Electro-microgelation of CS/PL spheres self-embedded with in situ AgNPs formation as a losartan delivery system

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Posted Date: December 1st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2271891/v1

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Additional Declarations: No competing interests reported.
Title
Electro-microgellation of CS/PL spheres self-embedded with in situ AgNPs formation as a losartan delivery system

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Highlights
- New hybrid colloidal microgel particles were electrochemically synthesized (Es AgNPs@CS/PL microgel)
- The key factors influence the morphology and size of the prepared microgel particles and the AgNPs were investigated.
- Effect of in situ formed AgNPs incorporation within the matrix on morphology and size of the chitosan/pullulan microgel particles.
- Examination of the in-situ incorporation of Losartan as a drug model within Es AgNPs@CS/PL microgel particles as a carrier vehicle.

Abstract:
Given rise to a green chemistry, in a single step, electro-microgelation of chitosan (CS)-pullulan (PL) hybrid system self-embedded with in situ formed silver nanoparticles (AgNPs) avoiding the presence of reducing and/or crosslinking agent was prepared and introduced as a drug carrier system of losartan as a model drug. The formation, morphology, size distribution, compositional and structural features of the microgel and AgNPs have been characterized by transmission electron microscopy (TEM), UV–visible measurements (UV–vis). The compositional and structural features of the microgels were revealed by Fourier transform infrared spectroscopy (FTIR), induced coupled plasma (ICP) and Zeta potential analysis. Losartan (LOS) entrapment within the microgel network possibility was examined using UV–Vis and TEM measurements. The results revealed that a uniform AgNPs with average narrow size distribution 5.4 nm and border to border interparticle distance 1.22 nm successfully self-embedded within in situ electrosynthesised chitosan-pullulan hybrid microgels of a dual size distribution (151.4 and 43.5 nm) with a positive surface charge +25 ± 5.18 mV guarantee the electrostatic repulsion between the particles. Besides, the incorporation of AgNPs during the electrosynthesis process played a crucial role in the morphology transition from pearl-necklace to microsphere structure. Furthermore, stability examination of the colloidal microgel particles introduced that the single particle contains Ag⁺ ions undergo a slow reduction rate in consistency with a nucleation favored over growth. On the other hand, the obtained microgel particles have a good antibacterial activity against Gram-positive and Gram-negative bacteria. Investigation of the ability of the losartan incorporation
inside the microgel particles during the microgel preparation explored that a stable core/shell like structure (core: losartan and shell: AgNPs@CS/PL) was effectively formed with average size 95 nm and zeta potential +39 mV promoting the particles stability.

**Keywords:**
Electrosynthesis, Microgel, AgNPs, Chitosan, Pullulan, Losartan and drug delivery.

1. Introduction

Recently, integration of colloid with gel features in polymeric microparticles with unique properties called microgels, in range of hundreds of nanometers to some micrometers has attracted much interest as a possible drug delivery vehicle. The dispersed colloidal microgel established due to the polymer chains intramolecularly crosslinked. Microgel particles represent fascinating aspects such as colloidal and structural stability in addition to swelling in response to external perturbation (solvency, temperature and pH...etc) for example, in aqueous system, the internal network of the hydrophilic polymer microgel particles can swell in response to an external stimulus. Particles possess of such characteristics have been receiving a considerable attention and became promising architecture to innumerable applications. These three dimensional soft, water swollen microgel networks are promising matrices to encapsulate and controlled delivery of one or more active drugs, where the microgels structure exhibits a favorable environment for the incorporation thanks to the presence of various functional groups. So, the payload has binding affinity for the polymer matrix.

Chitosan is a polysaccharide copolymer widely studied among biopolymer-based microgels. These microparticles have been utilized in drug delivery systems due to their natural abundant, nontoxicity, biodegradability, bioabsorbable, easy to tune and low cost and also shows antimicrobial activity. Besides, chitosan is comprising various functional groups attached to the chain’s backbone such as amino, hydroxyl, acetamide and can easily be functionalized with another different groups to promote the affinity of the chitosan matrix to the encapsulated drugs.

