Published in final edited form as:

Adv Ther (Weinh). 2020 November ; 3(11): . doi:10.1002/adtp.202000156.

Bi-functional nanoparticle-stabilized hydrogel colloidosomes as both extracellular matrix and bioactive factor delivery vehicle

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Abstract

Maintaining both cell-cell and cell-extracellular matrix (ECM) interactions is often a critical component of three-dimensional (3D) tissue regeneration. In high-density cell condensation systems, lack of appropriate cell-ECM interactions can result in limited and/or slow cell differentiation and tissue formation. To address these problems, a colloidosome microsphere system that is composed of a gelatin hydrogel core and a porous nanoparticle shell is developed. The colloidosome microsphere functions as an ECM and morphogen carrier for the induction of cartilage formation of high-density human mesenchymal stem cell (hMSC) in 3D cultures. With the protection of the nanoparticle shell, the colloidosome microspheres can be readily suspended in aqueous solution without clumping, thus incorporated homogeneously within high-density cell condensations. The gelatin-based colloidosome microspheres stimulate chondrogenesis of hMSCs and degrade rapidly to facilitate ECM remodeling for new tissue formation. When loaded with human transforming growth factor- β 1, a potent chondrogenic morphogen, the colloidosomes serve as a bioactive factor delivery vehicle as well. The dual functionality of the colloidosomes as an

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Supporting Information

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ECM and a growth factor carrier effectively supports the chondrogenic differentiation of high-density hMSC condensations. These capabilities render the colloidosomes a promising platform system amenable to large-scale production of high-density 3D tissue culture constructs.

Graphical Abstract

Gelatin microspheres have been used for tissue regeneration but they are easy to aggregate. This research coats traditional gelatin microspheres with a thin layer of nanoparticles to form a colloidosome structure so that they can readily suspended in aqueous solutions. The colloidosome microspheres stimulate chondrogenesis of hMSCs and degrade rapidly to facilitate ECM remodeling for new tissue formation.

Keywords

colloidosome; nanoparticle; 3D cell culture; chondrogenesis; tissue engineering

Establishment of a three dimensional (3D) microenvironment for cell differentiation is a key to the regeneration of various tissues.^[1] To mimic natural microenvironments, a 3D construct should promote both strong cell-extracellular matrix (ECM) and cell-cell interactions^[2] so that resident cells are supported for proliferation and functionalization. However, it is challenging to simultaneously enhance cell-ECM and cell-cell interactions in some tissue engineering strategies. For example, hydrogels^[3] and scaffolds^[4] promote cell-ECM interactions, but cell-cell interactions are restricted by the intervening biomaterial. On the other hand, in high-density cell spheroid and organoid systems, cell-cell interactions are abundant, but they may lack initial cell-ECM interactions that present in some in vivo developmental microenvironments.^[5]

Including ECM in high-density cell condensation systems produces a microenvironment rich in both cells and ECM, which can more accurately imitate in vivo cell condensations and enhance the function and differentiation capacity of the cells. For instance, integration of collagen or poly(lactic-co-glycolic acid) fibers enhances the formation of brain organoids from pluripotent stem cells,^[6] and addition of fragmented poly(L-lactide) fibers to human turbinate mesenchymal stem cell spheroids elevates their osteogenic differentiation ability.^[7] Similarly, ECM microspheres have been engineered for incorporation into cell condensations as well.^[8] Particularly, gelatin has been a widely base material for the

formation of hydrogel microspheres for biomedical applications.^[9, 10] As a derivative from collagen, gelatin contains abundant cell recognition sites that regulate and promote cell behaviors such as adhesion, growth and function,^[11] and can be easily degraded by cell secreted enzymes.^[12] Affinity interactions between gelatin and growth factors also permits local and sustained release of these bioactive factors from the microspheres.^[9, 13] Capitalizing on these properties, we and other groups have utilized gelatin microspheres within cell condensations to direct the differentiation of stem cells, and to drive the formation of tissues including bone,^[10, 14, 15] cartilage^[16, 17] and trachea.^[18] However, due to their “sticky” surface after interacting with water,^[19] gelatin microspheres tend to exhibit some clumping in aqueous solution. Although this tendency is a favorable characteristic of gelatin microspheres in specific biomedical applications, such as the formation of scaffolds,^[20] it makes their homogeneous suspension in cell culture media difficult. This problem can result in heterogeneous gelatin microspheres distribution and clumping in cell condensations, thus decreasing cell-ECM interactions by reducing the overall gelatin surface area. Therefore, a synthesis strategy that prevents gelatin microspheres from clumping would increase cell-ECM interactions in condensation constructs, and potentially lead to more uniform and enhanced signaling to cells for 3D tissue regeneration.

