Bull. Pharm. Sci., Assiut University, Vol. 45, Issue 1, 2022, pp. 53-62

Bulletin of Pharmaceutical Sciences
Assiut University
Website: http://bpsa.journals.ekb.eg/
e-mail: bullpharm@aun.edu.eg

CHITOSAN BASED POLYELECTROLYTE COMPLEX NANOPARTICLES: PREPARATION AND CHARACTERIZATION

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Recently, the use of biodegradable polymers for specialized applications such as controlled release of drug formulations gained considerable attention. Biodegradable nanoparticles have been widely utilized in drug delivery systems. In this work polyelectrolyte complexation was used to prepare chitosan nanoparticles. The preparation process and major parameters were discussed and optimized. The nanoparticles were prepared by the polyelectrolyte complexation of chitosan (CS) with hyaluronate (HA). Studies were performed to identify effect of chitosan and hyaluronate molecular weight, chitosan: hyaluronate mass ratio and the effects of the aqueous chitosan solution pH on the particle size of nanoparticles. Nanoparticles were characterized by particle size analysis, polydispersity index, zeta potential and transmission electron microscope (TEM). Results showed that nanoparticles could be prepared only within an optimal zone of appropriate chitosan and hyaluronate molecular weights and CS:HA mass ratio. Increasing the hyaluronate molecular weight from low to high led to a significant enlargement in the particle size of the prepared nanoparticles with different chitosan molecular weights. Besides, we found that increasing the hyaluronate content at mass ratio 1:2 of CS:HA led to precipitation of nanoparticles . The particle sizes of the chitosan/HA nanoparticles significantly decreased with the increase of chitosan solution pH. The spherical shaped chitosan/HA nanoparticles were confirmed by TEM. The study showed that systematic design and modulation of the particle size of chitosan/HA polyelectrolyte complexes can be readily achieved with right control of critical processing parameters.

Keywords: *Hyaluronate; Chitosan; Polyelectrolyte complex nanoparticles.*

INTRODUCTION

In the last few years, several works has been created on particulate drug delivery systems. Microparticles and nanoparticles, as particulate systems, have been extensively utilized to deliver wide variety of active pharmaceutical ingredients (APIs) to promote their pharmacokinetic and pharmacodynamic actions in the body[1]**.** Biodegradable polymers whichever natural or synthetic are usually degraded into biocompatible byproducts via enzyme catalysis or chemical hydrolysis, hence they can be implanted into the body with no need of consequent removal by surgical operation[2]**.** These polymers can be used to provide a controlled release pattern, thus the drug concentration within the target site is kept within the therapeutic window. Therefore, the encapsulation of drugs in biodegradable particles guards the drug from harmful conditions and sustain the release of the drug for the desired period; the administration frequency is thus reduced, and patient comfort and compliance are enhanced[3]**.**

Polyelectrolyte complexes (PECs) are widely investigated in pharmaceutical and medical fields as a drug delivery system. It is usually used in systemic and local applications and additionally it can be used for tissue

Received in 11/1/2022 & Accepted in 7/2/2022

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engineering and regeneration[4]. PECs are formed when electrostatic interaction takes place between two oppositely charged electrolytes when mixed in solutions [5]. The PECs has some particularly attractive advantages, particularly as carrier systems for drugs or gene delivery[6] such as: ease of preparation, no use of organic solvents during fabrication or chemical cross-linking agents and the reduction of toxicity and adverse side effects[7].

Several factors affect the structure and stability of PECs development; these include: the ionization degree of polyions and their charge density and charge distribution on the polymer chains, mass ratio, mixing order, polyelectrolytes molecular weights, interaction time, temperature, ionic strength polyelectrolytes concentration, and the pH of the medium[8].

Chitosan (CS) is a linear amino polysaccharide containing glucosamine and N-acetylglucosamine [9]. As a cationic polymer, chitosan can interact with negatively charged polymers forming the chitosan based PEC nanoparticles (PENPs). Because of its biocompatibility, biodegradability and nontoxicity properties, chitosan has been widely used [10]. Besides, it is agreeable to chemical modifications that can potentiate some of its properties for certain applications due to the existence of free –OH and –NH2 groups in its structure. These incredible physical, chemical, and biological properties have made CS as an excellent candidate for applications in cosmetics, food industry, medicine and pharmacy[11].