Despite the remarkable breakthroughs, at neutral pH, chitosan has low solubility nature which limits its application in the drug delivery field. The combination or modification of both hydrophilic and hydrophobic segments of chitosan considered as one of the magical strategies for the solubility enhancement of chitosan and facilitate the microgel particles formation moreover the drug encapsulation efficacy. herein, a microbial hydrophilic homopolysaccharide; pullulan was introduced to form synergy microgel network with chitosan. pullulan is, safe, biocompatible, biodegradable, non-toxic biopolymer with many potential applications in different fields including drug delivery, food packaging. So, based on the aforementioned background, the integration of pullulan and chitosan as a microscale gel is imagined to be a suitable solution as drug carrier vehicle using their particular advantages. Regarding to the microgel’s preparation methods, there are several routes to prepare, control size distribution, colloid stability of microscale hydrogel particles. Essentially, microgel preparation routes can be classified into homogeneous nucleation and polymerization, emulsification (emulsion polymerization, inverse microemulsion polymerization, membrane emulsification and heterogeneous controlled/living radical polymerization), polymer complexation,
radiation, or physical-based methods for microgel fabrication (photolithographic, micromolding, or microfluidic and droplet formation) methods\(^1\). Despite the remarkable advantages of these routes, there are drawbacks that didn’t fit well for the biomedical and the pharmaceutical applications including but not limited to chemical additives as initiators that cannot be recycled, unavoidable removal of the surfactant after the end of the reaction, certain polymerization methods complete at elevated temperatures which in turn is unsuitable for incorporating thermally unstable drugs\(^1\). Also, the expensive radiochemical synthesis could lead to degradation of the polymers into fragments\(^9\).

Herein, the suggested method to prepare micro-hybrid gel of chitosan and pullulan biopolymers based on the polymer complexation is the electrochemical method. The electrochemical method is an alternative in the formation of microgel colloidal particles due to simple equipment, no need to elevated temperatures, easy to adjust the process conditions, cost-effective, controllable products obtained, and avoiding long reaction time. In addition, the morphology of the prepared particles can be easily tuned and controlled by adjusting the applied potential. Besides, using electrosynthesis route is eco-friendly as there is no need to use toxic crosslinker agents\(^4\).

In this work, a facile and easy approach is introduced to develop efficient hybrid biopolymer based microgel colloidal particles. We report an electrochemical approach for the synthesis of polymer microgels. Under stirring at room temperature, electrochemical synthesis facilitated the microparticles formation in aqueous chitosan/pullulan solution in-situ during the Ag\(^+\) ions release using sacrificial silver anode. By applying an appropriate constant electrical potential on the electrolytic aqueous solution, the chain’s segments of the used polymers have the chance to crosslink by the help of long-lived in situ AgNPs formation without using any additives, that could be limit the probable future pharmaceutical applications of the prepared microgel. Losartan was used as a model drug and its affinity to incorporate into the microgel carrier as a payload was examined. Losartan potassium is orally active nonpeptide angiotensin II receptor antagonist used for the treatment of hypertension\(^10\). LOS has disadvantage like short half-life and side effects. So, there is an urgent need to introduce suitable strategies able to compromise these problematic issues and promote LOS therapeutic efficacy\(^11\).

2. Experimental

2.1. Materials

Pullulan was purchased from Alfa Aesar Karlsruhe (Germany), Chitosan (MW 100 to 300 K Da deacetylation degree 85%) obtained from Acros Organics New Jersey (USA), Losartan (LOS) (> 99.69%), was a generously gifted by Pharco Co. for Pharmaceutical Industries, Alexandria, Egypt. Anhydrous citric acid was bought from El Naser Pharmaceutical Chemical (Egypt). Platinum (Pt) and Silver (Ag) rectangular sheet (> 99.9%, Sigma Aldrich, St. Louis, MO, USA) and deionized water (resistivity > 2 × 10\(^8\) Ω cm). All reagents were of analytical grade and used as received.

2.2. Electrosynthesis of CS/PL (Es CS/PL) solution

Under the effect of electric potential, Chitosan/pullulan solution behavior was examined using the electrochemical method. Briefly, chitosan solution prepared by dissolving 0.5%(w/v) chitosan powder in 7% (v/v) aqueous citric acid (1M). A 0.5% (w/v) pullulan dissolved in deionized water. pullulan solution mixed with chitosan
solution and kept under stirring for 2 h. Now, the total ratio was 1% (w/v) CS/PL solution. Under the ambient condition, the prepared solution was perturbed electrochemically using a potentiostatic method at a constant potential of 1.5 V (BK, PRECISION 9201, Multi-Range Programmable DC Power Supply). The used cell consists of two platinum sheets electrodes (as an anode-cathode system) 1 cm apart were immersed vertically into the electrolytic solution. The process was conducted for 2 h. A system of abbreviation was used in the text (e.g., Es CS/PL where Es stands for electrosynthesis process of chitosan (CS) and Pullulan (PL) electrolytic solution to simplify samples identification.