Coating gelatin microspheres with a thin continuous film prevents them from being sticky. Although this strategy has been industrially applied,^[21] the continuous film coated on the surface would inhibit cell-gelatin interactions. To tackle this challenge, we hypothesized that clumping of gelatin microspheres in aqueous solution can be inhibited by coating them with a thin and porous surface layer. If this layer is thin and porous enough, the gelatin core will still be able to interact with surrounding cells, and potential bioactive factor loading and unloading will not be obstructed. This approach will enable important dual functionality of the microspheres as both an engineered ECM and a bioactive factor carrier without unwanted clumping. Engineering a gelatin-based colloidosome, which is a microcapsule with a colloidal particle shell, by coating the gelatin microsphere with a layer of suitable nanoparticles could be one potential way to form such layered structure. In this case, the gelatin core of the colloidosome can serve as an ECM and payload carrier similar to previously reported gelatin microspheres. The shell protects the encapsulated core from clumping, and is porous enough to allow the penetration of payloads. Colloidosomes are usually prepared with a Pickering emulsion, a type of emulsion that utilizes solid particles as the stabilizer.^[22] When prepared with crosslinkable macromer solution cores, the Pickering emulsion can be readily converted to solid hydrogel colloidosomes.^[23] Using the Pickering emulsion principle, we developed a colloidosome composed of a solid nanoparticle shell and a gelatin core. The colloidosome suspends much more readily in aqueous solution compared to gelatin microspheres without the nanoparticle shell. It is further shown that this colloidosome supports human mesenchymal stem cell (hMSC) viability and drive their chondrogenic differentiation in high-density cell spheroids through ECM support and the delivery of a potent chondrogenic morphogen, transforming growth factor-beta 1 (TGF- β 1).

The colloidosome preparation strategy is schematically summarized in Figure 1a. Briefly, the nanoparticles were first suspended in warm vegetable oil, while the gelatin was dissolved in warm aqueous solution. The gelatin solution was then gently added into the organic phase to form a water-in-oil Pickering emulsion. The solid particles moved to the water/oil

interface^[24] to reduce the interfacial energy of the whole system.^[25] Vigorous stirring and gentle warming was used during this step to avoid premature gelation of the thermosensitive gelatin. The emulsion was then gelled through sol-gel transition by reducing the temperature of the system in an ice-water bath under stirring. After complete gelation, gelatin core/silica nanoparticle shell hydrogel colloidosome microspheres were formed. Water was then extracted from the hydrogel colloidosomes with acetone. Dehydrated colloidosome microspheres were obtained by filtration and washing with acetone to fully remove the oil and unbound silica nanoparticles. As-formed colloidosome microspheres were air dried and stored until further use. 300 nm silica nanoparticles were used as the coating material to make the colloidosome microspheres following the above procedure. These colloidosome microspheres (Figure 1b, c) had an average size of $60 \pm 21 \mu\text{m}$ (Figure 1d). The buckling pattern of the shell of the dehydrated colloidosome (Figure 1c) indicates high binding affinity between the nanoparticle shell and the gelatin core.^[24] These colloidosome microspheres can be easily resuspended in aqueous solution by 5 seconds of vortexing (Figure 1e, 1f left tube). In contrast, gelatin microspheres without particle shells were observed to clump easily and were more difficult to resuspend under the same suspension conditions (Figure 1f right tube and 1g).

To confirm the core/shell structure, silica nanoparticles were fluorescently labeled with fluorescein isothiocyanate (FITC)^[26] and gelatin was fluorescently labeled with tetramethylrhodamine isothiocyanate (TRITC) prior to microsphere preparation. The labeled microspheres were visualized by laser scanning confocal microscopy (LSCM). Microspheres were loaded between two pieces of thin glass coverslips without spacers to visualize the core/shell structure. LSCM images (Figure 2a, b) demonstrated that silica nanoparticles formed a thin shell (green channel) coating the surface of a gelatin microsphere core (red channel). The shell layer appeared porous, evident by gaps between the silica nanoparticles observed with LSCM imaging (Figure 2c, d). Furthermore, limited fluorescence from the nanoparticles was found below the surface layer, indicating that the core was composed almost entirely of gelatin (Figure 2e). This unique structure results in a porous coating of nanoparticles with some exposure of the gelatin to the surrounding environment through the gaps in the nanoparticle layer. Consequently, individual colloidosome microspheres are protected from clumping in aqueous solution and can be suspended through simple agitation (Figure 1e).

