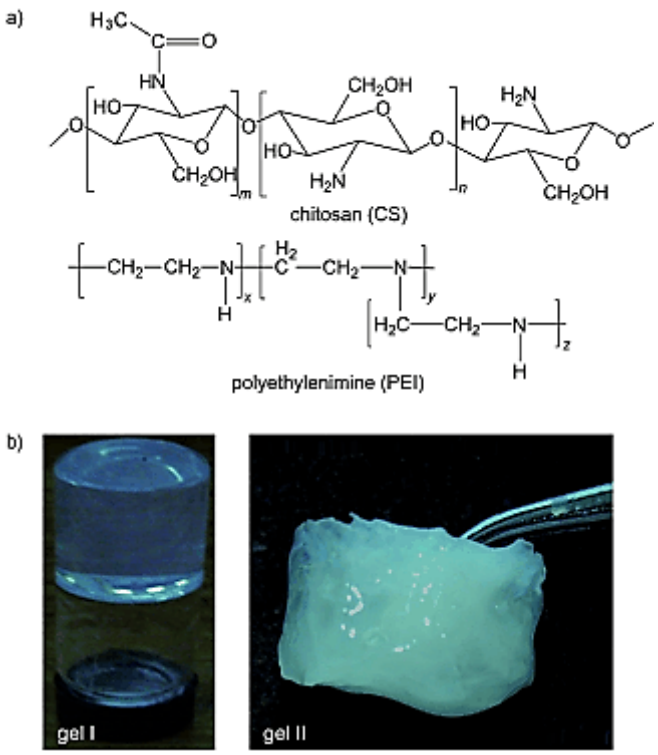
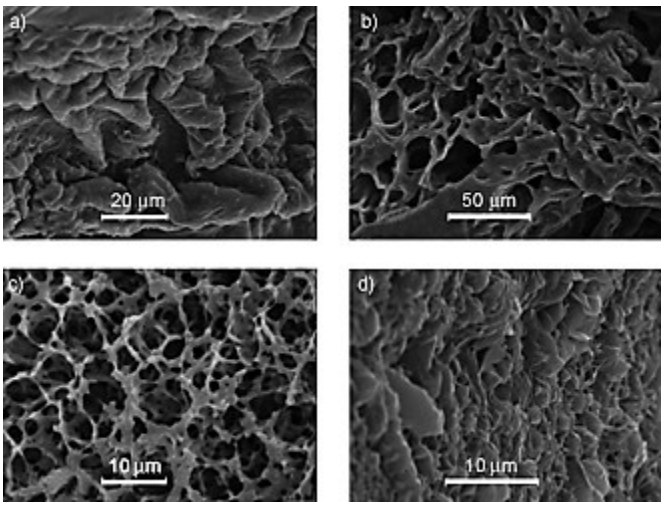


Figure 1. a) Structure of chitosan and poly(ethylenimine) (PEI). b) Hydrogels prepared by blending solutions of chitosan (CS) and PEI: gel I (chitosan/PEI 10:90) and gel II (chitosan/PEI 40:60).

The resulting hydrogels were examined by scanning electron microscopy (SEM), XRD, and IR (see Figure 2. and the Supporting Information). The hydrogel prepared from chitosan/PEI (40:60) displayed a spongelike, microporous morphology, which is radically different to that found in normal chitosan gels prepared by neutralization of solubilized chitosan (see Figure 2.)[16] and suggested possible application as a cellular support or scaffold.



← ***Figure 2.*** SEM images of hydrogels frozen in liquid nitrogen and freeze-dried: a) chitosan/PEI (10:90), b)–c) chitosan/PEI (40:60) and d) chitosan gel (chitosan solution neutralized with NaOH).

The mechanical analysis of the gels (see Figure 3. and Figure S4. in the Supporting Information) showed that the storage modulus (G') was significantly greater than the loss modulus (G'') up to 50 % strain, a property typical of a gel network,[20] in which all gels show very similar behavior. G′ and G″ were also evaluated for samples exposed to the cell culture conditions (up to 28 days). The results presented in Figure 3 b. indicated degradation of the gel over culture time with a reduction in storage modulus value of about 40 % after 28 days. Figure 3 c. shows that the compressive modulus and storage modulus values (Figure 3 a.) immediately following gel formation were significantly higher than the starting unmixed polymers (see Figure S4. in the Supporting Information), which indicated formation of a significant cross-linking network. However, the compressive modulus value decreased with time, as did the gel weight, which resulted in a gel with a progressively lower strength over time.