2.3. Electrosynthesis of CS/PL-AgNPs microgel (Es AgNPs@CS/PL) solution

Silver nanoparticles self-embedded chitosan/pullulan microgel particles solution was prepared using the above-mentioned method (2.2.) with one exception, the Platinum anode electrode replaced by silver anode. A system of abbreviation was used in the text (e.g. Es AgNPs@CS/PL where Es stands for electrosynthesis of silver nanoparticles AgNPs self-embedded within chitosan CS and Pullulan PL electrolytic solution to simplify samples identification.

2.4. Preparation of losartan-loaded microgel particles

Losartan-loaded AgNPs self-embedded chitosan /pullulan microgel particles solution was prepared using the above-mentioned method (2.3.). Briefly, a 0.005 % (w/v) freshly prepared aqueous losartan solution was mixed with the 1% (w/v) CS/PL solution and electrochemically synthesized under the same condition mentioned at (2.2.). The solution named Es LOS@Microgel.

2.4. Characterizations

2.4.1. UV-vis spectra measurement

UV-visible measurements were carried out in a Double Beam spectrophotometer (T80 double beam UV-vis spectrophotometer, pg instruments, UK) in the range of 190-1100 nm with wavelength accuracy ±0.3 nm. The measurements equipped with a 1 cm quartz cuvette holder for the samples at room temperature. quartz cuvette filled with 4 mL of the solution scanned to examine the presence of the surface plasmon resonance peak of AgNPs, Ag⁺ ions complexation and to investigate the change may happen to the characteristic bands of chitosan, pullulan and losartan during the synthetic procedure.

2.4.2. Transmission electron microscope (TEM) analysis

The formation, size and morphology of AgNPs, the structural change that may happen to the synthetic system and probability of losartan embedding or loading were examined by TEM (JEOL TEM-2100) attached to a CCD camera. The samples were examined via making a sonication for all samples using ultrasonic cleaner using sonicator (XH-E412). The samples for TEM were prepared by placing a drop of the synthesized solution onto a carbon coated Cu grid followed by slow evaporation of the solvent at ambient conditions before recording the TEM images.

2.4.3. FTIR analysis

To collect information about the functional groups, chemical characteristics of the formulated solutions, numerous bonding and chemical interactions among the constituents, infrared measurements were performed for CS/PL and Es AgNPs@CS/PL samples. The samples were characterized using Fourier transform infrared spectroscopy
(FTIR, NicoletiS10; Thermo Fisher Scientific Inc., USA) to investigate the impact of electrochemical synthesis method on the electrolytic solutions. FTIR spectrum collected over the range of 4000-500 cm⁻¹ at room temperature with spectral resolution 4 cm⁻¹.

2.4.4. Inductively coupled plasma optical emission spectroscopy (ICP-OES)

Concentration of silver in the Es AgNPs@CS/PL solution was quantitatively determined by using inductively couple plasma spectrometry (ICP–MS, Agilent 7700X, Australia).

2.4.5. Zeta potential analysis

Surface charge measurements were conducted using Zeta size Nano-zs90 (Malvern instruments, Malvern, UK) on a diluted as prepared solution with deionized water. Surface charge distribution was acquired at room temperature by averaging triplicate measurements.

2.4.6. Assessment of the antibacterial activity

The antibacterial activity of CS, PL, CS/PL and Es AgNPs@CS/PL microgel solutions was evaluated against Escherichia coli and Staphylococcus using the Mueller agar disc diffusion method with determination of the inhibition zones (mm). Briefly, sterile paper discs (6 mm) were impregnated in sample solutions and then left to dry at 37 °C for 24 h in sterile conditions. The bacterial suspensions were obtained by making a saline suspension of isolated colonies selected from 18 to 24 h of nutrient agar plating. The suspensions were adjusted to match the tube of 0.5 McFarland turbidity standards with the aid of spectrophotometry at 600 nm, which equals 10⁶ colony-forming units/ml. The surface of the Mueller agar was completely inoculated using a sterile swab, which was steeped in the prepared bacterial suspension. Then, the impregnated discs were placed on the inoculated agar and incubated for 24 h at 37 °C. After incubation, the diameters of the growth inhibition zones were measured.

