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Promotion of osteoblast proliferation on complex coacervation-based hyaluronic acid – recombinant mussel adhesive protein coatings on titanium

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Abstract

Many biological polyelectrolytes are capable of undergoing a fluid–fluid phase separation known as complex coacervation. Coacervates were prepared using hyaluronic acid (HA) and a recombinant fusion protein consisting of mussel adhesive motifs and the RGD peptide (fp-151-RGD). The low interfacial energy of the coacervate was exploited to coat titanium (Ti), a metal widely used in implant materials. The coacervate effectively distributed both HA and fp-151-RGD over the Ti surfaces and enhanced osteoblast proliferation. Approximately half of total fp-151-RGD and HA in the solution transferred to the titanium surface within 2 h. Titanium coated with coacervates having high residual negative surface charge showed the highest cell proliferation of preosteoblast cells (MC-3T3) compared to the treatments tested. Indeed, MC-3T3 cells on complex coacervate coated titanium foils exhibited over 5 times greater cell proliferation than bare, HA coated or fp-151-RGD coated titanium.

Keywords

Complex coacervate; Hyaluronic acid; Mussel adhesive protein; fp-151-RGD; MC-3T3; Biocoating

1. Introduction

Complex coacervation denotes a fluid–fluid phase separation that is brought about by the pairing and coalescence of oppositely charged polyelectrolytes in aqueous solution. The denser polymer-rich phase is the coacervate which gradually settles below the more dilute equilibrium phase [1]. In preparing complex coacervates from cationic and anionic polyelectrolytes the pH is adjusted to where their respective net charges are mutually neutralized to form a liquid, polymer-rich phase. Complex coacervates typically possess very low interfacial energy in aqueous solution, and it is this property that enables the coacervate to engulf a variety of particles in solution [1,2]. Consequently, coacervates have been utilized as encapsulants for flavors, oils, explosives, and cells to mention a few [3–5]. The biological origin of coacervation probably goes back to the origin of life, as it provided a means to separate proto-biomolecular clusters from the aqueous medium [6]. Recently, it was reported that marine sandcastle worms

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secrete their cement as a coacervate of adhesive proteins, and worm cement inspired complex coacervates made from synthetic polyelectrolytes are showing promise as wet adhesives [7–9].

Titanium based materials are widely used owing to desirable properties that include light weight, high strength, biocompatibility, and durability in extreme environments [10]. Titanium based materials are also inert to human body fluids, making them ideal for medical replacement structures such as orthopedic and dental implants [10]. Nevertheless, titanium based materials are still facing clinical challenges such as bacterial infections and implant loosening over time [11]. To improve the clinical performance of orthopedic implants, recent investigations have sought to promote specific cell binding to titanium based materials by surface modification with extracellular matrix (ECM) components or specific peptide sequences from ECM proteins, such as the Arg-Gly-Asp (RGD) peptide [12,13]. Various methods ranging from a simple adsorption coating of ECM molecules to deposition of poly-electrolyte multilayers (PEMs) with ECM molecules have been attempted to coat titanium based implants [12,13].

As many ECM molecules are highly charged, complex coacervation has promise as a coating method. When two ECM molecules of opposite net charge are condensed to form a coacervate phase, these molecules are concentrated in the coacervate phase and settle onto target surfaces due to their higher density. Thus, deposition of most ECM molecules onto a target surface is driven by gravity and interfacial energy. Also, when mass transfer is critical, the diffusion of cells, nutrients, and ECM molecules into 3-D porous scaffolds can be improved by the use of coacervate coatings given the low interfacial tension of complex coacervates. Indeed, Toh et al. showed cell seeding density on a 3-D porous scaffold was improved by complex coacervate coating [14].

For the purpose of this study, hyaluronic acid (HA) and a recombinant mussel adhesive fusion protein were selected as the anionic and cationic polyelectrolytes, respectively, for complex coacervation. HA has been used extensively as an ECM coating molecule due to its antifouling, antimicrobial and cell proliferation activities [12,15]. fp-151-RGD is a recombinant RGD-fusion of a mussel adhesive protein. fp-151-RGD improves cell adhesion, spreading and proliferation for various types of cells [16]. Here, the complex coacervation of HA and fp-151-RGD has been investigated as a coating strategy for titanium with HA and fp-151-RGD with the aim of promoting cell proliferation.