Chitosan-based PEC can be established to different forms, including hydrogels/beads[12], fibrous membranes/films[13], micro/nanoparticles[14]. Chitosan-based PEC has been proven to have various uses in pharmaceutical and biomedical areas, for instance drug delivery for nutrients with controlled release and delayed digestibility[15], gene delivery system for nonviral vector[16], three-dimensional scaffold to mimic tumor microenvironment[17] and engineering of bone tissues[18].

Hyaluronic acid (HA), also identified as hyaluronan, has a linear structure with repeating disaccharide units bound by ß-1,4-glycosidic bonds as its primary structure [19]. Each disaccharide is made up of ß 1,3-glycosidic bonds connecting N-acetyl-D-glucosamine and D-glucuronic acid[20]. HA is found in all the soft tissues of the body especially in higher concentrations in the vitreous humor of the eye and synovial fluid. HA has remarkable physicochemical properties like biodegradability and biocompatibility, non-immunogenic behavior, non-toxicity and non-inflammatory[20&21]. HA is a familiar material in varies medical applications, as eye surgery, visco-supplementation and drug delivery[22]. Many essential functions of HA include cell signaling and wound healing/tissue regeneration[23], assistant device in ophthalmic surgeries[24], treatment of joint disease as a lubricant and/or shock-absorbing fluid, treatment of bone and skin inflammatory diseases[25]. Because of their biological and physicochemical properties, as well as their safety profile, native HA and many of its derivatives are important biomaterials for a variety of medical, medicinal, food, and cosmetic applications[23]. Owing to the previously mentioned properties of both of these biopolymers, CS:HA polyelectrolyte complex nanoparticles(PENPs) were fabricated in this work.

The goal of this study was to create a drug delivery system by employing PECs of chitosan and hyaluronic acid, two polysaccharides of the family of glycosaminoglycans. The prepared particles provide the opportunity to formulate carriers with tunable geometries, and generally with good capacity of active ingredients encapsulation. An added advantage for the CS:HA systems is the complete hydrophilic environment and the mild preparative conditions[26]. Indeed, the avoidance of high shear forces or organic solvents makes easier encapsulation of labile drugs[27]. Alterations were made to chitosan concentration and its molecular weight, chitosan to HA mass ratio, chitosan solution pH and HA molecular weight to find out the optimal conditions to produce chitosan based PENPs. The design of the screening experiments was done based on sequential stepwise study by changing one variable while keeping all the others constant. The obtained particles were assessed for their particle size, polydispersity index, zeta potential, solution pH and transmission electron microscopy.

MATERIALS AND METHODS

Materials

Chitosan (CS) low molecular weight (viscosity of 500 cps, and > 95% deacetylated) and medium molecular weight (viscosity of 200000 cps, and 75-85% deacetylated) were purchased from Primex®, Siglufjordur, Iceland. Hyaluronic acid (HA) low molecular weight (0.5-1 MDa) and high molecular weight (1.8 MDa) were purchased from Bloomage Freda Biopharm Co., Ltd, China. Potassium bromide (KBr), sodium hydroxide and acetic acid were attained from El Nasr Pharmaceutical Chemical Co. Egypt.

Preparation of the plain chitosan/hyaluronate polyelectrolyte complex nanoparticles (PENPs)

The plain PENPs were prepared by physically mixing chitosan (containing positively charged amino groups) and hyaluronic acid (containing negatively charged carboxylic group) solutions[28]**.** Chitosan solutions of different molecular weights (low and medium) (0.1%, w/v) and solutions of different molecular weights of hyaluronate (low and high) (0.1%, w/v) were prepared in 0.1% acetic acid and distilled water respectively. Briefly, aqueous solutions of HA were added slowly to CS solution under magnetic stirring at 450-500 rpm at room temperature for 30 min.

Effect of hyaluronate molecular weight on the particle size of the plain PENPs with different chitosan molecular weight

Chitosan-hyaluronate PENPs were prepared using different molecular weights (low and high) of HA at specific CS and HA concentration (0.1%, w/v), keeping other factors constant viz. CS:HA mass ratio (2:1), chitosan solution pH (5.0).