3. Results and discussion

3.1. Electrosynthesis of CS/PL

3.1.1. Morphology of the CS/PL using TEM

The nature of the chitosan/Pullulan system and its fine internal structure were studied by TEM measurement. Figure 1 explores the morphological change that take place when the synthetic system is placed under the electrochemical action for 120 min. Representative TEM image revealed that chitosan and pullulan assemblies with Pearl-necklace architecture. Apparently, it is believed that the presence of the synthetic system under the effect of the electric field plays a magical role in the chain transition to pearl-necklace conformation. In the literature it is generally believed that the semirigid polyelectrolyte characteristics and the medium molecular weight of chitosan employed in this study the extended polymer chain is long enough to interlink chitosan/pullulan chains. At the cathode-polyelectrolyte interface, the charge of the positively charged groups were screened accompanied with collapse the coiled chains into spherical globule. This in turn gives the chance for inter- and intra-chains interaction among hydrophobic, polar, and cationic residues to take place. Besides, even residual N-acetyl groups can participate such a compaction behavior. In particular, the CS/PL segments appear engaged with each other and form an overall pearl necklace structure. On the other hand, Figure 1c reveals the presence of a minor separated CS/PL nanospheres. A prolong study needed to further understand the anticipated impact.
of electrosynthesis conditions on the morphology of the synthetic system used in this study.

Figure 1 (a and b) TEM images represents Cs/Pl pearl-necklace structure in low and high magnification, respectively and c TEM image represents a completely separated Cs/Pl nanospheres.

3.1.2. UV–visible spectra analysis.

The formation of the pearl-necklace structure and the presence of CS/PL nanoparticles was further examined through UV-vis spectrophotometer. As shown in figure 2, UV-Vis patterns introduced two emerged peaks with a progressive increase in the absorption intensity as a function of the electrosynthesis time. A narrow peak centered at 255 nm may be assigned to the formation CS/PL nanoparticles. The other peak appeared at 340 nm has a red shift 365 nm with a progressive increase in the maxima as the electrosynthesis time varied. This result was consistent very well with the TEM measurements.

Figure 2 UV-vis absorption spectra of Electro-synthesized CS/PL solution recorded as a function of time and inset schematic diagram represents the formation of the CS/PL nanospheres.
3.2. Electrosynthesis of Es AgNPs@CS/PL microgel colloidal particles
(Studying the effect of the in-situ formation of AgNPs during the electrosynthesis of the polymeric microgel particles)

3.2.1. Morphology and size distribution study using TEM

Figure 3. (a and b) TEM images of Es AgNPs@Cs/Pl microgel in low and high magnification, respectively c TEM image of Ag NPs distribution within Cs/Pl nanosphere and d High resolution TEM image demonstrating the presence of Ag NPs in respect to the polymer matrix.

Figure 4. size distribution curve of the Es AgNPs@Cs/Pl microgel particles.
Figure 5. size distribution (left image) and border to border interparticle distance curves of the AgNPs within single Es AgNPs@CS/PL microgel particle.

Figure 6. a High-resolution TEM image demonstrating the lattice pattern of AgNPs (inset image represents the SAED pattern of AgNPs), b corresponds to AgNPs lattice fringes and c corresponds to and IFFT of AgNPs.

The impact of the preparation method on the morphology of the synthetic system was examined by TEM measurements as shown in figure 3. Apparently, it seems that we successfully synthesized a microgel spheres. Figure 3a and b show TEM images in low and high magnification of electrosynthesised colloid Es AgNPs@CS/PL, which revealed the formation of roughly spherical particles with a broad size distribution. It can be clearly seen that spheres have a bimodal distribution (151.4 and 43.5 nm) as shown in figure 4. By deep looking, Figure 3c explored a highly uniform distribution of small AgNPs fully embedded within the formed CS/PL microspheres in a quite narrow size distribution with average size 5.2 nm as represented in figure 5(left image) and border to border interparticle distance 1.22 nm (figure 5 (right image)). Nevertheless, despite slower reduction process in our case, due to complexation of the silver ions with chitosan
chains, results formation in well separated nanoparticles i.e. a good dispersion of the particles within the matrix\textsuperscript{19}. This proves a high stabilizing potential of chitosan with medium molecular weight as it has been previously established. In figure 3d, as marked by red and blue arrows, it is clear that a low contrast layer is capped on the surface of the formed AgNPs, which provides visual evidence for the stabilizing role of the CS/PL polymer chains. HRTEM images have given us further insight into the microstructure and crystallinity of the as-prepared Ag NPs. A representative HRTEM image is shown in Figure 6a where the lattice fringes are clearly visible with a space about 0.194 nm Figure 6b,c corresponding to the lattice space of the (111) planes of Ag \textsuperscript{20}. This observation confirmed the formed Ag NPs are highly crystalline\textsuperscript{20}. The SAED pattern shows obvious ring patterns mixed with several diffraction spots, underlying that the formed Ag nanoparticles were polycrystalline structure\textsuperscript{20-22} as shown in the inset of figure 6a.