The gelatin core can be crosslinked to enhance the stability of the colloidosome for biomedical applications. As a natural crosslinker, genipin derived from *Genipa americana* fruit extract has been widely studied and used for both in vitro and in vivo use due to its biocompatibility.^[27] To verify whether the crosslinked colloidosomes can take up biomolecules, we tested the adsorption/absorption ability of the genipin crosslinked colloidosomes using FITC labeled ovalbumin (FITC-OVA) as a model molecule (Figure S1, Supporting Information). For this test, 20 μL of FITC-OVA solution at a given concentration was added to 1 mg of crosslinked colloidosome, followed by 1 h of incubation at 37 °C. The mixture was then centrifuged at 14000 rpm for 1 minute to retrieve the supernatant. The percentage adsorbed and absorbed was determined by the fluorescence decrease from the supernatant compared to the original FITC-OVA solution. It was found that 1 mg colloidosome held approximately 80% - 90% of the FITC-OVA in solutions of original

concentrations up to 10 mg/mL. This result demonstrated that the colloidosomes can maintain a high carrying capacity for biomolecular payload, suggesting the strong potential of the colloidosomes as a bioactive factor carrier for biomedical applications.

The ability of the colloidosomes to support and/or induce the chondrogenic differentiation of hMSCs in 3D was then examined. High-density cell condensation culture with abundant cell-cell interactions supports chondrogenic differentiation of hMSCs and new matrix production and remodeling.^[28] The introduction of gelatin microspheres to high-density hMSC aggregates has been previously reported to promote their chondrogenic differentiation.^[16, 29] We expected that, relative to gelatin microspheres without shells, the colloidosomes would facilitate better individual suspension in media and incorporation into the hMSCs aggregates, which would increase their interactions with the cells and enhance chondrogenesis. The chondroinductive potential of the colloidosomes incorporated within of hMSC aggregates was compared against control aggregate groups with gelatin microspheres without nanoparticle shells and groups with no microspheres. TGF- β 1 was used as a soluble chondroinductive signal, and was supplemented in the differentiation medium at 10 ng/mL. We first examined the behavior of the cells with or without microspheres using LSCM. High-density cell aggregates were cultured in a V-bottom 96 well plate for 2, 7 and 14 days. Five minutes prior to imaging, live cell indicator fluorescein diacetate was added to the culture for viable cell staining. Due to red autofluorescence of genipin crosslinking, incorporation of microspheres into the cell aggregates was imaged through Z-stacks of green fluorescence of live cell and red fluorescence of genipin crosslinked microspheres (Figure 3, Figure S2, Supporting Information, for cells without microspheres). After 2 days, hMSC aggregates successfully incorporated with microspheres regardless of whether the microspheres had a nanoparticle shell. However, the microspheres without the nanoparticle shell were not as well dispersed throughout the cell aggregate (Figure 3b and Figures S3 and S4, Supporting Information). Notably, empty spaces were observed not occupied by cells presumably due to the poor separation of gelatin microspheres (arrows in Figure S3, Supporting Information), which indicate less uniform integration of microspheres within the cell aggregates. After 7 days, apparent degradation of colloidosome microspheres was observed (Figure 3c, Figures S5 and S6, Supporting Information). Although degradation of gelatin microspheres without shells can also be seen, it was to a lesser extent (Figure S5, Supporting Information). There are two possible reasons that contributed to faster degradation of the colloidosome microspheres. First, colloidosome microspheres were more uniformly incorporated into the cell aggregates, which would increase the surface area in contact with cells and cell-microsphere interactions. Second, silica nanoparticles stimulate the expression of matrix metalloproteinases^[30] that can degrade gelatin. After 14 days, due to persistent degradation of microspheres, the formation of new ECM and/or construct thickness limiting fluorescence excitation and emission, no intact microspheres were observed under LSCM (Figure S7a b, Supporting Information). These histologic findings indicate that the gelatin-based microspheres gradually degraded and were potentially taken up by the cells during chondrogenesis.

