Synthesis of Hyaluronic acid-Tyramine Microgels for Sustained Protein Release

Elaheh Jooybar *
Chemical Engineering department
Sharif University of Technology
Tehran, Iran
jooybar@che.sharif.edu

Mohammad J. Abdekhodaie
Chemical Engineering department
Sharif University of Technology
Tehran, Iran
abdmj@sharif.edu

Pieter J. Dijkstra
Developmental BioEngineering
University of Twente
Enschede, The Netherlands
p.j.dijkstra@utwente.nl

***Abstract*— Microgels are hydrophilic polymer matrix with high water content suitable for encapsulation and delivery of biomolecules. In this study, hyaluronic acid (HA) microgels were synthesized using a water in oil emulsion method. First, hyaluronic acid was modified with tyramine, and then the microemulsion was produced by homogenizing the polymer solution in isooctane as an oil phase. HA microdroplets were crosslinked via enzymatic method by addition of horseradish peroxidase (enzyme) and hydrogen peroxide, and stable microgels were produced in a mild crosslinking reaction. According to the results, larger microgels were achieved by increasing the initial polymer concentration. Two sample proteins, Bovine serum albumin (BSA) and lysozyme, were incorporated in the polymer network to investigate the encapsulation efficiency of the microgels. The results demonstrated that the proposed method has a high efficiency for protein encapsulation (> 70%). The release profiles showed that lysozyme, as a cationic protein, was released in a sustained manner over a period of two weeks. However, BSA, as a negatively charged protein, showed a faster release rate. The simple method of microgel fabrication, besides the sustained release of the encapsulated proteins, makes the HA microgels a promising vehicle for delivery of cationic proteins.**

Keywords*— *Microgels; Hyaluronic acid; Inverse microemulsion; Enzymatic crosslinking; Protein delivery

I. INTRODUCTION

One of the major issues in the treatment of many diseases is the delivery of the therapeutic proteins, like hormones, enzymes, and antibodies, in a sustained way. Proteins have a short half-lives in the body due to proteolysis so that the bolus injection of the proteins may not work[1]. One strategy to extend the presence of the proteins and slow down their release rate is to incorporate them physically within a polymeric network. Microgels are a crosslinked polymer network in micrometer size with high water content which has been shown to be a suitable platform for delivery of the hydrophilic molecules[2]. It has been shown that microgels have special properties in loading and releasing therapeutic molecules. High surface area, injectability, control over particle size, and structural integrity are important factors which make the microgels an intresting vehicle for delivery purposes[3, 4]. Also, the microgels can be incorporated in the structure of other scaffolds to enhance their potential for tissue regneration purposes by providing a prolong delivery of target bioactive molecules [5, 6].

Both synthetic and natural polymers have been employed for developing microgels and encapsulating biomolecules[7]. Among those, hyaluronic acid (HA), a natural glycosaminoglycan present all over the body, has been widely investigated in biomedical applications because of its excellent biocompatibility, biodegradability, and low immunological risks [8-12].

The microgel network can be formed by physical interactions (e.g. ionic crosslinking) or covalent chemical crosslinking (e.g. free radical reaction). In physical interaction, the networks are not stable like chemical crosslinking. However, one major concern regarding chemical crosslinking is the presence of cytotoxic reagents or exposure to harsh experimental conditions which may affect the bioactivity of encapsulated proteins [13, 14]. Recently, enzymatic crosslinking has been extensively explored in various biomaterial fields [15, 16], tissue engineering [17, 18] and drug delivery [13, 19]. In this method, the network is formed under mild condition (pH=7.4, room temperature) by addition of an enzyme, like peroxidases, and hydrogen peroxide (H_2O_2). The use of enzymatic crosslinking for developing hydrogels for encapsulating cells or proteins was investigated in several studies. The encapsulated cells remained viable and functional and also the encapsulated proteins remained bioactive [1, 20, 21].

Herein, HA-based microgels were prepared by enzymatic crosslinking of tyramine conjugated HA in an inverse microemulsion system. The advantages of the current method of microgel fabrication is using a mild crosslinking condition and facile preparation method. Two model proteins were encapsulated in the microgels during fabrication procedure, and the passive release of the encapsulated proteins was studied during a period of 2 weeks. High loading capacity, sustained release of the encapsulated proteins, and degradability of the microgels make them a promising carrier for delivery of proteins.