3.2.2. UV-VIS measurements

During the electrochemical process, the transparent color of the electrolyte solution turned to pale yellow and gradually become yellowish with increasing the electrosynthesis time. This may be a pre indication on the silver nanoparticles formation. The UV-visible spectroscopy is commonly used as an analytical tool for studying the silver nanoparticles formation during synthesis owing to the characteristic surface plasmon resonance observation\textsuperscript{24,25}.

![UV-vis absorption spectra of Electro-synthesized Cs/Pl/Ag solution recorded as a function of time.](image)

The UV-vis patterns were collected during the electrosynthesis of the CS/PL Ag solution at different stages (15, 30, 45, 60, 75, 90, 105 and 120 min.) as shown in figure 7. According to the nanoparticle size, shape and the surrounding, the surface plasmon resonance band (SPR) of silver nanoparticles reveals in the range 350–600 nm\textsuperscript{26}. The
presence of such characteristic band come from the excitation of free conducting electrons upon the interaction with the incident light\textsuperscript{27}. As shown in figure 7, the presence of a single maxima peaked at 420 nm was due to the surface plasmon resonance of Ag NPs which is in agreement with the previous studies \textsuperscript{19,28,29}, indicates the presence of spherical nanoparticles which is also confirmed later by TEM results. As expected, the UV-vis patterns introduced a progressive increase in the absorption intensity of the localized SPR band centered at 420 nm as a function of the electrosynthesis time figure 7, confirming the reduction progress of the Ag\textsuperscript{+} ions within the chitosan/pullulan matrix solution during the electrosynthesis process. In addition, remaining of the SPR band maxima almost constant, signifying that the most formed AgNPs size is quite similar in all experimental conditions. Also, in parallel, there is an emerged absorption peak appeared at 250 nm, which may be assigned to the formation of [chitosan/pullulan]-Ag\textsuperscript{+} complex\textsuperscript{30,31}. Next, it is important to follow the behavior of the Ag\textsuperscript{+} ions and Ag NPs in the polymer matrix solution against the aging\textsuperscript{19}.

![Absorbance vs Electrosynthesis time](image)

Figure 8 absorption intensity of 270 and 420 nm as a function of electrosynthesis time

To go further in the examination of the spectra and taking advantage from recording the UV-vis spectra of the electrolyte solution during the electrosynthesis process. In this aim, Figure 8 introduces the intensity variation of bands centered at 270 and 420 nm as a function of the electrosynthesis time\textsuperscript{32}. In general, along the electrosynthesis time, the complexation and reduction processes of Ag\textsuperscript{+} ions occur simultaneously, where the amount of Ag complexation was higher than the amount of Ag reduction. Essentially, this behavior divided into two stages, stage I and II as represented in figure 8. stage I continued with complexation and reduction rates of $2 \times 10^{-4}$ and $1.3 \times 10^{-4}$, respectively. At a critical time, the rates raised to $6 \times 10^{-4}$ and $8 \times 10^{-4}$, respectively. The presence of such behavior indicates a change happened to the synthetic system properties. The stage II may be considered as a good evidence on the onset of polymer nanospheres formation,
where the polymer chain starts to interconnect and compact into a globule form\textsuperscript{14}. This behavior increases the chance of the Ag ions reduction. This hypothesis is supported by the rapid reduction rate of the stage II in addition to the localization of the SPR bands as introduced in figure 7, a feature indicating that nucleation is more favored than growth\textsuperscript{27}.

### 3.2.3. Stability

Long-term stability considered as one of the biggest challenges in the synthesis of AgNPs, where the variations in the size and surface charge may stimulate the AgNPs instability. From this point, the stability of AgNPs and the behavior of Ag\textsuperscript{+} ions in the polymer matrix solution as a function of time was evaluated by the investigation of UV-vis spectra of colloidal microparticles for a period of 60 days post-synthesis as shown in figure 9. Referring to the previous studies\textsuperscript{33-35}, the presence of the nanoparticles in an aqueous environment makes the size change and agglomeration to be unavoidable phenomenon during the long-term preservation\textsuperscript{36}. On the basis of our experimental findings, one can see a low level of SPR red shift, which refers to that AgNPs was quite stable. On the other hand, UV-vis patterns explored another phenomenon, increase in the intensity of SPR band centered at around 420 nm as a function of the storing time accompanied with decrease in the intensity of [Ag\textsuperscript{+}-polymer] complex band. It seems that this behavior reflects the slow reduction rate of the remained Ag\textsuperscript{+} ions to Ag\textsuperscript{0} atoms in the self-assembled microgel without size enlargement and agglomeration of AgNPs.