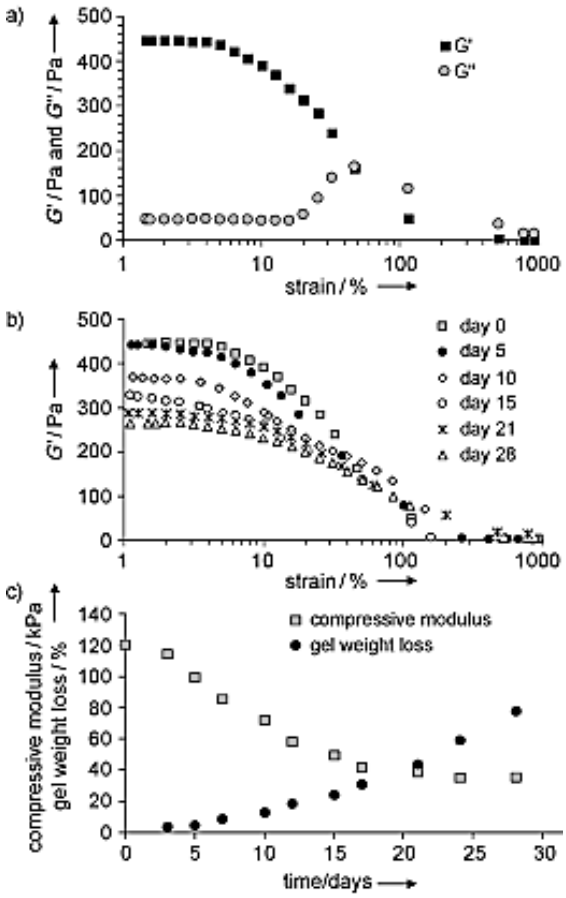
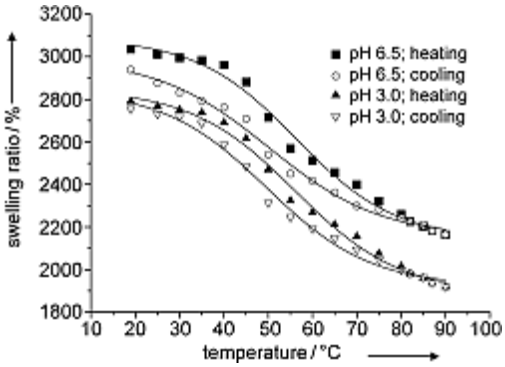


Figure 3. Mechanical and degradation properties of the chitosan/PEI 40:60 hydrogel (all measurements were conducted at 37 °C). a) Storage modulus (G') and loss modulus (G'') plotted as a function of strain. b) Storage modulus versus strain (%) over time and c) compressive modulus and weight loss as a function of time (gel samples for all these studies were 1 cm (height)×2 cm (diameter).

To examine responses to external stimuli, the swelling ratio of the gel (chitosan/PEI 40:60) were investigated as a function of temperature and pH (Figure 4.). This showed that the gel underwent significant volume changes with temperature, with the gel shrinking when the temperature was increased from 45 °C to 90 °C, but not collapsing or fragmenting at any of these temperatures.[7, 15] pH-induced changes (3.0–6.5) were limited, with pH values greater than 9 or less than 3 leading to rapid collapse. The gels were found to be stable at physiological pH for several weeks and could be formed using PEI dissolved in culture media, which was then mixed with the chitosan solution. The hydrogels (90:10 to 10:90) were evaluated for their ability to support the attachment and growth of skeletal mammalian cells by using a polymer-blending microarray platform[21] alongside chitosan and PEI. Results showed that under these conditions the homopolymers alone did not support cell attachment or spreading, while most of the chitosan/PEI blends showed significant cell attachment (see the Supporting Information). The optimal polymer blend (chitosan/PEI 40:60) was the most effective for cell encapsulation and proliferation, which could perhaps be attributed to the open-network structure displayed by the hydrogel (Figure 2 b–c.) that favored cell growth and proliferation.