II. MATERIALS AND METHOD

A. Materials

Sodium hyaluronate (MW=25 kDa) was purchased from Contipro Pharma, the Czech Republic. Tyramine hydrochloride (TA.HCl) (99%), anhydrous N,N-dimethylformamide (DMF) (99.8%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), hydrogen peroxide

978-1-5386-7952-4/18/$31.00 ©2018 IEEE

(H_2O_2), horseradish peroxidase (HRP), Hyaluronidase from bovine testes (lyophilized powder, 400-1000 units/mg) and MES hemisodium salt dry powder, Tween 80, Span 80, FITC-BSA, and FITC-lysozyme were purchased from Sigma-Aldrich. All other solvents were purchased from Biosolve (Valkenswaard, The Netherlands) and used as received. 2,2,4-Trimethylpentane (isooctane) was purchased from Honeywell.

B. Chemical modification of hyaluronic acid with tyramine

Sodium hyaluronate was dissolved in 30 mL of PBS. EDAC (1.0 g) and NHS (1.3 g) were added and the solution was stirred at room temperature. The product was precipitated in ethanol, filtered, and dried in the fume hood. 1 g of the synthesized polymer was dissolved in 30 ml of 0.1 M MES buffer (pH 6.1). TA.HCl (100 mg) was added to the solution and the pH was maintained constant at 6.1. After overnight stirring, the resulting solution was precipitated in cold ethanol, filtered and washed with ethanol and finally diethyl ether. The resulting white powder was dissolved in MilliQ-water and dialyzed (MWCO 1000) against 100 mM sodium chloride solution for 2 days, a mixture of distilled water and ethanol (5:1) for 1 day and distilled water for 1 day. The hyaluronic acid tyramine (HA-TA) conjugate was obtained after freeze drying. The degree of substitution (DS), defined as the number of substituted groups per 100 disaccharide units, was calculated from ^{1}H NMR (D_2O) spectral data by comparing the integral values of the aromatic protons of tyramine (6.8 and 7.2 ppm) and the methyl protons of HA (1.9 ppm).

C. Hydrogel formation

The HA-TA was dissolved in PBS at concentrations of 5% or 10% w/v. A freshly made HRP and H_2O_2 solution in PBS were added and the mixture was gently mixed to form a gel. In a typical example, to 1ml of HA-TA solution (5% w/v), 20 µL of H_2O_2 (1.5 % v/v) and 10 µL HRP (2 mg/ml) were added. Gelation were determined with the vial tilting method. When the polymer solution does not flow upon inverting the vial was considered as gel state.

D. Microgel fabrication

HA microgels were prepared using water in oil emulsion method. To prepare the microdroplets, first 2 mL of the HA-TA solution contained HRP with a mg HRP/mmol TA ratio of 0.6 was added dropwise to 25 mL of the oil phase (isooctane) contained 2.5% v/v Span 80 as surfactant. The mixture was homogenized on ice using an Ultra-Turrax (T25, IKA Works Inc., USA) at a 13500 rpm speed. Subsequently, 20 µL of a H_2O_2 solution was added to the microemulsion to solidify the droplets. After 30 min homogenization the particles were collected by centrifugation at 8000 rpm for 5 min. Particles were subsequently washed with acetone and ethanol and were dried under reduced pressure at room temperature.

E. Scanning electron microscopy

The morphology of the microgels were visualized using high resolution scanning electron microscopy, with a voltage of 1kV. Dry microgels were placed on a SEM mounting stud and Imaged with Zeiss MERLIN HR-SEM.

F. Protein loading and release

Two model proteins, FITC-lysozyme (14 kDa, p*I*=11.35[22]) and FITC conjugated bovine serum albumin (FITC-BSA, 60 kDa, p*I*=4.7 [23]) were used for encapsulation and release study. The proteins were dissolved in polymer precursor solutions prior to microgel fabrication. The mg protein/mg HA-TA ratio was 1/5 w/w. The HA microgels were prepared as described above.

To determine the amount of proteins loaded in the microgels, the matrix was degraded by incubating them overnight with 1 mL of a hyaluronidase solution (200 U/ml) at 37 °C. The encapsulation efficiency was calculated as the amount of protein loaded to initial amount of protein used * 100. The amount of the proteins was measured using a fluorescent plate reader (excitation: 485 nm; emission: 535 nm, Perkin-Elmer, Victor 3, USA).