Our findings lead us to suggest that the lessening in the absorbance intensity of the [Ag\textsuperscript{+}-polymer] complex band due to the reduction of the ions embedded within the nanospheres, which in consistency with the trend of a nucleation gradually favored over growth drawn from figure 9.

![Figure 9](image)

**Figure 9** UV-vis absorption spectra of Cs/Pl/Ag solution as a function of storage time at 4\textdegree{} C and a schematic diagram explored the aging effect on the formed microparticle.

### 3.2.4. ICP

Inductively coupled plasma analysis (ICP) was conducted to measure the concentration of AgNPs. The result presented that the concentration of silver in Es AgNPs@CS/PL solution was 494 mgL\textsuperscript{-1}, appropriate amount of electro-released silver within the polymeric matrix which considered as an electrolytic solution.
3.2.5. Fourier transform infrared spectroscopic analysis

FTIR was measured to evaluate the chemical structural changes from CS/PL system to Es AgNPs@CS/PL microgel and investigate the probable functional groups of the synthetic system responsible for the coil to spherical globule transition, formation and stabilization of the AgNPs. FTIR spectra (figure 10) represents the basic characteristic bands of CS/PL system. The main band located between 3000-3600 cm\(^{-1}\) with maximum at 3567 cm\(^{-1}\) (a broad band attributed to \(-\text{OH} \text{ and } -\text{NH}_2\) stretching\(^{37-39}\), 2900 cm\(^{-1}\)(C-H symmetric stretch)\(^{31,40-42}\), 2524 cm\(^{-1}\), 2175 cm\(^{-1}\), 1972 cm\(^{-1}\) and 1625 cm\(^{-1}\) (stretching frequencies of carbonyl (C=O) and C=C groups)\(^{31,43,44}\), 1525 cm\(^{-1}\) (NH\(_2\) bending vibrations)\(^{41,45,46}\), 1409 cm\(^{-1}\) (deformation from C-OH vibration)\(^{38,41,47}\), absorption bands in the range 1270-800 cm\(^{-1}\) belong to ((1-4) C-O-C)the glycosidic ring. In particular, the bands at 1228, 1081 and 1029 cm\(^{-1}\) corresponds to the glycosidic linkage\(^{37,39,41,45}\). After electrosynthesis process, FTIR spectra of Es AgNPs@CS/PL sample showed that O-H, NH\(_2\) stretching vibration shifted from 3567 to 3594 cm\(^{-1}\) may be assigned to the Ag\(^{+}\)-Polymer matrix complex formation and the C-H symmetric stretching peak at 2900 cm\(^{-1}\) disappeared. Also, C-OH vibration at 1409 cm\(^{-1}\) showed a relative decrease in absorbance indicating that the hydroxyl groups participate in the reaction. Moreover, a relative reduction in the intensity of peaks at spectral range 1280-990 cm\(^{-1}\) has been observed due to the deformation stretching vibration of the glycosidic bond. This outcome proposed that, amino, amide and hydroxyl groups involved in the intra-interchain interaction, helping in the formation of CS/PL microgel incorporated with AgNPs (as depicted by TEM measurements) stabilized by protonated amine groups. The explored peculiar nanostructures with different hydrogen bonding network were likely to be formed.

![Figure 10. FTIR spectroscopy of the CS/PL and Es AgNPs@CS/PL microgel films.](image-url)
3.2.6. zeta potential

Zeta potential is a crucial parameter for determining the stability of nanoparticle suspensions. Also, it gives a valuable information about the distribution of charged groups on the polymer chains around the nanoparticles. Zeta potential measurements of the Es AgNPs@Cs/Pl microgels presented in Figure 11 introduced that microgels have a positive surface charge $+25 \pm 5.18$ mV. The positive surface charge of the microgels come from the greater availability of the protonated NH$_3^+$ groups directed to the solution. This will produce good electrostatic repulsion between the particles, resulting in better stability.$^{18,36}$

![Zeta Potential Distribution](image)

**Figure 11.** Zeta potential distribution of Es AgNPs@Cs/Pl microgels exhibited at a mean charge with $+25$ mV

3.2.7. Mechanism of AgNPs@CS/PL nanospheres

It is essential to investigate possible mechanisms responsible for the conformational transition from a coil to a dense spherical globule like structure (microgel) during the electrochemical process within chitosan and pullulan synthetic matrix. The synthetic matrix was rich by functional groups such as amine, amide, Hydroxyl, alcoholic OH and protonated amine groups, which promote the interlink between chitosan and pullulan chains. During the electrochemical synthesis of AgNPs in the solution of CS/PL polymers, the next successive steps of electrode and chemical reactions can be featured:

1- Electro-release of Ag$^+$ ions

During the electrochemical synthesis process, the anodic dissolution of Ag$^+$ ions in the electrolyte that contains complexing ligands started with almost constant release rate.$^{49}$

At the anode-solution interface:

$$n \text{Ag}^0 \rightarrow n \text{Ag}^+ + n \text{e}^- \quad \text{equation (1)}$$

2- Reaction of Ag$^+$ ions with the functional groups
At the sacrificial Ag anode, the Ag ions immediately undergoes the formation of [Ag\(^+\) - polymer] complex through the functional groups as confirmed by UV-vis and FTIR measurements\(^{50}\).

At the anode-solution interface:
\[ \text{Ag}^+ + \text{CS/PL} \rightarrow [\text{Ag}^+ - \text{CS/PL}] \text{ complex} \quad \text{equation (2)} \]

Ag\(^+\) act as a cross linker between the chain segments, which increases the probability of the chains compact with overall positive charge. In addition, the semirigid polyelectrolyte characteristics and the extended polymer chains are long enough to interlink and form a dense network \(^{51}\). Now, the (the positively charged segments) network started its journey directing towards cathode.

3- Coil to spherical globule transition
At the cathode surface, the cathodic reduction of the Ag ions began, in addition to the screening of the protonated amine groups helps in the coil to spherical globule transition\(^{14}\). In deep discussion, synthetic solution used in our experiment was a functional groups rich polyelectrolyte, neutralization of the positively charged groups at the cathode surface promote the chance of more interconnection between the chain’s segments\(^{16}\). Besides, the normal interaction between CS/PL chains. Moreover, the presence of acetamide groups, even a small quantity of residual acetamide groups may participate in intra- or interchain links due to e.g. hydrogen bonding, which increase the chains collapsing feature. Herein, AgNPs and some Ag\(^+\) ions confined inside the spherical globule as confirmed by the UV-vis and stability measurements. Now, we can imagine the formation of a microgel network of CS/PL self-embedded with a homogenous distributed AgNPs.

Figure 12 UV-vis absorption spectra of electro-release of Ag\(^+\) ions through citric acid solution as a function of time.
It was necessary to examine if the citric acid participate in the reaction as a reducing agent or stabilizer. So, electro-release of Ag\(^+\) ions in the presence of the citric acid only was performed under the same experiment condition. Figure 12 represents the variation of the UV-vis absorption patterns as a function of time collected every 15 min for 1h. The patterns revealed that there are no absorption bands related to any complexation of citrate with Ag\(^+\) ions or reduction. But an Ag layer deposited on the Pt cathode surface. Hence, these results are good evidence that citrate ions mayn’t be participate in the process of complexation or reduction of Ag\(^+\) ions. Because of the presence of some Ag\(^+\) ions unreduced to AgNPs, we analyzed the stability of microgel/silver hybrids over two months. We found that the Ag\(^+\) ions reduced with a slow rate as shown in figure 9, where the Ag-complex band undergoes a decrease in absorption intensity accompanied with increasing in the intensity of the SPR band. This action is not followed by any spectral shift of the SPR band due to the appropriate interparticle separation 1.22 nm between AgNPs (as represented in figure 5) provided by microgel network and prevention of particles enlargement. It is considered as good evidence that embedded Ag\(^+\) ions within the microgel sphere reduced to AgNPs favorite nucleation over the growth.

4- Effect of the electrosynthesis time

As the time of the electrosynthesis goes on, the complexation and reduction of Ag\(^+\) ions inside the synthetic solution occurred simultaneously but in two different stages. In general, the amount of complexation of the Ag\(^+\) ions with the polymeric system are higher than the reduction of it as depicted in figure 8. On the other hand, the amount of the Ag\(^+\) ions reduction and the rate at the II stage is higher than that of the I stage. Moreover, the ratio of complexation/reduction of the I stage was higher than that of II stage. The presence of such behavior indicates a conformational change prevailed the synthetic solution. This puzzling behavior can be properly explained in terms of the change take place over the amino groups to Ag\(^+\) ions ratio. At the I stage, Electro-release of Ag\(^+\) ions continuously consumed the amino groups leads to a scenario of the predominance of complexation over reduction. Up to critical time, the expanded CS/PL chains were inter, intrachain interacted in such a manner changing the coil like structure to dense spherical globule. So, Ag\(^+\) ions confined uniformly and homogenously inside the spherical globule (microgel), which promote the reduction rate. Moreover, the AgNPs migration and agglomeration probability were avoided due to a sufficient dense CS/PL structure, a feature indicating that nucleation is more favored than growth. Finally, it must be highlighted that electrochemical process with sacrificial Ag anode has a clear impact on the morphology of the CS/PL matrix as presented by TEM measurements as represented in figure 13. Under the perturbation of the applied electric potential, the electro-release of Ag\(^+\) ions increase the opportunity of chains interlink points, making the coil structure of the polymeric matrix chains compact into a dense microsphere. From this point of view, in the future, it is necessary to examine more parameters to propose a model in making sense of experimental evidence results.
3.2.8. Antibacterial studies

