

Localized delivery of DNA to the cells by viral collagen-loaded silica colloidal crystals

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Low-molecular-weight colloidal crystals with enhanced biocompatibility and ordered porous structure are used in drug-delivery systems. The objective of our study is to demonstrate the use of silica nanoscale colloid particles for localized recombinant DNA release. The colloids were coated with collagen-containing viral vector constructs of lentiviral green fluorescent protein (GFP), and solidified at 37°C. The colloid-collagen-viral vector platform (CCP) was transferred to cell monolayer cultures of human lung fibroblasts. Results show specific infection of cells directly beneath the platform, as evidenced by positive GFP in their cytoplasm, while neighboring cells show no cytoplasmic GFP. The infection of specific cells is probably due to the gradual release of viral particles from the collagen matrix by cell-secreted collagenase, which avoids overdosing the cells with viral particles, resulting from the cytopathic effect often seen with high-titer viral infection. Cells infected with the lentiviral-GFP or lentivirus alone, not incorporated into the colloid-collagen device, show caspase 3-associated apoptotic cell death. This suggests that colloidal crystal-coated collagen may be used as a powerful platform to deliver genes of choice to localized subgroups of specific cells of interest. This specificity in the delivery mode is beneficial for functional studies of gene-directed impact on a particular cell population of interest in a heterogeneous cell culture.

INTRODUCTION

Recently, extensive interest has been devoted to the study of nanoparticles for use as biomedical agents for disease diagnosis and treatment (1). However, efficient intracellular delivery has not developed the ability to avoid nonspecific effects, such as toxicity. Therefore, a strategy of cell-specific delivery is urgently needed to obtain the beneficial effects of using dosage levels sufficient for predicted results with minimal systemic damage to detoxifying tissues, such as liver, with the final successful outcome accompanied by better patient acceptance. The recent development of innovative methods for specific drug delivery has received significant attention globally, primarily in cancer (2,3); promising strategies include liposomes (4,5), antibodies (6,7), and viral particles (8–10). Among these approaches, ionically cross-linked chitosan nanoparticles prove to be efficient vehicles for transporting peptides across the nasal mucosa (11). This approach has laid the foundation

for the present studies with nanosized colloidal crystals, which we describe here.

So far the uses of nanocrystals were primarily focused on the area of drug delivery via cell membrane permeation. Gold microparticles coated with DNA are introduced into cells by high-velocity acceleration to penetrate the cell membrane (12). However, this method is problematic due to the obvious difficulties in controlling either the precise amount of DNA or the precise cell penetration modality. In this report, we demonstrate that colloidal crystals are promising candidate materials for this type of biological application. A colloidal crystal is created when a dispersion of uniformly sized spheres (or colloids) is allowed to sediment out into a closely packed crystalline structure. Known primarily as a template for making three-dimensional (3-D) photonic crystals, colloidal crystals have also received attention in bioengineering applications; inverted colloidal crystals can be used as 3-D cell culture scaffolds (13). Advances in

intracellular drug delivery, coupled with unique capabilities of colloidal crystals, provide an essential technology needed to overcome the selective barrier of the cell membrane.

We report here the combined use of viral vectors embedded in a collagen matrix adhering to a nanocolloid platform for precision gene delivery to a subpopulation of cells in culture. The virus mixture, packed into the voids of the colloid crystal, is released into the cells by collagenase enzyme secreted from the cells into the proximal supernatant medium. Our delivery system avoids cell death, since the final infection of the specific subpopulation of cells is time-dependent on cell-secreted collagenase, rather than overdosing the entire monolayer culture with all the lentiviral particles, as traditionally done, often resulting in cell death. Our colloid-collagen-viral vector platform (CCP) offers promise for applications (8,14,15) to deliver a broad range of agents, such as bacteriophages (16), plant (17,18), insect (19), and animal viruses (20,21).