The release of the proteins into PBS (pH=7.4) containing 0.05% w/v sodium azide was monitored over a period of two weeks. At selected time intervals, the microgels were collected and centrifuged at 7000 rpm for 10 min. The supernatant was removed and replaced with a fresh medium. Samples were stored at -20°C in dark vials for further analysis. The amount of the proteins was measured using a fluorescent plate reader (excitation: 485 nm; emission: 535 nm, Perkin-Elmer, Victor 3, USA).

III. RESULTS AND DISCUSSION

A. HA-TA synthesis and hydrogel formation

The HA-TA conjugate was synthesized through EDC/NHS chemistry. First, some carboxylic acid groups on the hyaluronic acid backbone were converted to NHS esters. In the second step, the NHS esters were reacted with tyramine to provide the HA-TA. According to the ^{1}H NMR spectral data, the degree of substitution calculated from the integral ratio of the tyramine phenolic protons and the hyaluronic acid methyl protons was 13% (Figure 1).

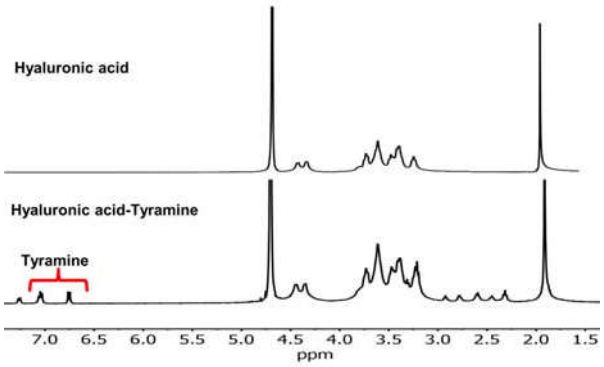

Figure 1. ^{1}H NMR spectra of hyaluronic acid and hyaluronic acid conjugated tyramine in D_2O.

HA-TA hydrogels were prepared by addition of HRP and H_2O_2 to the polymer solutions. HA-TA hydrogel formation by an enzyme-mediated oxidation reaction is schematically presented in Figure 2a. The macroscopic view of the HA-TA hydrogel which was made by 5% w/v HA-TA in PBS is shown in Figure 2b.

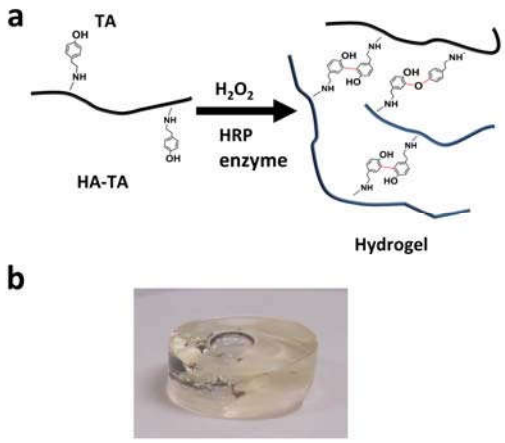

Figure 2. a) Schematic representation of HA-TA hydrogel formation. b) Macroscopic appearance of HA-TA hydrogel.

B. *Microgel size and morphology*

To determine the size of the microgels in the swollen state they were dispersed in water and imaged by fluorescent microscope (EVOS digital microscope, Invitrogen, Netherlands). The microgels were loaded with FITC-lysozyme to be able to be depicted properly. Image J software was used to analyze the size distribution, and the histograms are shown in Figure 3. As it was shown, by decreasing the polymer concentration from 10% to 5% w/v, the microgels average diameter decreased. The reason could be the viscosity of the polymer solution as the viscosity will increase by increasing the polymer concentration and thus less efficient emulsion would be achieved. 5% microgels have a size distribution of 1 to 12 µm, while the size of 10% microgels change from 1 to 50 µm.

SEM micrographs of 5% and 10% microgels were shown in Figure 4. The microgels have a spherical shape with a smooth surface. The size of the dry microgels were measured using SEM images and Image J software. The diameter size of the 5% and 10% microgels in dry state ranged from almost 700 nm to 7 µm and 1.5 µm to 16 µm, respectively.