← ***Figure 4.*** The swelling behavior of the hydrogels as determined at two different pH values with a temperature cycle from 20 to 90 °C (see the Supporting Information for experimental details).

HeLa or primary human fetal skeletal cells,[22] labeled with Cell-tracker green, were seeded (simply by mixing with the appropriate chitosan/PEI mixture before full gelation had occurred) and cultured within chitosan/PEI hydrogels over a period of 28 days. The cells were homogeneously distributed throughout the hydrogel scaffold (Figure 5.) and were viable, healthy, and proliferated throughout the four-week culture period, although it was noticed that the gels started to disintegrate when cell culture was prolonged beyond this.

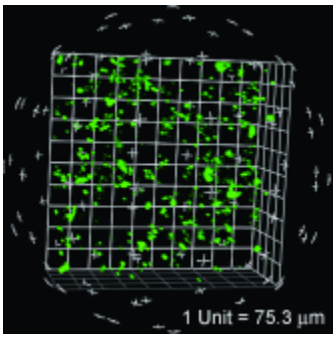


Figure 5. → A confocal image, taken on day 21, of HeLa cells (labeled with CellTracker Green) growing within the chitosan/PEI (40:60) hydrogel scaffold.

Throughout the course of the 28 day culture period within the chitosan/PEI hydrogels, the human skeletal cells, derived from predominantly cartilaginous fetal femora,[22] exhibited a chondrocyte-like spherical morphology (Figure 6.), as opposed to the fibroblastic morphology found in monolayer culture. Expression of *Pcna*, a marker of cell proliferation,[23] by fetal skeletal cells was analyzed (qPCR; see the Supporting Information for details) to determine the effect of culture within chitosan/PEI hydrogels on cell proliferation (Figure 6 e.). Culture, in the absence of the chondrogenic differentiation media, resulted in a significant increase in *Pcna* expression on days 14 ($P<0.01$), 21 ($P<0.001$), and 28 ($P<0.05$) in comparison to day 7. Thus skeletal cells proliferated actively over the 28-day culture period within chitosan/PEI hydrogels.

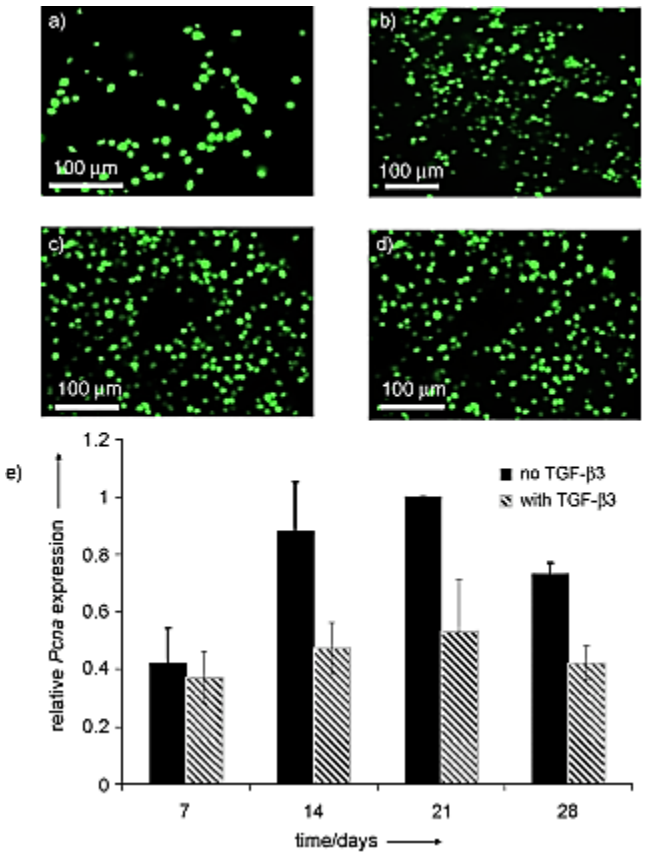


Figure 6. a) Human fetal skeletal cells, labeled with CellTracker Green, grown in the hydrogel scaffold (chitosan/PEI 40:60): a) day 7 (magnification 20×), b) day 14 (10×), c) day 21 (10×), and d) day 28 (10×; see Figure S6–S7 in the Supporting Information). e) Real-time qPCR analysis for expression of*Pcna* (a marker of cell proliferation). Fold relative expression levels for the genes of interest were normalized to β-actin expression which served as a housekeeping gene. The group with the highest expression was assigned a value of 1 and expression levels in the remaining groups were determined relative to that group (n=3). Note: values for *Pcna* gene expression in the presence of TGF-β3 at days 7, 14, 21, and 28 were not statistically different, which suggested that these cells had not proliferated; instead, they had undergone chondrogenic differentiation in response to TGF-β3.