MATERIALS AND METHODS

Silica Colloids

The silica colloids were obtained from Dr. A.A. Zakhidov, University of Texas at Dallas. Colloidal crystals were grown using a slow crystallization process of monodispersed aqueous silica colloids in a glass cylinder, typically for 10 months at ambient temperature (22). Resulting deposits were polycrystalline with rod-like single crystals, which were sintered for several hours at 700°–750°C. The crystals thus obtained are closely packed, interconnected silicon dioxide spheres arranged in a face-centered cubic (FCC) lattice structure (Figure 1A). The diameter of such spheres can range from 20 nm to 10 μm with interconnected voids between the spheres; the silica colloids used in this investigation had sphere diameters between 200 and 400 nm. The crystals were cut using a low-speed diamond saw and polished to a thickness of <1 mm to ensure that when used they would be suspended above the monolayer cultures; thicker colloidal crystals would not be able to float above the cell culture, and their weight would cause them to adhere to the cell cultures and destroy the cells beneath them. Inverse carbon replicas of the colloidal crystals were fabricated using the phenolic resin route (22), followed by dissolution of the silica spheres in 2% hydrofluoric acid (HF).

Antibodies

Peroxidase-conjugated sheep immunoglobulin G (IgG) fraction to rabbit IgG was purchased from MP Biomedicals (Solon, OH, USA), and goat anti-mouse IgG, peroxidase-conjugated, from Pierce Biotechnology (Rockford, IL, USA). Monoclonal mouse matrix metalloprotease-1 (MMP-1; i.e., collagenase-1) antibody came from Lab Vision (Fremont, CA, USA), rabbit polyclonal to active caspase 3 from Abcam (Cambridge, MA, USA), and polyclonal rabbit caspase-3 and β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture and Lentiviral Transduction

Human lung embryonic fibroblasts (WI-38 cell strain) were cultured in minimum essential medium (MEM; Hyclone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone); cells at early passages [population doubling level (PDL 30–35)] were used for all experiments described in this study. The viral constructs were packaged in VSV-G pseudoviral particles using feline immunodeficiency virus (FIV) vectors (System Biosciences, Mountain View, CA, USA) and transfected to packaging cells by Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's specifications. Lenti-luciferase virus was made from the package of this gene construct (System Biosciences) using the 293-T17 culture system. Two milliliters of the culture supernatant containing the packaged lenti-luciferase viral particles were used to infect WI-38 fibroblast cultures in 35-mm glass-bottomed culture dishes, maintained for observation for 96 h in a humidified atmosphere containing 5% CO₂ at 37°C.

Preparation of Colloid-Collagen-Viral Vector Platform

Colloidal crystals were rinsed in 70% ethyl alcohol and exposed to ultraviolet (UV) light overnight inside the cell culture hood for sterilization. All procedures were performed on ice under sterile conditions. Rat-tail collagen type I was used for the experiment, 4.79 mg/mL mixed with 0.02 N acetic acid to a final concentration of 2.85 mg/mL. This collagen stock was mixed with the vehicle buffer [0.75 M NaCl and 50 mM phosphate-buffered saline (PBS) in 50 mL H₂O] and neutralization buffer (260 mM NaHCO₃, 200 mM HEPES, and 50 mM NaOH in 50 mL H₂O) in the ratio 7:2:1 to produce a final collagen concentration of 2 mg/mL. pH was confirmed at 7.0 using pH paper.

Polybrene® (Sigma-Aldrich, St. Louis, MO, USA) was added to the lentivirus to a final concentration of 5 mg/mL, which was then added to the collagen in the ratio of 15:100 μL, and

50 μL collagen-virus mixture were micropipetted into Petri dishes. The sterilized colloidal crystals were slowly immersed in the collagen-virus mixture and incubated at 37°C for 30 min to 1 h to form a collagen gel. The crystalline samples, coated with collagen and virus, were transferred onto monolayer cultures in glass-bottom Petri dishes. The cells with the CCP were incubated at 37°C for 24–96 h. After 96 h, the CCPs were removed, and the cells were observed under a fluorescent microscope to determine the infection level of the virus.