Effect of different CS: HA mass ratio on the particle size of the plain PENPs

Different CS:HA mass ratio (1:2, 2:1 and 4:1) at specific concentrations of chitosan and hyaluronate (0.1%, w/v) were used for the preparation of the plain CS-HA PENPs with different molecular weight of hyaluronate (low and high). Chitosan solution pH was kept at (5.0) and chitosan with low molecular weight was used for this study.

Effect of different chitosan solution pH on the particle size of the plain PENPs

Two different pH values of the chitosan solution (4.5 and 5.0) were tested using different molecular weight of hyaluronate (low and high) at specific concentrations of chitosan and hyaluronate (0.1%, w/v) and CS:HA mass ratio of 1:2.

Characterization of the chitosan-hyaluronate polyelectrolyte complex nanoparticles (PENPs)

Determination of the particle size (PS), polydispersity index (PDI) and zeta potential (ZP)

The mean PS, PDI and ZP of the plain PENPs formulations were measured after dilution with deionized water at 25 °C applying a Zetasizer Nano Instrument (ZS, ZEN3600, Malvern Instruments, UK) with photon correlation spectroscopy[29]

Determination of pH

The pH meter was used to measure the pH values of the plain PENPs formulations. All measurements were performed in triplicates of different batches[30]**.**

Determination of the morphology of PENPs using transmission electron microscope (TEM)

Transmission electron microscopy (Model JEM- 100S, Joel, Tokyo, Japan) was utilized to examine the morphology of the PENPs [31]**.** A droplet of 0.1% (v/v) washed nanoparticles dispersion was deposited on to copper 300-mesh grid and stained with 2% phosphotungstic acid. Excess solution was carefully removed via using filter paper and air-dried at room temperature for imaging[32]**.**

Statistical analysis

All findings were recorded as mean ± standard deviation. Study of one-way variance followed by a post-hoc test (Tukey 's test) using GraphPad® Instat software version 3.06 (GraphPad Software, Inc., La Jolla, CA) was carried out for multiple comparison (n= 3), and statistical significance was described as $P< 0.05$[33]**.**

RESULTS AND DISCUSSION

Preparation of the plain chitosan/hyaluronate polyelectrolyte complex nanoparticles (PENPs)

Polyelectrolyte complexes (PECs) are produced when a polycation and a polyanion are mixed together in an aqueous solution. In this study, PECs were prepared by the interaction between the ionized amino groups of chitosan ($NH3^{+}$) and the ionized carboxylic acid groups (COO^{-}) of hyaluronate. The objective of this work was to investigate whether it is possible to integrate different molecular weight of hyaluronate (high and low m.wt.) into chitosan (CS) to produce the polyelectrolyte complex nanoparticles (PENPs), as well as to investigates how different molecular weight of HA affect the particle size of PENPs with different chitosan molecular weights (low and medium), CS:HA mass ratios (1:2, 2:1 and 4:1) and different CS solutions pH (4.5 and 5.0) (Table 1).

Table 1: Investigated variables in the screening study for the preparation of plain CS-HA PENPs.

Variable	Variable Name
Molecular weight of hyaluronate	Low
	High
pH of chitosan solution	4.5
	5
Molecular weight of chitosan	Low
	Medium
CS and HA Mass Ratio	1:2
	2:1
	4:1

- All experiments were done at room temperature.

Characterization of the plain polyelectrolyte complex nanoparticles (PENPs)

Determination of the particle size (PS), polydispersity index (PDI) and zeta potential (ZP)

Effect of hyaluronate molecular weight on the particle size of the plain PENPs with different chitosan molecular weights

As shown in Table (2), increasing the hyaluronate molecular weight from low to high led to a significant increase (p-value < 0.05) in the particle size of the plain PENPs at all the studied chitosan molecular weights. These results were in accordance with previous reports[34]**.** The capability of a polycation-polyanion pair to produce stable PECs is affected by the polymer chain flexibility depending on the relative molecular weights [35]**.** This may be due to the fact that high molecular weight polymers could not disentangle sufficiently enough for electrostatic attraction between different particles, this leads to aggregate formation and larger particle sizes[36]**.** Also the spatial distribution of hyaluronan molecules became bigger at high molecular weight, leading to, larger particle size formation**.** Together with the fact that using hyaluronate and chitosan of medium or high molecular weights led to viscous solution of both polymers that may impair the mixing process and favor aggregate formation[37]**.**

The polydispersity index (PDI) value of the PENPs ranged from 0.33 ± 0.02 to 0.47 ± 0.3. It was noted that, the plain PENPs prepared with low hyaluronate molecular weight resulted in particles with low PDI than prepared with high hyaluronate molecular weight which might be ascribed to the small particle size range, where low value of PDI, mean high uniformity between the nanoparticles.