To investigate the influence of the 3D chitosan/PEI hydrogel system on the culture of fetal skeletal cells compared to the standard monolayer culture, analysis of key genes involved in cellular differentiation was carried out (see Figure 7. and the Supporting Information).[24] The 3D gel facilitated cellular proliferation and prevented dedifferentiation of these cells into fibroblasts as demonstrated by their spherical morphology and low levels of *Col1a1* expression (a gene prolifically expressed by fibroblasts, see Figure 7.). In addition, there was a steady increase in the expression of two chondrogenic markers *Col2a1* (type-II collagen) and *aggrecan* (see the Supporting Information). In contrast, expression of *Col1a1* (type-I collagen) by fetal skeletal cells cultured in a monolayer increased steadily with time (Figure 7 c.), with negligible expression of the two chondrogenic genes (*Col2a1* and *aggrecan*; Figure S7 in the Supporting Information), which is indicative of dedifferentiation[24] of the fetal skeletal cells under conditions of monolayer culture. Culture within a 3D gel environment as opposed to a 2D monolayer culture environment thus prevented dedifferentiation of the fetal skeletal cells into fibroblasts by maintaining these cells in a chondrocyte-like spherical morphology. Furthermore, the 3D chitosan/PEI hydrogel culture system in combination with the chondrogenic growth factor TGF-β3 was essential to stimulate the differentiation of the fetal skeletal cell population along the chondrogenic lineage (see Figure S7a , b in the Supporting Information).

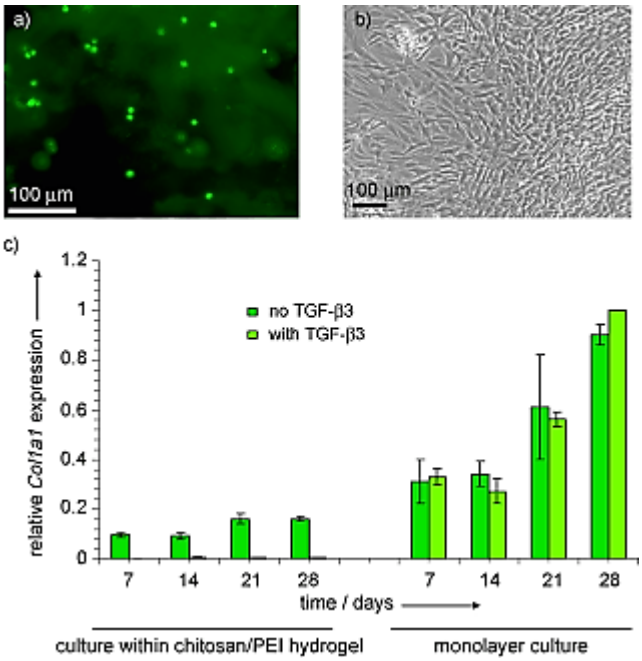


Figure 7. Human skeletal cells: a) cultured in the 3D chitosan/PEI hydrogel exhibiting a chondrocyte-like spherical morphology and b) grown as a monolayer (culture flask) exhibiting a distinct fibroblastic morphology. c) qPCR Analysis of *Col1a1* (type-I collagen), expression by fetal skeletal cells cultured within the chitosan/PEI hydrogels (3D culture) and in monolayers (2D culture) over the course of 28 days in the presence and absence of TGF-β3. Relative gene expression levels were normalized to the expression of β-

actin. Fold relative expression levels were expressed as (mean±standard deviation) for plotting as bar graphs, *n*=4 for monolayer cultures and *n*=3 for hydrogel cultures.