Scanning Electron Microscopy

Imaging of colloidal crystals and colloidal crystals coated with collagen was carried out on a Zeiss SUPRA™ 35VP ultra-high performance variable pressure field emission scanning electron microscope, at an accelerating voltage of 3–5 KV. The vacuum of the chamber was kept around 9 × 10⁻⁹ Torr. For scanning electron microscopy (SEM) analysis, the colloidal crystalline samples were adhered onto sample stubs.

Immunofluorescence Microscopy

WI-38 cells were plated in 35-mm glass-bottom Petri dishes (MatTek, Ashland, MA, USA). Three days later, the cells were infected with virus delivered with or without the CCP for 96 h, as described in the section entitled Preparation of Colloid-Collagen-Viral Vector Platform. Cells were then fixed with 4% paraformaldehyde in PBS, and directly imaged with a Zeiss Axiovert 200M epi-fluorescence microscope, using a 63 × 1.3 numerical aperture (NA) Plan Apo objective. Figures were formatted using Adobe® Photoshop®.

Western Blot Analysis

Fibroblast cultures of the WI-38 strain with and without infection with the viral vector construct were collected with cell scrapers in the medium along with ice-cold PBS and centrifuged at 250× g for 5 min. Cell extracts were prepared in radioimmunoprecipitation (RIPA) buffer [20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2% Triton® X-100, 2 mM EDTA, and 0.2% sodium dodecyl

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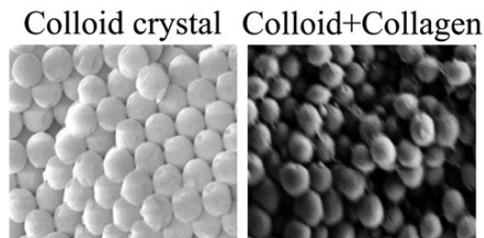
sulfate (SDS)] containing protease inhibitors (Calbiochem, San Diego, CA, USA) with constant agitation at 4°C for 30 min, followed by centrifugation at 15,000× *g* for 20 min. The cellular polypeptides from the protein extract were released by boiling in double-strength Laemmli SDS-sampling buffer at 95°C for 5 min. Equal amounts of proteins were separated on (9%–12%) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membrane with a transfer buffer containing 25 mM Tris base, 192 mM glycine, and 20% methanol for 3.5 h with a constant current of 325 mA. Nitrocellulose membranes were blocked in 5% nonfat dry milk (LabScientific, Livingston, NJ, USA) in Tris-buffered saline Tween®-20 (TBST; 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, and 0.1% Tween-20). The blots were washed and incubated with indicated primary antibodies on a rotary shaker at 4°C overnight. After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated second antibodies for 1 h at room temperature, then with enhanced chemiluminescence reagent (Amersham ECL™; GE Healthcare, Piscataway, NJ, USA) for 1 min, and visualized by exposure to Kodak X-OMAT film.

For collagenase immunoblots, media from cells incubated for different time intervals were collected. Protease inhibitors were added, and the samples were concentrated using Centrplus® centrifugal filter devices (Millipore) according to the manufacturer's instructions. The concentrated media were loaded onto 9% SDS-PAGE as described previously.

Trypan Blue Exclusion Assay for Cell Viability

Cultures were processed initially to collect medium supernatant containing the detached cells, which were harvested by further centrifugation to generate cell pellets. They were then added to the fraction of attached cells, which were collected by treating the adherent remaining monolayer cultures from previous steps with 0.05% trypsin, 0.02% EDTA. The combined adherent and detached cell populations were then processed to determine cell

A



B

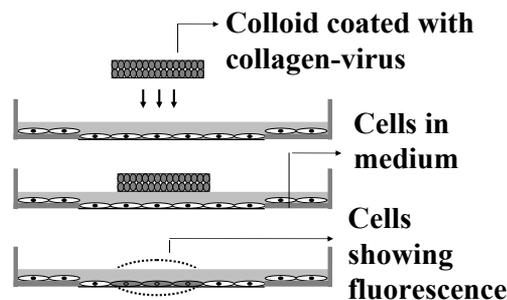


Figure 1. Structure of colloidal crystals, and a graphic representation of the experimental design.