After 1 and 2 weeks, the chondrogenesis of the cell aggregates was determined by quantifying the amount of glycosaminoglycan (GAG) and DNA within each cell condensation. GAG is an extracellular matrix molecule marker of neocartilage formation,

[16, 31] whereas DNA levels are indirect measures of the number of cells per aggregate. After 1 week, cells incorporated with colloidosome microspheres contained significantly higher levels of GAG compared to the group containing gelatin microspheres without a nanoparticle shell and the group containing no microspheres (Figure 4a). In contrast, the DNA levels in all three groups were similar, indicating maintained cell viability in the presence of microspheres. GAG to DNA (GAG/DNA) ratio indicated significantly higher chondrogenesis on a per cell basis in the colloidosome group compared to control groups. After 2 weeks, the level of GAG within the non-colloidosome gelatin microsphere group was close to that of colloidosome group, and both microsphere groups had significantly higher levels of GAG and GAG/DNA than the group without microspheres (Figure 4a). Notably, both groups with microspheres had slightly greater DNA amounts than the group without microspheres, indicating improved proliferation and/or survival of cells in the presence of microspheres regardless of the nanoparticle coating. Additionally, GAG formation was evaluated qualitatively via histological analysis using Safranin O (SafO) staining at 2-weeks with Fast Green used to counterstain cells and microspheres (Figure 4b). From the histological images, it was observed that gelatin microspheres without a nanoparticle shell were not well separated in the constructs whereas the colloidosome microspheres were more homogeneously distributed and displayed improved incorporation within the cell condensation. The size of the colloidosomes was smaller compared to the microspheres without a nanoparticle shell (Figure 4b), which indicated an increased rate of degradation, as both microparticles were of comparable size prior to culture (Figure 1d). This phenomenon is consistent with LSCM findings discussed above. Together, these results suggest both colloidosomes and gelatin microspheres without a nanoparticle shell enhance the chondrogenic differentiation of hMSCs. With easier suspension ability and improved incorporation within cell condensations, colloidosomes provided enhanced cell-microsphere interactions and accelerated chondrogenesis of hMSCs in high-density aggregates.

As demonstrated above, the colloidosome is also an excellent carrier for proteins. We and others have previously reported that gelatin microspheres have high affinity to TGF- β 1. In the absence of cells in aqueous solution, minimal TGF- β 1 is released,^[17, 29] but when microspheres are incubated with the addition of soluble collagenase, the gelatin is degraded and TGF- β 1 is released in an enzyme-dependent fashion.^[15] Therefore, we hypothesized that TGF- β 1 could be loaded into the gelatin-based colloidosomes the nanoparticle coating and released during their degradation by cell-secreted collagenases within the condensations for induction of hMSC chondrogenic differentiation. To test this, we loaded TGF- β 1 into the colloidosomes and evaluated their ability to support chondrogenesis in high-density hMSC condensations compared to those cultured with gelatin microspheres without a nanoparticle shell. Condensations cultured with TGF- β 1-loaded colloidosomes were not supplemented with further TGF- β 1, unlike those cultured with non-loaded microspheres, which were regularly supplemented with TGF- β 1 in the culture media. After 1-week culture, hMSCs mixed with colloidosomes expressed significantly higher levels of GAG, both in absolute amount and when normalized by DNA content (Figure 4c). After 2 weeks, the GAG amount and GAG/DNA of the colloidosome group was still significantly higher than the gelatin microsphere without nanoparticle group and the control group lacking microspheres. Similar to the results from exogenous TGF- β 1 study above, colloidosome microspheres after 2

weeks culture were smaller than the microspheres without nanoparticle coating (Figure 4d). Taken together, the higher GAG level of the hMSC condensations with incorporated colloidosome microspheres may be a result of enhanced cell-ECM interactions with the microspheres, faster gelatin degradation, and consequently more rapid TGF- β 1 release to stimulate the chondrogenesis.

To verify the robustness of the trends we discovered, both experiments supplying exogenous and endogenous TGF- β 1 with incorporated colloidosome or gelatin microspheres without shells were repeated two additional times with the same hMSC donor under the same conditions (Figures S8 and S9, Supporting Information). The overall trends of the GAG expression, DNA levels and GAG/DNA ratios were quite consistent among all three repeats.