The values of zeta potential for the plain PENPs were in the range of 6.93 ± 0.44 to 21.8 ± 2.2 mV. The zeta potential values were, in all cases, positive showing the prevalence of the polycationic nature of chitosan on the surface composition of the system[38]**.** Increasing the molecular weight of HA led to a significant decrease in the values of ZP of PENPs which might be elucidated by reduction of the surface area carrying charge as particle size increased. The low-zeta-potential nanoparticles can produce aggregates and precipitates through low electrostatic repulsion[39]**.** Also, the differences in the zeta potential values by changing the molecular weight of hyaluronate can be elucidated by the limited ability of the polymer chains to mix effectively due to high viscosity, thus producing large entities having different arrangement of polymers on the surface[36] suggesting, the unsuitability of polymers with high molecular weights for the manufacture of stable nanoparticles.

Table 2: Effect of hyaluronate molecular weight on the particle size of the plain PENPs with different chitosan molecular weights

HA M (w)	CS M (w)	PS ± S.D. (nm)	PDI ± S.D.	ZP ± S.D. (mV)	pH of Formulae ± S.D.
Low	Low	190.7 ± 13.05	0.33 ± 0.02	20.7 ± 0.75	4.89 ± 0.001
	Medium	227.2 ± 20.3	0.35 ± 0.01	21.8 ± 2.2	5.3 ± 0.002
High	Low	536 ± 18	0.45 ± 0.25	18.6 ± 0.55	5.2 ± 0.0
	Medium	589.5 ± 30.7	0.47 ± 0.3	6.93 ± 0.44	5.19 ± 0.0

- All formulae contain 2:1 (CS:HA), chitosan solution pH of (5.0) and 0.1% CS and HA

Effect of different CS:HA mass ratio on the particle size of the plain PENPs

Distinct zones for the PENPs formation, phase separation and aggregation were also examined visually based on different tested CS:HA mass ratios using different hyaluronate molecular weights.

The results in Table (3) showed that there is a positive correlation between increasing the chitosan amount relative to hyaluronate and the particle size of the prepared PENP's at all the studied hyaluronate molecular weights. Increasing the HA content at mass ratio of CS:HA (1:2) led to precipitation of PENPs. This may be explained by the fact that the number of negative carboxylic groups of HA are approximately equal to number of positive amino groups of CS at 1:2 mass ratio of CS:HA thus, the net charge of PENPs was close to 0 resulting in formation of unstable particles since one mole of HA contains 2.6 micromoles of the HA monomer or carboxylic groups whereas one mole of CS contains 5.6 micromoles of CS monomer (degree of deacetylation (90%) was considered) or amine groups[25&28].

Generally, zeta potential results revealed that decreasing the charge of PENPs takes place by increasing HA content as in 2:1 relative to 4:1 CS:HA mass ratios with values of 20.7 ± 0.75 and 24.7 ± 1.36 mV respectively.

Effect of different chitosan solution pH on the particle size of the plain PENPs

The solution pH controls the ionization of amino groups of CS and carboxylic acid groups of HA, which might influence the particle size, zeta potential, and the development of PENPs. Below the pKa of chitosan (6.5), the amino groups are protonated with a positive charge. Also, the pKa of HA is close to 2.6, where pH elevation leads to a more negative charge of carboxylic acid[40]**.** The pH of the CS solution was adjusted with dilute acetic acid solution at pH 4.5 and 5.0, then the HA solution was added into the CS solution to form PENPs. Table (4) illustrate a significant ($P< 0.05$) decrease in the particle size with the increase of chitosan solution pH (from 4.5 to 5.0) which might be due to the reduction of the charge density of HA on decreasing the pH, hence, less interaction happens between the two polymers, resulting in formation of PENPs with large particle size [41]**.**

As can be seen from the results, the polydispersity index (PDI) values of the plain PENPs ranged from 0.39 ± 0.03 to 0.7 ± 0.14. The same results were noted as the plain PENPs prepared with low hyaluronate molecular weight resulted in particles with low PDI than prepared with high hyaluronate M (w) at different chitosan solution pH.