(A) Scanning electron microscopy (SEM) images of colloidal crystal with and without collagen. There are no significant changes in the morphological structure of the colloid when coated with collagen. (B) The experimental setup is represented here. The sterilized colloidal crystals were slowly immersed in the collagen-virus mixture and incubated at 37°C for 30 min to 1 h to form a collagen gel. The colloidal crystal-collagen-viral vector platform (CCP) was transferred to monolayer cultures of WI-38 human fibroblast cells, plated in 35-mm glass-bottom Petri dishes; the medium acts as the interface between the cells and the CCP. The cells with the colloid-collagen virus were incubated at 37°C for 24–96 h. After 96 h, the CCP was removed, and the cells were observed under a fluorescence microscope for cell-associated cytoplasmic fluorescence intensity to substantiate successful infection.

viability in the cultures by Trypan blue exclusion assay. Trypan blue (Sigma-Aldrich) was mixed with cells (1:1), and live-cell indices were determined by counting cells that did not take up this dye manually on an inverted microscope.

RESULTS

Colloidal Crystals

The colloidal crystals were coated with collagen, a natural biopolymer, as a surfactant carrying the viral vector construct. SEM pictures of the colloid (Figure 1A) show a regularly spaced arrangement of voids within a colloidal crystal array. Colloidal crystals coated with collagen show no significant changes in colloid morphology, represented by Figure

1B; the collagen seems to pack the voids of the colloidal crystal.

Localized Transfer of Viral DNA to Subpopulations of Cells in Monolayer Culture

As described in the Materials and Methods section, rat-tail collagen in solution containing the lenti-luciferase construct was first added to the colloid in a precursor soluble form at low temperature (4°C); as the temperature rises to 37°C, the virus-containing liquid collagen gels fill the voids of the nanoscale porous colloid (Figure 1B). These CCPs were then positioned above confluent cultures of WI-38 human fibroblasts; infection efficiency of luciferase gene expression was evaluated by fluorescence microscopy. As seen in Figure 2A, the colloid-collagen-lenti-luciferase infects

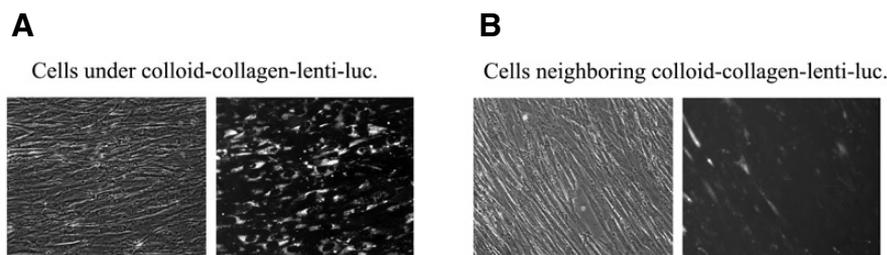


Figure 2. Localized steady transfer of recombinant viral vector gene to the cells. (A) Cells directly under the colloid-collagen-viral vector platform (CCP) region exhibit maximum fluorescence. The infection associated fluorescence activity is close to 90% efficiency for that specific subpopulation of human lung fibroblasts. The fluorescence intensity in cells reaches a maximum at 96 h postinfection, perhaps reflecting the time required for the fibroblasts to secrete sufficient collagenase to digest the collagen encapsulating the CCP, releasing the lenti-luciferase virus to infect the cells. (B) Regions neighboring the CCP, where the viruses are not transferred and the cells exhibit least infection and minimum fluorescence. This shows a precise and slow release of the lentiviruses to the cells.