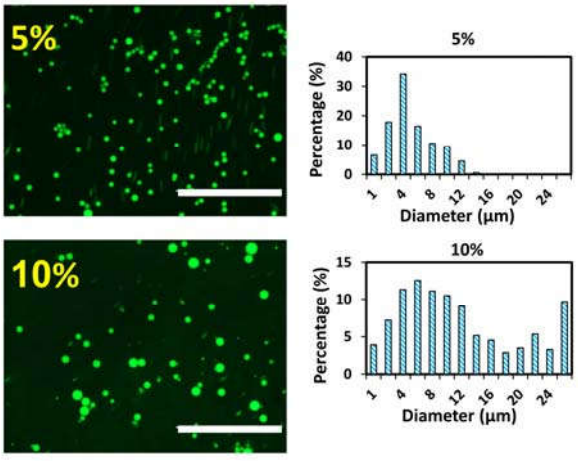

Figure 3. Microscopic images and size distribution of 5% and 10% HA microgels loaded with FITC-lysozyem. Scale bar is 200 µm.

The swelling ratio is expressed as the ratio of the wet weight to the dry weight of the microgels. For 5% and 10% microgels the swelling ratio was almost 25 and 15, respectively. By increasing the polymer concentration, the swelling ratio was decreased which can be attributed to the presence of a denser network in 10% microgels.

C. *In vitro protein release*

The cumulative release profiles of FITC-lysozyme and FITC-BSA from 5% and 10% HA-TA microgels over 14 days were shown in Figure 5. As it was depicted, lysozyme was released from microgels in a sustained manner after an initial burst release. However, the cumulative release of BSA showed that ~90% of the BSA was released within four days. Comparing the release profiles of lysozyme and BSA, it is obvious that lysozyme was released with a slower rate than

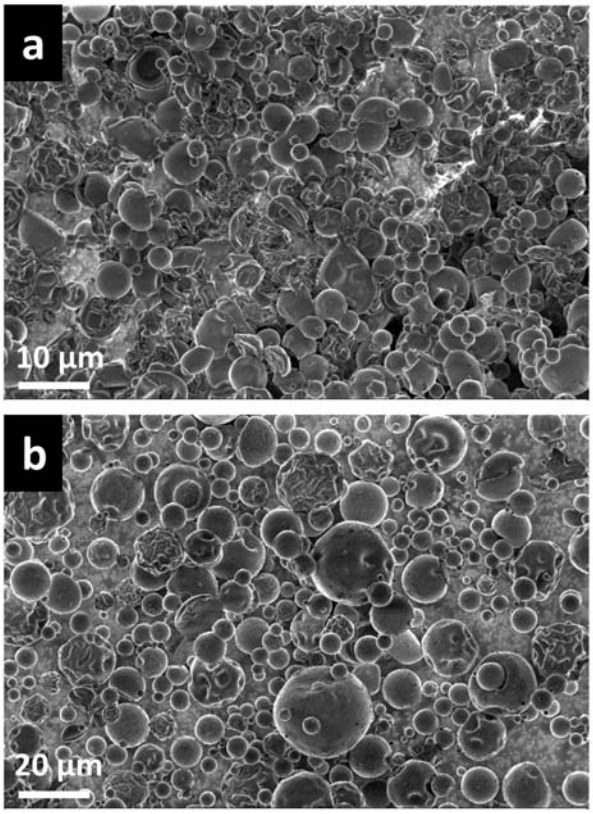

Figure 4. SEM micrographs of dry microgels. a) 5% and b) 10% microgels.

BSA. The reason could be the electrostatic interaction between HA and the proteins. HA is a negatively charge polysaccharide[24], while lysozyme has a positive charge. Therefore, the electrostatic attraction will keep the lysozyme in the microgel structure. However, BSA is a negatively

charged protein and the repulsive force between the molecule and HA resulted in a faster release rate. There is no significant difference between the release profiles of proteins from 5% and 10% microgels.

At the first stages of the release, diffusion is controlling the release rate. After one week, the microgels started to swell which shows the degradation of the polymer network. Figure 6 shows that microgels (10%) swell a lot during incubation at 37 ºC and after two weeks they will completely disappear. Therefore, degradation also plays a role in the release rate of the proteins from the microgels. As it was mentioned, the other important parameter in controlling the rate of release is the electrostatic interaction. Although the size of the lysozyme (MW 14 kDa) is smaller than BSA (MW 60 kDa) and a faster release is expected, we can see a slower and sustained release profile for this protein due to the electrostatic interaction of the lysozyme with the substrate.