In addition, cells could be efficiently transfected by encapsulation of a lipoplex (DNA and cationic lipid) within the gel during its preparation (see Figure 8. and the Supporting Information).

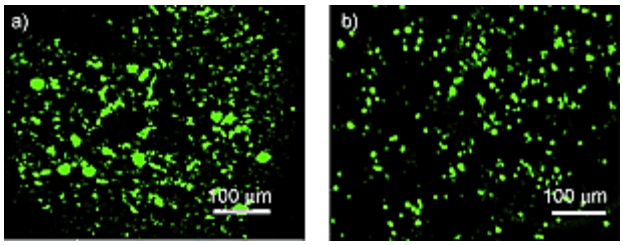


Figure 8. Transfection of HeLa cells with pEGFP-C1 (a plasmid carrying the gene for enhanced green fluorescence protein) using Lipofectamine2000. a) Transfection in the chitosan/PEI (40:60) hydrogel and b) control transfection without gel. Clumps of cells are seen within the gel.

In conclusion, we have demonstrated the generation of a novel hydrogel by the simple expedient of mixing two cationic polymers, chitosan and poly(ethylenimine), in the presence of cell culture media to give a 3D scaffold. The hydrogels were stable under cell culture conditions and were fully capable of supporting cell attachment and proliferation. This gel represents a new class of materials prepared in a simple, robust, and scalable manner and offers the ability of manipulating the cells in their 3D state, with the ability to generate gels of any desired shape by using a mold. A variety of additional materials such as DNA, proteins, peptides, and small drug molecules can be stably incorporated into the gel network offering the potential of modulating cellular growth/behavior within the gel. Culture of primary skeletal cells within the 3D gel environment facilitated cell proliferation and prevented dedifferentiation of the skeletal cells into fibroblasts by maintaining these cells in a chondrocyte-like spherical morphology.

Experimental Section

Hydrogel preparation: Chitosan (75 % deacetylated) and PEI were purchased from Aldrich. Chitosan (2 %) was dissolved in aqueous acetic acid (1 %), PEI was dissolved in water (10 % w/v), and both solutions were filtered (0.22 µm filter) and sterilized. These solutions were simply mixed in varying proportions at 25 °C; gelation was complete within a few minutes.

A scanning electron microscope (Philips XL30CP SEM) was used to examine the surface morphology of the dried polymer gels. The samples were covered with a thin layer of Au and glued to a metal-base specimen holder to achieve good electrical contact with the grounded electrode. The micrographs were taken at 20 kV in a secondary electron imaging mode.

Swelling behavior of hydrogels: The swelling behavior of the hydrogel chitosan/PEI (40:60) was investigated by measuring the swelling ratio as a function of temperature. The swelling ratio (SR) of the hydrogels was determined as: SR %=[(swollen mass−dry mass)/dry mass]×100.

Rheological measurements: The rheological studies of the hydrogels using an Advanced Rheometer-AR 2000 (TA Instruments) with a cone and plate fixture geometry (see the Supporting Information).

Cell culture: Human skeletal cells[22] were harvested from day 12 monolayer cultures grown in α-MEM (MEM=minimum essential medium) supplemented with 10 % fetal calf serum (FCS) and labeled with CellTracker Green/CMFDA (Invitrogen) following the manufacturer's instructions. 100 000 labeled cells were suspended in the sterile PEI solution and this solution was added immediately to the sterile chitosan solution, which had been added to wells of a 24-well plate. Gelation of the mixture of the two solutions (chitosan/PEI 40:60) took place within two minutes and encapsulated the cells within the gel. Once this was achieved, the media (α-MEM containing 10 % FCS, 1 mL) was added to each well containing the gel (n=3; Figure S6 in the Supporting Information), and the cells were cultured for a period of 28 days in this culture system. The media was replaced every two days, and cell viability and growth monitored on days 3, 7, 14, 21, and 28 of culture by fluorescence microscopy. For HeLa cell culture, RPMI-CM media (supplemented with 10 % fetal bovine serum (FBS), glutamine (4 mm), and antibiotics (penicillin and streptomycin, 100 units mL^{-1}) was used. The media was changed every two days. Images of HeLa cells in chitosan/PEI gel were captured by Confocal microscopy at 20× magnification and analyzed using Velocity software.

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