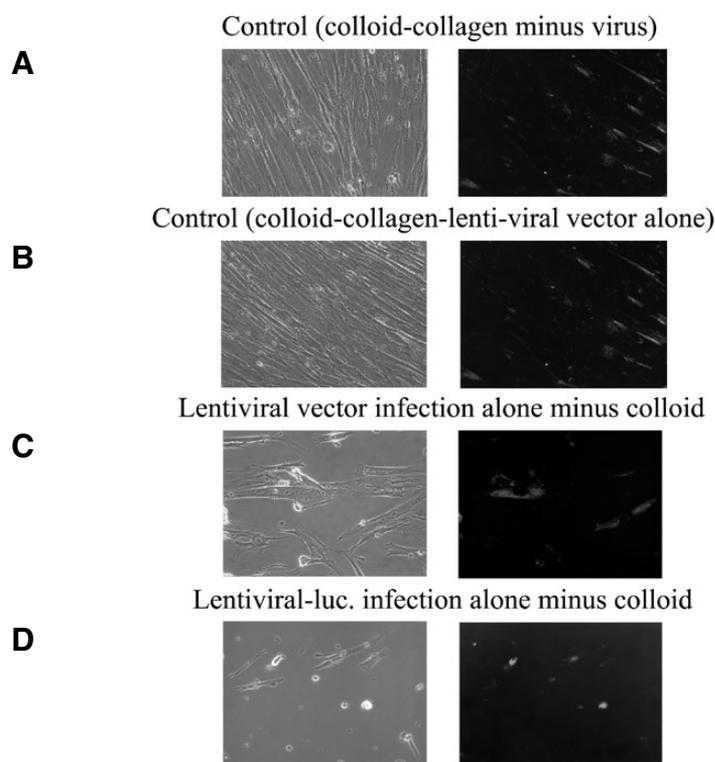


Figure 3. Apoptosis in cells treated with virus directly. (A) Control experiments exhibit no fluorescence at all. Cells were cultured in a 35-mm glass-bottom Petri dish, and then incubated with colloid-collagen platform without lentivirus for 96 h, and observed under a fluorescent microscope after removing the colloid-collagen-viral vector platform (CCP). (B) Another control with cells exposed to colloid-collagen platform bearing the lentiviral vector alone, without the luciferase and green fluorescence protein constructs; the cells exhibit almost no fluorescence. (C) Cells cultured in 35-mm glass-bottom Petri dishes treated with viral vector alone undergo slow apoptosis, and die eventually by 96 h. (D) Slow apoptotic death is observed to begin at 24 h postinfection when WI-38 cells are treated directly with lenti-luciferase, and reaches the maximum level at 96 h, as shown. Left hand panels of the above four sets of figures are phase-contrast images, while the right hand panels are fluorescence images of the same fields.

specific subpopulations of confluent fibroblasts with close to 90% efficiency, while neighboring cells show very little infection-associated luciferase fluorescence activity (Figure 2B). This result

shows that at least 96 h is needed to reach maximal detectable fluorescence intensity, since the lenti-luciferase gene viral particles need to be released from the CCPs before infecting the

cells beneath them and express gene-associated fluorescence. This delay may reflect the interval required for cells to secrete sufficient collagenase to digest the collagen that encapsulates the viral particles. Negative controls of colloid-collagen-lentiviral vector alone, or colloid-collagen minus virus, show no fluorescence activity at all (Figure 3, A and B); in sharp contrast, in control infections with either lentiviral vector alone at the same titer or lenti-luciferase alone (uncoupled with colloidal crystal-collagen support), the infection kills most cells in the monolayer culture bearing the viral expression construct by 96 h (Figure 3, C and D), and apoptotic death is observed to begin at 24 h postinfection and reaches its maximal level at 96 h.

Release of Collagenase into the Medium by WI-38 Cells

The plain MEM (without FBS) used for incubating the WI-38 cells was assayed for secretion of collagenase into the culture medium. Media were collected from cells at 24, 48, 72, and 96 h; collagenase protein was quantified by Western blot analysis, as seen in Figure 4A. This experiment confirms the release of collagenase by cells, reported in Reference 23. To investigate whether collagenase is the means of releasing the viral particles and not the culture medium itself, we incubated the CCP in MEM containing 10% FBS and antibiotics for 96 h in the absence of cultured cell monolayer; colloidal crystals with collagen alone were used as a control. After 96 h, media from these colloidal crystals were used to infect the cells directly; 96 h after infection, the cells were observed in the fluorescent microscope. The results, shown in Figure 4B, illustrate that the virus is not released from the collagen by incubation in cell-free, 10% FBS medium, even after 96 h. Thus, the release of the viral particles is due to the active participation of cell-secreted collagenase, rather than any biological activity of the FBS in the culture medium.