2. Materials and methods

2.1. Materials

Titanium (Ti) foils (Alfa Aesar, MA, USA, purity > 99.5%) of 0.25 mm thickness were cut into 10 × 10 mm pieces and washed with acetone, ethanol and water to eliminate impurities. After autoclaving the Ti foils in liquid cycle, the Ti foils were dried in vacuum chamber and stored in the cell culture laminar flow bench. Hyaluronic acid (Average molecular weight, $M_w = 35,000$) was purchased from Lifecore (Chaska, MN, USA) and recombinant mussel adhesive protein, fp-151 and fp-151-RGD were generously supplied by Kollodis Biosciences (MA, USA). fp-151 is a fusion of an mefp-5 sequence sandwiched between two domains each consisting of 6 repeats of the decapeptide AKPSYPPTYK from mefp-1. fp-151-RGD has an additional GRGDSP fusion sequence [16]. 3,4-dihydroxyphenyl-L-alanine (DOPA) containing fp-151-RGD was prepared by modification with mushroom tyrosinase [17].

2.2. HA/fp-151-RGD coacervation

By varying the ratio between 0.1% (w/v) of HA with 0.1% (w/v) of fp-151-RGD at pH 4.0 (adjusted with dilute HCl) in 10 mM NaCl solution, coacervation of the two polyelectrolytes

was monitored by turbidity. Total polymer concentration was fixed at 0.1 % (w/v). Turbidity measurements were done by UV–Vis spectrophotometry at 600 nm, at which absorbance by fp-151-RGD was negligible. The relative turbidity is defined as $-\ln(T/T_0)$ where T and T_0 are light transmittance with and without sample, respectively [18]. Turbidity associated with coacervation was monitored by observing liquid droplet formation by inverted microscopy.

2.3. Zeta potential measurement

The zeta potential of both polyelectrolytes and complex coacervates was measured by a Malvern 3000 Zeta sizer instrument at 25 °C. The instrument uses a 10 mW He – Ne laser operating at 632.8 nm. The points of zero charge for fp-151-RGD and HA were determined by measuring the zeta potential at different pHs for the polyelectrolytes dissolved in 10 mM NaCl at an initial concentration of 0.1% (w/v). The pH was varied by the addition of 10 mM NaCl and 10 mM HCl solution. Similarly, changes in the zeta potential during coacervation of HA/fp-151-RGD were investigated by incremental additions wt % of HA at pH 4.0.

2.4. Absorption of HA/fp-151-RGD monitored by quartz crystal microbalance with dissipation (QCM-D)

A quartz crystal microbalance in dissipation mode (QCM-D, Q-Sense AB, Göteborg, Sweden) was used to follow the adsorption of fp-HA/fp-151-RGD coacervates onto a titanium coated quartz crystal (Q-Sense AB). Prior to use, the quartz crystals were rinsed in ethanol, blown dry with nitrogen and placed in a plasma oven for 10 min to prepare a clean surface. The crystal is excited at its fundamental frequency (about 5 MHz) and readings were made at the third ($\nu = 3$) and fifth ($\nu = 5$) overtones corresponding to 15 and 25 MHz, respectively. The buffer (10 mM sodium chloride, pH 4.0) was circulated into the QCM flow cell (internal volume of 100 μ l and temperature of 24 °C), for 20 min at a flow rate of 0.1 ml/min using a peristaltic pump. After stabilization of the signal, coacervate adsorption ($C_p = 0.01\%$ (w/v), HA:fp-151-RGD = 3:7) was monitored by the shifts in frequency (Δf) and energy dissipation (ΔD).

2.5. Surface energy measurement using dynamic contact angle

Dynamic contact angles (DCA) on bare or coacervate coated Ti foils were measured using a modified the Wilhelmy plate technique (Cahn Radian 315, Thermo, Waltham, MA, USA). The sample surface was immersed in the test liquid at a pre-selected rate of 10 μ m/s until 7 mm of the slide was submerged, and was withdrawn at the same velocity. The DCA measured the forces on the test surface during immersion, submersion and then emersion. The DCA software calculated the corresponding advancing and receding angles. In order to ensure the accuracy of the data, an average of five measurements was taken for each liquid–solid combination.