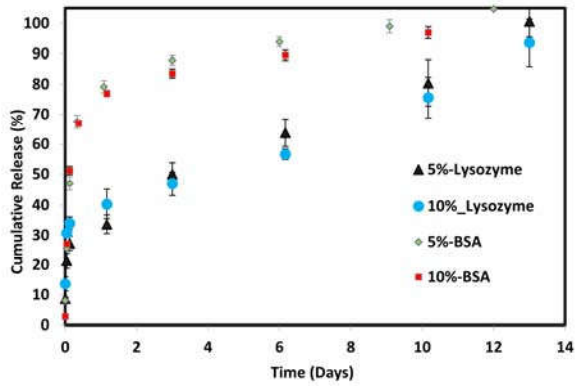

Figure 5. Cumulative release of FITC-lysozyme and FITC-BSA from 5% and 10% microgels.

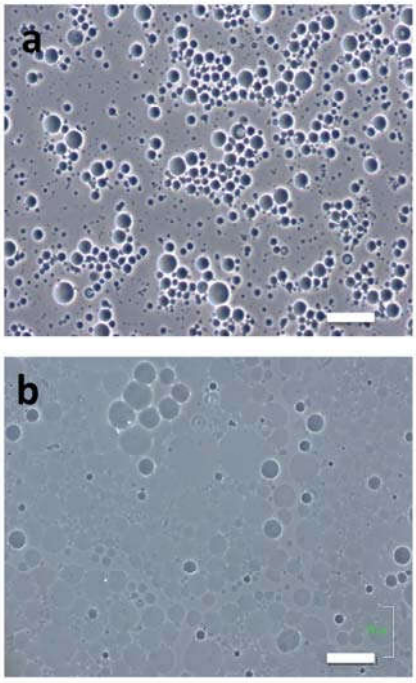

Figure 6. Degradation of the 10% microgels. a) Microgels immediately after fabrication, b) Microgels after two weeks of incubation at 37 ºC. Scale bars are 100 µm.

IV. Conclusions

In this study, hyaluronic acid base microgels were prepared by in situ crosslinking of HA-TA in an inverse microemulsion. The microdroplets were crosslinked enzymatically using HRP and H_2O_2. The average diameter of the microgels decreased by decreasing the initial polymer concentration, and narrower size distribution was achieved for 5% microgels. The microgels exhibited a spherical shape with a smooth surface. BSA and lysozyme were encapsulated in the microgels and the release profiles showed that the negatively charged HA microgels was a good platform for the delivery of cationic proteins like lysozyme. The enzymatic crosslinking is performed under mild condition and thus minimizes damages to the encapsulated proteins. The results demonstrate that the proposed HA-TA microgels have a great potential for protein delivery to be used in disease treatment.

Acknowledgment

This project has received funding from Iranian National Science Foundation (INSF) under grant number 94004082, and the European Union's Horizon 2020 research and innovation program under grant agreement No 691128.

References

[1] F. Lee, J. E. Chung, and M. Kurisawa, "An injectable hyaluronic acid–tyramine hydrogel system for protein delivery," *Journal of Controlled Release,* vol. 134, pp. 186-193, 2009.

[2] H. Bysell, R. Månsson, P. Hansson, and M. Malmsten, "Microgels and microcapsules in peptide and protein drug delivery," *Advanced drug delivery reviews,* vol. 63, pp. 1172-1185, 2011.

[3] A. Pepe, P. Podesva, and G. Simone, "Tunable uptake/release mechanism of protein microgel particles in biomimicking environment," *Scientific Reports,* vol. 7, p. 6014, 2017.

[4] X. Xu, A. K. Jha, R. L. Duncan, and X. Jia, "Heparin-decorated, hyaluronic acid-based hydrogel particles for the controlled release of bone morphogenetic protein 2," *Acta biomaterialia,* vol. 7, pp. 3050-3059, 2011.

[5] D. Sivakumaran, D. Maitland, and T. Hoare, "Injectable microgel-hydrogel composites for prolonged small-molecule drug delivery," *Biomacromolecules,* vol. 12, pp. 4112-4120, 2011.

[6] T. A. Holland, Y. Tabata, and A. G. Mikos, "Dual growth factor delivery from degradable oligo (poly (ethylene glycol) fumarate) hydrogel scaffolds for cartilage tissue engineering," *Journal of Controlled Release,* vol. 101, pp. 111-125, 2005.

[7] V. Sinha and A. Trehan, "Biodegradable microspheres for protein delivery," *Journal of controlled release,* vol. 90, pp. 261-280, 2003.

[8] G. Bhakta, B. Rai, Z. X. Lim, J. H. Hui, G. S. Stein, A. J. van Wijnen, *et al.*, "Hyaluronic acid-based hydrogels functionalized with heparin that support controlled release of bioactive BMP-2," *Biomaterials,* vol. 33, pp. 6113-6122, 2012.