Apoptotic Effect upon Cells Directly Infected by Virus

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Caspase-3 blots and Trypan blue exclusion assay were used to prove that cell death after infection with virus directly, not embedded in the CCP, is due to apoptosis. Caspase-3 activity was quantified using Western blot analysis (Figure 5A); Trypan blue staining was used to estimate the percentages of dead and live cells. By 96 h, the percentage of dead cells was >60% (Figure 5B). Control for this experiment shows that when lenti-luciferase particles are embedded in CCPs, the observation as shown by Figure 2 (i.e., the release of virus and localized infection of specific cell groups in a gradual fashion without inducing the cell death) is again observed (Figure 5C). Thus the CCP provides not only localized focal delivery, but also the time-dependent release of infectious particles, to avoid rapid infection-induced cytopathic effects.

DISCUSSION

In this report, we present a strategy applying advances in nanoparticles to demonstrate porous crystals composed of nanoscale particles as a powerful enabling platform for direct delivery to cells. Our approach, inexpensive to produce, with a colloid substrate as the carrier to packaged viral vector, produces a complete, biocompatible gene-delivery system. We show that (i) delivery is localized to a cell subpopulation immediately proximal to the CCP; and (ii) the release of the viral particles is time-dependent, thus avoiding the cell death usually associated with high titer viral infection.

Our rationale for using collagen as the material to encapsulate the viral particles is (i) this protein is readily cleaved by ubiquitous cell-associated collagenases; (ii) collagen is in liquid form at 4°C, allowing even suspension of the mixture with the viral particles to form a coating on the colloidal crystal; and (iii) finally, the coated viral-collagen can be easily solidified by transfer to 37°C, thus firmly affixing it to the colloid crystal-backbone substratum. The entire setup can then be easily used in monolayer tissue cultures for the experiments described here.