It was found that the PENPs had positive zeta potential values which were in the range of 18.6 ± .55 to 25.1 ± 0.32 mV. Similarly as stated before, increasing the molecular weight of HA led to a significant decrease in the values of ZP of the PENPs with different chitosan solution pH. Also, as presented in the Table (4), there is a significant reduction of zeta potential with pH 5 because when the pH increased, the positive amino groups decreased, and the negative carboxylic group increased.

Table 3: Effect of different CS:HA mass ratio on the particle size of the plain PENPs.

HA M (w)	CS:HA mass ratio	PS ± S.D. (nm)	PDI ± S.D.	ZP ± S.D. (mV)	pH of Formulae ± S.D.
Low	4:1	219.3 ± 41.6	0.39 ± 0.03	24.7 ± 1.36	4.91 ± 0.003
	2:1	190.7 ± 13.05	0.33 ± 0.02	20.7 ± 0.75	4.89 ± 0.001
	1:2	Precipitation	-	-	-
High	4:1	1545 ± 98	0.56 ± 0.22	29.1 ± 4.1	5.14 ± 0.0
	2:1	536 ± 18	0.45 ± 0.25	18.6 ± 0.55	5.2 ± 0.0
	1:2	Precipitation	-	-	-

- All formulae contain low M (w) of CS, chitosan solution pH of (5.0) and 0.1% CS and HA

Table 4: Effect of different chitosan solution pH on the particle size of the plain PENPs.

HA M (w)	CS solution pH	PS ± S.D. (nm)	PDI ± S.D.	ZP ± S.D. (mV)	pH of Formulae± S.D.
Low	4.5	440.3 ± 41.5	0.6 ± 0.13	25.1 ± 0.32	4.55 ± 0.0
	5.0	190.7 ± 13.05	0.33 ± 0.02	20.7 ± 0.75	4.89 ± 0.001
High	4.5	659.2 ± 71	0.7 ± 0.14	22.8 ± 1.4	4.7 ± 0.0
	5.0	536 ± 18	0.45 ± 0.25	18.6 ± 0.55	5.2 ± 0.0

- All formulae contain low M (w) of CS, 2:1 (CS: HA) and 0.1% CS and HA.

Determination of pH

The pH values of the PENPs formulae in all our studies were ranging from 4.55 ± 0.0 to 5.3 ± 0.002 which is considered appropriate for most of drug delivery systems.

Transmission electron microscope (TEM)

The morphology of the CS:HA PENPs developed by the optimal preparation conditions was observed by the TEM. Results shown in (Figure 1) reveal spherical particles but in some cases, deformation can be observed. The observed sizes were smaller than those obtained by the dynamic light scattering results due to the drying process before TEM imaging while the dynamic light scattering reflected the hydrodynamic diameter of the PENPs[34].