[9] T. A. Debele, S. L. Mekuria, and H.-C. Tsai, "Polysaccharide based nanogels in the drug delivery system: Application as

the carrier of pharmaceutical agents," *Materials Science and Engineering: C,* vol. 68, pp. 964-981, 2016.

[10] X. Jia, Y. Yeo, R. J. Clifton, T. Jiao, D. S. Kohane, J. B. Kobler, *et al.*, "Hyaluronic acid-based microgels and microgel networks for vocal fold regeneration," *Biomacromolecules,* vol. 7, pp. 3336-3344, 2006.

[11] Y. H. Yun, D. J. Goetz, P. Yellen, and W. Chen, "Hyaluronan microspheres for sustained gene delivery and site-specific targeting," *Biomaterials,* vol. 25, pp. 147-157, 2004.

[12] C. Luo, J. Zhao, M. Tu, R. Zeng, and J. Rong, "Hyaluronan microgel as a potential carrier for protein sustained delivery by tailoring the crosslink network," *Materials Science and Engineering: C,* vol. 36, pp. 301-308, 2014.

[13] C. Wu, C. Böttcher, and R. Haag, "Enzymatically crosslinked dendritic polyglycerol nanogels for encapsulation of catalytically active proteins," *Soft Matter,* vol. 11, pp. 972-980, 2015.

[14] A. L. Liu and A. J. García, "Methods for generating hydrogel particles for protein delivery," *Annals of biomedical engineering,* vol. 44, pp. 1946-1958, 2016.

[15] F. Lee, J. E. Chung, and M. Kurisawa, "An injectable enzymatically crosslinked hyaluronic acid–tyramine hydrogel system with independent tuning of mechanical strength and gelation rate," *Soft Matter,* vol. 4, pp. 880-887, 2008.

[16] R. Jin, C. Hiemstra, Z. Zhong, and J. Feijen, "Enzyme-mediated fast in situ formation of hydrogels from dextran–tyramine conjugates," *Biomaterials,* vol. 28, pp. 2791-2800, 2007.

[17] R. Jin, L. M. Teixeira, P. J. Dijkstra, M. Karperien, C. Van Blitterswijk, Z. Zhong, *et al.*, "Injectable chitosan-based hydrogels for cartilage tissue engineering," *Biomaterials,* vol. 30, pp. 2544-2551, 2009.

[18] W. S. Toh, T. C. Lim, M. Kurisawa, and M. Spector, "Modulation of mesenchymal stem cell chondrogenesis in a tunable hyaluronic acid hydrogel microenvironment," *Biomaterials,* vol. 33, pp. 3835-3845, 2012.

[19] B. Y. Kim, J. W. Bae, and K. D. Park, "Enzymatically in situ shell cross-linked micelles composed of 4-arm PPO–PEO and heparin for controlled dual drug delivery," *Journal of controlled release,* vol. 172, pp. 535-540, 2013.

[20] S. Henke, J. Leijten, E. Kemna, M. Neubauer, A. Fery, A. van den Berg, *et al.*, "Enzymatic Crosslinking of Polymer Conjugates is Superior over Ionic or UV Crosslinking for the On‐Chip Production of Cell‐Laden Microgels," *Macromolecular bioscience,* vol. 16, pp. 1524-1532, 2016.

[21] T. Kamperman, S. Henke, B. Zoetebier, N. Ruiterkamp, R. Wang, B. Pouran, *et al.*, "Nanoemulsion-induced enzymatic crosslinking of tyramine-functionalized polymer droplets," *Journal of Materials Chemistry B,* vol. 5, pp. 4835-4844, 2017.

[22] D. E. Kuehner, J. Engmann, F. Fergg, M. Wernick, H. W. Blanch, and J. M. Prausnitz, "Lysozyme net charge and ion binding in concentrated aqueous electrolyte solutions," *The Journal of Physical Chemistry B,* vol. 103, pp. 1368-1374, 1999.

[23] S. Ge, K. Kojio, A. Takahara, and T. Kajiyama, "Bovine serum albumin adsorption onto immobilized organotrichlorosilane surface: influence of the phase separation on protein adsorption patterns," *Journal of biomaterials science. Polymer edition,* vol. 9, p. 131, 1998.

[24] M. Kong and H. J. Park, "Stability investigation of hyaluronic acid based nanoemulsion and its potential as transdermal carrier," *Carbohydrate polymers,* vol. 83, pp. 1303-1310, 2011.