2.6. Cell culture and cell proliferation measurement

Mouse preosteoblast MC3T3-E1 subclone 4 cells (CRL-2593, ATCC) were maintained in MEM- α medium without ascorbic acid (Invitrogen) supplemented with 10% fetal bovine serum (FBS; ATCC) and penicillin/streptavidin (Sigma) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Titanium foils (1 $\times$ 1 cm) were placed in 12-well culture plates and incubated with HA/fp-151-RGD solution ($C_p = 0.1\%$ (w/v)) in 10 mM NaCl, pH 4.0 varying concentration weight ratio of HA from 0 to 1. Uncoated Ti foils were used as controls. After 1–8 h coating with the biopolymer solutions, the foils were washed with PBS for 30 min and placed on another 12 well plates. MC3T3-E1 cells ($\sim 1 \times 10^5$) were seeded on each Ti plated. 24 h after seeding, the foils were transferred to another 12 well plates to avoid cell propagation on cell culture dish rather than sample surfaces. 2–4 days after cell seeding, the culture medium was aspirated, and 500 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the wells to allow the formation of formazan crystals for 2 h. Finally, absorbance was measured at 570 nm using a microplate reader (Perkin–Elmer). For cell spreading analysis, the

actin cytoskeleton was labeled with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma) and analyzed by fluorescence microscopy.

3. Result and discussion

3.1. Coacervate formation from HA/fp-151-RGD mixtures

Before coacervating fp-151-RGD and HA, the surface charge of each polyelectrolyte was measured in the pH range 2–11. The isoelectric points of HA and fp-151-RGD deduced from surface charge measurements were 2.5 and 9.3, respectively (Fig. 1A). Since fp-151-RGD precipitates from solution $\geq$ pH 7.0, the pH range of 3.5–6.5 was selected for inducing complex coacervation. Parameters for coacervation are pH, ionic strength, temperature, molecular weight, concentration and ratio of polymers in solution [2]. Varying buffer pH (3.5–6.5), ratio between HA and fp-151-RGD (0–1), and total polymer concentration (C_p , 0.01–1% (w/v)), coacervates were formed, and surface charge of the solution was monitored. Coacervation dependent phase separation was observed by light microscopy and by turbidity increase of the solution. The coacervate droplet size was influenced by total polymer concentration, HA: fp-151-RGD ratio, pH and time. In the present study, the pH and C_p of the sample solution were fixed at 4 and 0.1% (w/v), respectively, for titanium foil coating. When HA and fp-151-RGD were mixed in solution (10 mM sodium chloride, pH 4.0), turbidity ensued (Fig. 1B) and formation of spherical coacervate micro-droplet was observable by optical microscope (Fig. 1C). After varying the weight ratio of HA and fp-151-RGD from 0 to 1, turbidity and zeta potential of the solutions were measured. At a HA: fp-151-RGD ratio of 3:7, maximum turbidity occurred and surface charge dropped to zero (Fig. 1B). Optimal coacervate yield was directly correlated with maximum turbidity and zero surface charge [2].

3.2. Coacervate coating of Ti foil and surface characteristics

Due to coalescence of coacervate droplets and given their higher density, phase separated droplets settled [19]. Settling during complex coacervation was monitored by microscopy and is available as a video file (Supplement data1). Adsorption of the coacervate was also detected by QCM-D (Fig. 2). HA/fp-151-RGD coacervates adsorbed on Ti coated quartz electrode were detected by frequency reduction and film compliance by ΔD increase when the coacervate accumulates on the Ti surface. To quantitatively measure deposition of HA/fp-151-RGD on Ti foil, fp-151-RGD concentration in the solution was measured before and after complex coacervation. At a ratio of 3:7 (HA and fp-151-RGD), over ~85% of total fp-151-RGD was recruited to the coacervate. The coacervate, which consists of roughly equal parts of HA and fp-151-RGD, completely coated Ti foil after 2 h. Since HA and fp-151-RGD were efficiently transferred to Ti surface by complex coacervate formation, we speculate that most ECM components capable of forming coacervate e.g. HA/chitosan, collagen/HA would also be effectively presented to target surfaces without significant loss.

To monitor changes in hydrophilicity following deposition of coacervate on Ti, the dynamic contact angle of the coated surface was determined. Consistent with a previous report [20], the average advancing contact angle of bare titanium was 81.6 ± 1.5 degrees. However, the advancing contact angle of Ti coated with coacervate of HA/fp-151-RGD (HA:fp-151-RGD = 4:6, at which preosteoblast cell growth was optimal) decreased to 49.6 ± 2.0 degrees. With surface energy (τ) defined as $\tau = \gamma \cos\theta$ (γ = water surface tension, θ = contact angle), Ti coated with coacervate increased hydrophilicity and surface energy versus the bare Ti surface. Hydrophilic surfaces are reported to be favorable for short-term cell adhesion and proliferation for various cell types and that the higher surface energy is preferred for better osteoblast growth and mineralization [21,22]. Thus, the coacervated HA/fp-151-RGD coating on Ti makes it a more attractive surface to osteoblasts.