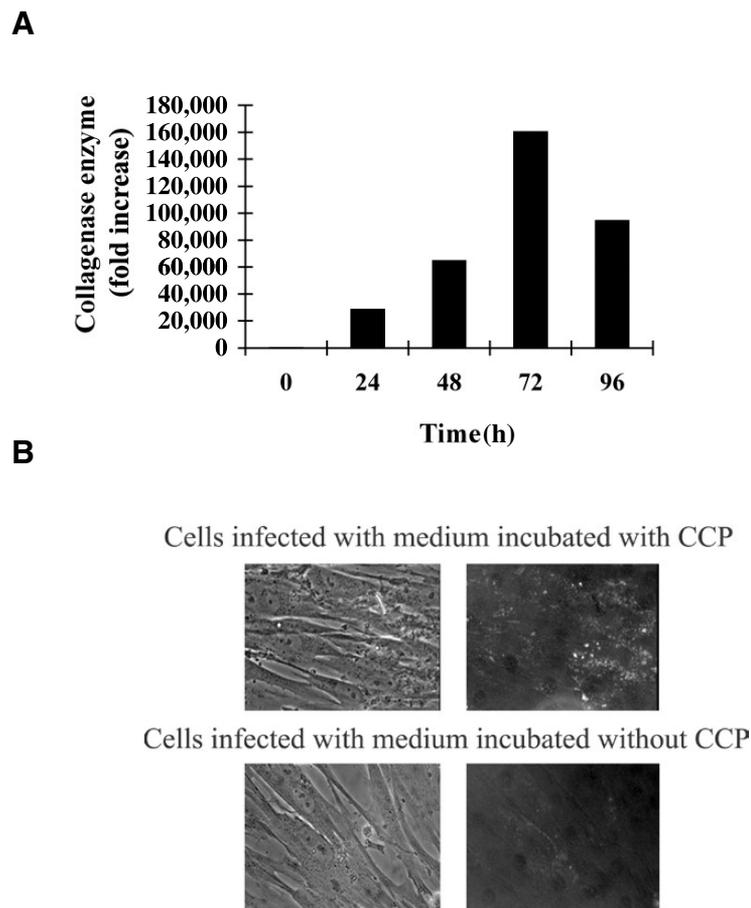


Figure 4. Collagenase enzyme disintegrates collagen. (A) Minimal essential medium (MEM) without fetal bovine serum (FBS) was used to incubate WI-38 cells; this medium, collected at different time points, was assayed for the level of secreted collagenase in the medium. The collected medium was concentrated using Centriplus centrifugal filters, followed by Western blot analysis with anti-collagenase-1 antibody. Densitometric quantitation in the lower panel shows that the maximum release of collagenase is at 72 h; this may be the reason for the delay of disintegration of the collagen in the colloid-collagen-viral vector platform (CCP), releasing the lentiviruses. (B) CCPs, with and without viral vector constructs, were immersed in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS for 96 h, and then the medium was used to infect WI-38 cells. Cells infected with this medium showed no fluorescence, demonstrating that the collagenase enzyme released by the WI-38 cells into the medium is requisite for the release of entrapped viral vector constructs from the collagen.

As depicted in Figure 4A, the release of the viral particles from the encapsulated collagen matrix seems to be accomplished by collagenases secreted by the recipient cell cultures. These enzymes are metalloendopeptidases; they mediate the initial and rate-limiting step in interstitial collagen degradation, by cleaving the three collagen polypeptide chains at a single locus three-fourths of the distance along the collagen molecule from its N-terminal end (24). Upon collagenase digestion, the collagen disintegrates, and the embedded virus is thus released into the culture medium immediately adjacent to the subpopulation of cells.

Failure to release the viral particles by medium containing FBS only illustrates the need of cell cooperation in viral delivery by secreting the essential enzyme, collagenase (Figure 4B).

Comparing cultures processed for lenti-luciferase viral infection with and without the colloid-collagen delivery system shows that in the former case, the time-limited release of the virus particles avoids the cytopathic effects seen in the latter scenario; this illustrates a further advantage of our colloidal crystal-collagen delivery system. In general, using lentiviral particles as the gene-delivery system must balance the need for sufficient