In summary, we have developed a novel gelatin core/nanoparticle shell colloidosome microsphere composites using the Pickering emulsion mechanism. In these composites, nanoparticles assemble onto surfaces of gelatin emulsions for the stabilization of the system. The obtained colloidosome microspheres can be readily suspended and mixed with cells for 3D cell spheroid culture. Due to the permeable nature of the nanoparticle shell, the gelatin core and its payloads can be easily accessed by cells, endowing the colloidosomes dual functionality as both a support for cell function through enhanced cell-ECM interactions and for delivery of a bioactive factor payload. Without the involvement of chemical bonds, silica nanoparticles may be replaced with a variety of substitutes, such as CaCO_3 and hydroxyapatite nanoparticles, for specific applications. In addition, due to the ease with which they can be dispersed in aqueous solution, the colloidosome microspheres can be readily adapted for large scale production of engineered tissue constructs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

EA gratefully acknowledges the funding support from the NIH (R01AR063194, R01AR074948, R01EB023907). RT acknowledges NHLBI T32 grants (T32HL134622, T32HL007829) for financial support. The authors appreciate the CWRU Light Microscopy Imaging Core supported by NIH (S10OD024996) for LSCM imaging.

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Figure 1.

Preparation and characterizations of gelatin core/particle shell microspheres. (a) Schematic representation of the preparation procedure. (b) SEM image of the colloidosome microspheres. Bar: 10 μm. (c) SEM image of surface silica on colloidosome microspheres. Bar: 2 μm. (d) Diameter distributions of gelatin microspheres without shells (top; 122 individual microspheres were analyzed; mean size $\pm$ standard deviation: 58 ± 24 μm) and the colloidosome microspheres (bottom; 121 individual microspheres were analyzed; mean size $\pm$ standard deviation: 60 ± 21 μm). (e) Macroscopic image of 2 mg microspheres in 1 mL water after 5 s of vortexing. Left: colloidosomes; Right: gelatin microspheres without silica nanoparticles. (f) Microscopic image shows 2 mg colloidosomes in 1 mL water after 5 s of

vortexing. Bar: 100 μm . (g) Microscopic image shows 2 mg gelatin microspheres without silica nanoparticle shell in 1 mL water after 5 s of vortexing. Bar: 100 μm .

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Figure 2.

LSCM images of silica shell/gelatin core microspheres. (a) Scheme of sample preparation for LSCM imaging of center and surface of colloidosomes. Microspheres are compressed between a glass coverslip and slide for imaging of the surface coating. (b) Low magnification images show core/shell structure. Bar: 100 μm . (c) Z-stack LSCM image shows surface of microspheres. (d) Green channel of (c) showing the porous surface composed of FITC labeled silica nanoparticles. (e) Same z-stack LSCM image shows center of microspheres. Bars: 50 μm . Green: FITC labeled silica; Red: TRITC labeled gelatin. Cross-sectional profiles of the z-stacks show the core/shell structure of the colloidosomes.

Figure 3. Colloidosome microspheres degrade faster than gelatin microspheres without shells in the high-density hMSC condensations. (a) Scheme of the preparation of a hMSC aggregate with microspheres. LSCM z-stack images of hMSC aggregates with colloidosome or gelatin microspheres without a nanoparticle shell after (b) 2 and (c) 7 days. Bars: 100 μm .

Figure 4.

GAG and DNA content analysis of cell condensations with exogenously or endogenously supplied TGF- β 1 after 1 week and 2 weeks ($n = 4$ samples per group). (a) GAG and DNA analysis of hMSC condensations with exogenously supplied TGF- β 1 after 1 and 2 weeks. GAG amounts and GAG/DNA ratios within all groups are significantly different between the two time points ($p < 0.05$ Tukey with one-way ANOVA). (b) GAG staining with Safranin O and Fast Green counterstain of hMSC condensations with exogenously supplied TGF- β 1 after 2 weeks. (c) GAG and DNA analysis of hMSC condensations with endogenously supplied TGF- β 1 after 1 and 2 weeks. GAG amounts and GAG/DNA ratios within all groups are significantly different between the two time points ($p < 0.05$ Tukey with one-way

ANOVA). (d) GAG staining with Safranin O and Fast Green counterstain of hMSC condensations with endogenously supplied TGF- β 1 after 2 weeks.

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