3.3. MC-3T3 cell proliferation on coacervate coated surfaces

To investigate the effect of HA/fp-151-RGD coacervate coatings on MC-3T3 cell proliferation, we performed the MTT assay, which measures metabolic activity of viable cells. Typically, activity is linearly correlated with the number of live cells attached to the surfaces [23]. To optimize MC-3T3 cell proliferation on a coacervate coating of HA/fp-151-RGD, it was first necessary to adjust the HA:fp-151-RGD ratio for complex coacervation. Cell proliferation was not optimal at 30% (w/w) HA – the HA concentration for the highest observed coacervate yield. Instead, cell proliferation was the greatest at 40% (w/w) HA – over 7 times greater than on bare Ti (Fig. 3A). MC-3T3 cells reportedly prefer negatively charged surfaces and bone-like crystal growth is increased on negatively charged surfaces such as hydroxyapatite [24, 25]. Consistent with previous reports, 40% (w/w) HA exhibits the highest negative surface charge of the HA/fp-151-RGD coacervates. As the GRGDSP peptide localizes to the surface, the negatively charged coacervate deposition led to higher MC-3T3 cell proliferation. Coating time was optimized at a ratio of 4:6 (HA:fp-151-RGD) where MC-3T3 cell proliferation was the greatest. Interestingly, a 2-h coating with the coacervate showed maximal cell proliferation, but there was no improvement with an 8-h coating time (Fig. 3B). Excess coating time apparently inhibits cell adhesion on coacervate coated surfaces.

The effects of the coacervate coating (HA: fp-151-RGD = 4:6) on MC-3T3 cell proliferation were investigated by comparing different treatments of Ti foil. Bare Ti foil, HA coated Ti foil, and fp-151-RGD coated Ti foil were controls (Fig. 4). Two days after seeding with MC-3T3 cells, cell proliferation on the coacervate coated Ti was a little lower than the control group. However, after 4 days, cell proliferation on the coacervate coated Ti was the highest among all samples tested and 5 times higher than the second highest control (fp-151-RGD coated Ti, Fig. 4). To test the reproducibility of MC3T3 cell growth pattern on coacervate composition & coating time, replicate experiments were performed and similar results were obtained. Enhanced cell proliferation might have been caused by a higher mass transfer of target ECM molecules (HA and fp-151-RGD) or the higher hydrophilicity of the coacervate coating. The coacervation thus provides an effective vehicle for delivery of ECM such as HA and fp-151-RGD to the Ti (~50% delivered in 2 h).

3.4. GRGDSP peptide effect on MC-3T3 cell proliferation

To explore whether the RGD peptide (GRGDSP) is necessary for enhancement of MC3T3 cell proliferation, recombinant mussel adhesive protein fp-151[26] without the RGD peptide was coacervated with HA at the ratio of 6:4 and coated onto Ti surfaces for MC-3T3 cell growth. The RGD sequence is well known as cell binding factor and widely used in biomedical research [27,28]. Coatings based on the coacervation of fp-151 without GRGDSP and HA showed lower cell adhesion than bare titanium, negative control and cell spreading on complex coacervate of fp-151/HA was hardly observed (Fig. 5). This result confirms that enhancement of MC-3T3 cell proliferation requires the presence of GRGDSP peptide in the recombinant mussel adhesive protein. The result also suggests that coacervates of HA and recombinant mussel adhesive protein without the RGD peptides HA may have potential as anti-foulants as previously noted [29].