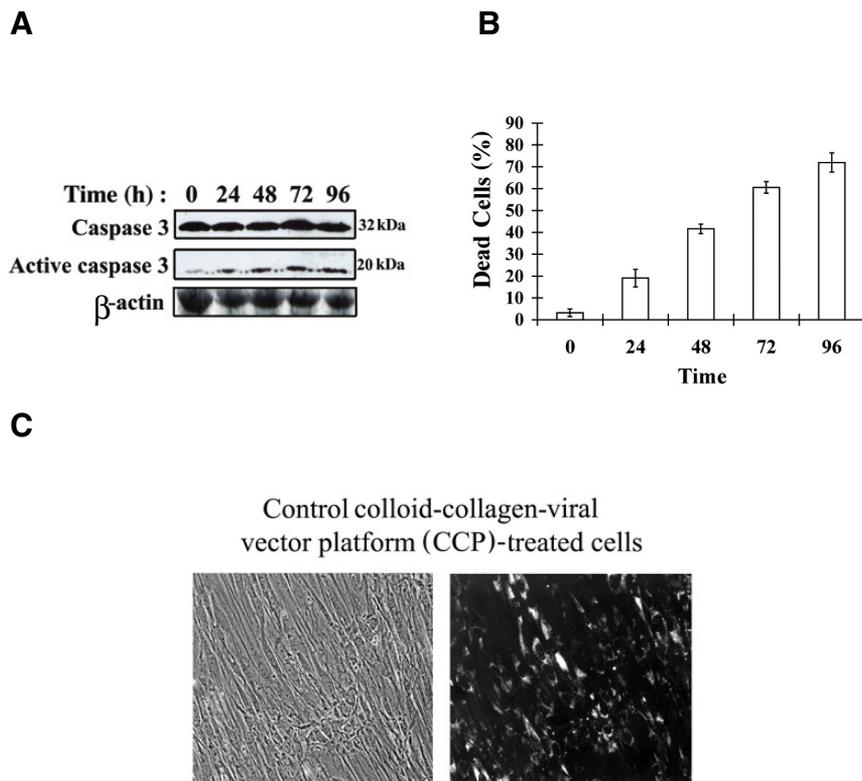


Figure 5. Apoptotic effect mediated through caspase-3 cascade. (A) Caspases are key mediators of programmed cell death, or apoptosis. Fifty micrograms of cell extracts of WI-38 cells infected by lenti-viruses (or not) were immunoblotted against active caspase-3 and caspase-3 antibodies. The lower panel demonstrates equal loading of β -actin antibody. The precursor forms of caspases are composed of a prodomain and large and small catalytic subunits; the active form of the subunits is 17–20 kDa. Caspase activity increases with time, as is shown here. (B) Cell viability assay quantifies the cytotoxic level of the cells; adherent and detached cell populations were combined for this assay. The percentage of dead cells was determined by counting the cells taking up Trypan blue stain, scored manually in an inverted microscope. The percentage of cells dying increased with time. (C) Control cultures with the colloid-collagen-viral vector platform (CCP) show that cells do not die as seen by the phase-contrast microscopy (left panel) which show fluorescence positivity (right panel) reflecting their infection by the lenti-luciferase viral particles similar to those demonstrated in Figure 2.

dosage of infecting viral particles to induce effective actions by delivered gene expression against the usual induction of cell death associated with virus infection. The result shown in Figure 3D demonstrates that infection with lenti-luciferase viral particles directly, not embedded in the colloidal crystal-collagen delivery system, indeed causes cells to commit apoptotic death, as evidenced by caspase activation, and hallmarked by 10-kDa autocatalytic end products (25).

Our colloidal crystalline technique produces a robust, biocompatible, porous matrix with an extremely large surface-to-volume ratio. There are other ways to create highly porous structures that could be used in the same way as we use the colloidal crystals; however,

none of these techniques has actually been applied as a substrate for holding, and slowly releasing, virus into cells in this way. Moreover, existing competing technologies creating porous platforms all have intrinsic disadvantages. For example, alumina templates (26) do not have sufficient surface-to-volume ratio to deliver the virus controllably; our pilot studies with this template generally result in cell death (data not shown). Another method uses porous silicon (27) electropolished with an electrolyte containing hydrofluoric acid; however, the etching results in disordered pores with nanocrystals remaining in the inter-pore regions, which are often not biocompatible, and may result in toxic effects to the cells. Block copolymers (28) have been used to create micro-

spheres, but not a porous substrate, for drug-delivery applications. Bulk silicon-based integrated circuits need packaging in a biocompatible material if they are to be used in, and linked to, living tissues. Microfabrication has been used to create complicated microelectromechanical systems-based drug-delivery devices (29), whose main disadvantages are biocompatibility and biofouling (referring to the impact of surrounding tissue on the function of an implanted device). These devices are also difficult to fabricate, and their extended use in vivo may cause infections or other undesired immune reactions. Thus, based on the criteria of maximum biocompatibility, high-porous surface and density ratio, and ease of fabrication, colloidal crystal is our platform of choice; so far no other such delivery system exists.

Future applications, such as using colloidal crystal-collagen in animal studies for localized delivery, will reveal whether our approach provides a new strategy for tissue site-specific drug or gene-based delivery of therapeutic countermeasures. The benefit of our gene-delivery platform with colloidal crystals coated with collagen provides special advantages over the current technology because (i) it is localized delivery to very narrowly confined tissue sites with focal pathology, such as injury sites, avoiding the systemic administration of gene- or small molecule-based therapeutics that may create off-site side effects; and (ii) the focused and gradual release modality avoids the need of large doses of drugs or genes which are diluted by systemic administration. Clinically, the localized delivery and gradual release of genes of interest may be especially powerful in economic terms, avoiding the need of repetitive use of expensive therapeutic gene-based drugs, not to mention their side effects on neighboring non-involved tissues, a situation often observed in cancer chemotherapy, as well as wound healing involved in sustained burn injury.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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