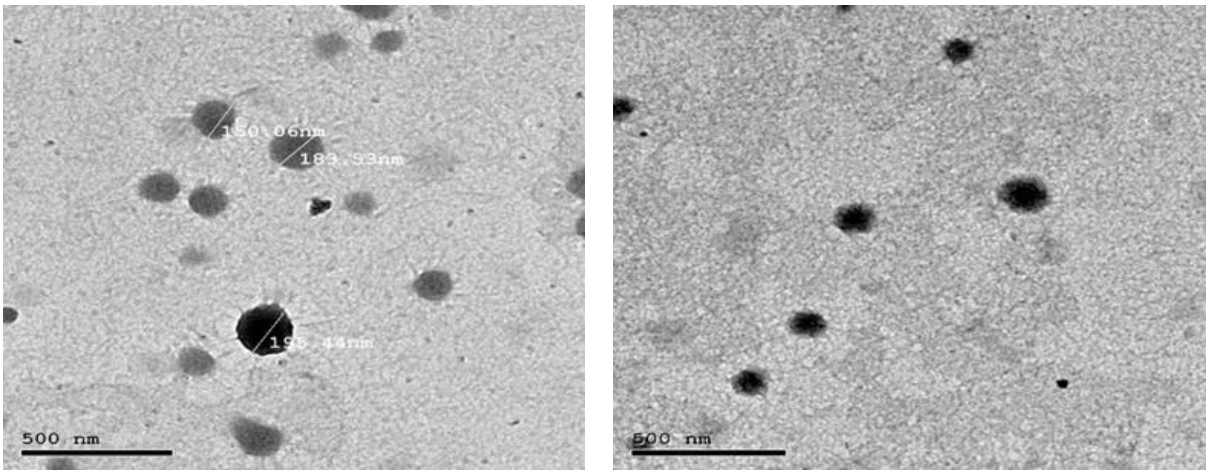

Fig. 1: TEM of the plain PENPS containing low m.wt. of HA and medium m.wt. of CS, 2:1 (CS:HA) and chitosan solution pH of (5.0

CONCLUSION

PECs, being biodegradable, stable and biocompatible formulations, are able to combine the unique properties of different polymers without losing their inherent characteristics and widely investigated in pharmaceutical and medical fields as a drug delivery system. It is usually used in systemic and local applications and additionally it can be used for tissue engineering and regeneration. Hyaluronate molecular weights (low and high), CS:HA mass ratio and pH value of the solution were the prominent parameters significantly affecting the particle size of the plain PENPs. Results revealed that formulae prepared with low molecular weight hyaluronate are smaller in size compared to those prepared with high molecular weight hyaluronate with different CS:HA mass ratios and chitosan solutions pH. Consequently, low molecular weight hyaluronate is more suitable for drug delivery systems than high molecular weight hyaluronate and can be used for various medical applications such as skin disease, ocular surgery, etc. Therefore, future studies will be carried out using low molecular weight hyaluronate to prepare PENPs suitable for topical and intra-articular drug delivery systems and considered as viable and non-toxic carriers for formulating bioactive compounds due to their favorable physicochemical and biopharmaceutical properties.

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Bull. Pharm. Sci., Assiut University, Vol. 45, Issue 1, 2022, pp.53-62.

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جامعة أسيوط

BULL. PHARM. SCI. Assiut Univ.

الجسيمات النانومترية المتعددة الإلكتروليت القائمة على الكيتوزان: التحضير والتوصيف التقييم

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في الآونة الأخيرة ، اكتسب استخدام البوليمرات القابلة للتحلل الحيوي لتحضيرالجسيمات النانومترية اهتمامًا كبيرًا.لذلك كان الهدف من هذه الدراسة هو تحضير الجسيمات النانومترية عن طريق خلط الكيتوزان وحمض الهيالورونات مباشرة وتم تشكيلها من خلال التفاعل بين المجموعة الأمينية موجبة الشحنة للكيتوزان ومجموعة الكربوكسيلات سالبة الشحنة لحمض الهيالورونات. تم ايضا دراسة تأثير الوزن الجزيئي للكيتوزان والهيالورونات ، نسبة كتلة الشيتوزان:الهيالورونات وتأثيرات الأس الهيدروجيني لمحلول الشيتوزان المائي على حجم الجسيمات، معامل التشتت المتعدد ، فرق جهد والمجهر الإلكتروني الماسح. أظهرت النتائج أنه لا يمكن تحضير الجسيمات النانومترية إلا باستخدام أوزان جزيئية مناسبة من الكيتوزان والهيالورونات وبنسب معينة من كتلة الشيتوزان:الهيالورونات. وقد أدت زيادة الوزن الجزيئي للهيالورونات من منخفض إلى مرتفع إلى زيادة كبيرة في حجم الجسيمات النانومترية المحضرة بأوزان جزيئية مختلفة من الكيتوزان. إلى جانب ذلك ، وجدنا أن زيادة محتوى الهيالورونات بنسبة كتلة ١:٢ أدت إلى ترسيب الجسيمات النانومترية. كما انخفضت أحجام الجسيمات النانومترية بشكل ملحوظ مع زيادة الرقم الهيدروجيني لمحلول الشيتوزان. ايضا تم التأكد ان الجسيمات النانومترية للكيتوزان وحمض الهيالورونات كروية الشكل بواسطة المجهر الإلكتروني الماسح. أوضحت هذة الدراسة أن التصميم المنهجي والتعديل لحجم الجسيمات للجسيمات النانومترية متعددة الإلكتروليت يمكن تحقيقه بسهولة من خلال الاختيار المناسب للمعاملات المختلفة.