3.5. Cell spreading on coacervates and the effect of DOPA cross-linking on cell proliferation and cell spreading

The spreading of MC-3T3 cells on a coacervate coated surface (HA: fp-151-RGD = 4:6) was observed by FITC-conjugated phalloidin by confocal microscopy. However, cell shapes on coacervate coated surfaces were mainly round (~over 50%), and actin filaments remained dispersed inside of the cells. The relationship of fluidic coacervates to cell shape and motility requires further study. Possible scenarios include that MC-3T3 cells were themselves encapsulated by the coacervate phase or that the cytoskeleton failed to grow in specific

directions due to a uniform distribution of RGD peptides over the entire cell surface. Alternatively, MC-3T3 spreading may be discouraged by the fluidity of the coacervate coating given that soluble RGD peptides can inhibit cell adhesion [30]. Also, it was reported that mechanical forces are fundamental determinants for cell shape [31]. To enable solidification of coacervate of HA and fp-151-RGD, we modified 30% of the tyrosine residues in fp-151-RGD to 3,4-dihydroxyphenyl-L-alanine (DOPA) [17], a cross-linker of mussel adhesive and marine sandcastle worm cement [7,17,32]. After coacervating HA with the DOPA containing fp-151-RGD at a ratio 4:6, the Ti foil was coated with DOPA containing coacervate, and MC-3T3 cells were allowed to proliferate for 4 days as before. Cell spreading judged by actin filament formation visualized by FITC-conjugated phalloidin was better than controls but cell proliferation was not significantly improved vis-à-vis the coacervate coating without DOPA (data not shown). DOPA cross-linking of coacervate helped restore cell spreading but it requires further optimization (e.g. coating time, washing methods, pH, concentration and ratio of each polymers) to achieve better cell proliferation.

Our study provides a basis for the complex coacervation of HA and fp-151-RGD as a useful coating strategy for osteoblast cell proliferation. Solidification of these coacervates by cross-linking (e.g. DOPA) may be necessary to restore cell spreading while maintaining high levels of cell proliferation. Finally, a better understanding of complex coacervates would help to more rationally engineer coacervate coatings with bioactive signals for implants or scaffolds.

4. Conclusions

We have investigated the complex coacervation of HA and a recombinant mussel adhesive fusion protein and demonstrated their potential for endowing implant coatings with improved cell proliferation of preosteoblast, MC-3T3 cells. As coacervates, HA and fp-151-RGD efficiently deposited onto Ti within 2 h. Moreover, the coacervate coating of Ti enhanced hydrophilicity and surface energy, both favorable for MC-3T3 proliferation. Notably, coacervated HA/fp-151-RGD on Ti provides a 5-fold improvement for MC-3T3 cell proliferation over the control group. Combining the complex coacervation of HA and recombinant mussel adhesive protein with GRGDSP peptide represents a promising strategy for the implant or scaffold coatings, especially given the unique surface spreading property of the coacervate phase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi: 10.1016/j.biomaterials.2009.10.041.

Fig. 1.

(A) Isoelectric point detection of HA (filled circle) and fp-151-RGD (Unfilled circle) by surface charge measurement (B) Complex coacervation yield as indicated by turbidity (filled circle) and surface charge by zeta potential measurement (Unfilled circle). Each value and error bar represents the mean of duplicate samples and its standard deviation. (C) Light microscopy image of micro-droplets of HA and fp-151-RGD coacervate. Scale bar is 25 μ m.

Fig. 2.
Coacervated coating of HA and fp-151-RGD on a titanium coated quartz crystal electrode with energy dissipation.

Fig. 3. Optimization of coacervate mixing ratio and coating time (A) HA and fp-151-RGD mixing ratios during coacervate formation and (B) coacervate coating time HA/fp-151-RGD on Ti foil for maximizing MC-3T3 cell proliferation. Cell viability was measured 4 days after seeding MC-3T3 cells. Abbreviations: NC, non-coated. Each value and error bar represents the mean of triplicate samples and its standard deviation.

Fig. 4.

Comparison of the MC-3T3 cell proliferation on bare, HA coated, fp-151-RGD coated, and coacervate (HA/fp-151-RGD) coated Ti foils for 4 days. Black bar, bare Ti foil; Red bar, HA coated Ti foil; Green bar, fp-151-RGD coated Ti foil; Yellow bar, complex coacervate of HA/fp-151-RGD (HA:fp-151-RGD ratio of 4:6 (w/w)) coated Ti foil. Each value and error bar represents the mean of triplicate samples $\pm$ standard deviation.

Fig. 5. Dependence of MC-3T3 cell proliferation on GRGDSP sequence. Abbreviations: NC, non-coated Ti foil; W/O RGD, Ti coated with coacervated HA/fp-151 (HA:fp-151 ratio of 4:6 (w/w)); W/RGD, Ti coated with coacervated HA/fp-151-RGD (HA:fp-151-RGD ratio of 4:6 (w/w)). Cell viability measured 4 days after seeding MC-3T3 cells. Each value and error bar represents the mean of triplicate samples $\pm$ standard deviation.