As expected, the chitosan demonstrated inhibition zone against S. aureus (12.3 mm) and E. coli (9 mm). Such antimicrobial potential against both gram-positive and gram-negative bacteria is derived from the protonated amino groups in chitosan. It is well known that the cationic charge of the quaternary ammonium interacts with the phospholipid components in the cytoplasmic membrane of bacteria. This in turn leads to lysis of the cell wall and leakage of the cell components leading to cell death. Introducing PL to the polymeric matrix of CS exhibited bad effect on the antimicrobial potential against both S. aureus and E. coli. Incorporation of in-situ AgNPs formation into electro-prepared CS/PL microgel has considerably enhanced the antimicrobial potential of the realized CS/PL against both gram-positive and gram-negative bacteria as represented in figure 14. For gram-positive bacteria, AgNPs displayed a synergetic biocidal potential with chitosan leading to increase the inhibition zone to 19 mm as depicted in table 1. Similarly, the gram-negative bacteria recorded increase of inhibition zones to 13 nm as depicted in table 1. Particularly, the moderate promotion of antimicrobial potential in the presence of the AgNPs may be come from the incorporation most of AgNPs within the microgel particles during the preparation procedure.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gram-positive (S. aureus)</th>
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<tr>
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Table 1 Antimicrobial activity of CS, PL, CS/PL and Es AgNPs@CS/PL by disc diffusion assay.
3.3. Characterization of Es LOS@CS/PL microgel spheres

3.3.1. UV-visible spectra analysis

Figure 15 shows the absorption spectrum of the LOS, Es LOS@Microgel and Es AgNPs@Cs/Pl solutions. LOS pattern exhibits peak centered at 265 nm after electrosynthesis, LOS characteristic band completely disappeared and a new broad band with high intensity emerged at around 480 nm. At this point, it is to be mentioned that the Es AgNPs@Cs/Pl absorption band (420 nm) red shifted by around 60 nm in the presence of the LOS. Hence, it seems straightforward to conclude that LOS incorporated inside the microgel network during the electrosynthesis process.

Figure 15. UV-visible spectra of Es AgNPs@Cs/Pl recorded for different times (15, 30, 45, 60, 75, 90, 105 and 120 min.)
3.3.2. TEM and Zeta potential

TEM images revealed that LOS was successfully in situ incorporated within the microgel assembled as highly compact and uniform core shell like structure (dark core: LOS and grey shell: AgNPs@Cs/Pl/) as shown in figure 16a and b. A well-defined uniformly distributed spheres confirmed the good homogeneous incubation of losartan within the formed microgel. As shown in figure 16 c, the size distribution curve explored that the average size of the microgels equals to 92.98 nm. LOS occupied approximately 70% of the overall size of the microgel particle. In addition, figure 16 d and the inset represent the AgNPs lattice fringes and The SAED pattern shows obvious ring patterns mixed with several diffraction spots, underlying that the formed Ag nanoparticles were polycrystalline structure.

Figure 16 (a and b) TEM images of Es LOS@microgel particles in low and high magnification, c the size distribution curve of the LOS@microgel particles, and d High-resolution TEM image demonstrating the lattice pattern of AgNPs (inset image represents the SAED pattern of AgNPs).

Zeta potential value helps to examine the stability of the drug embedded microgel solution Es LOS@Microgel. Figure 17 shows a zeta potential value of +39 mV indicating high electrostatic repulsion, which promoting the microgel particles stability. +39 mV
of Es LOS@Microgel was greater than +25 mV of Es AgNPs@CS/PL, this may be assigned to the presence of the potassium ions.

Figure 17. Zeta potential distribution of Es LOS@Cs/Pl microgel particles exhibited at a mean charge with +39 mV.

Another important point is that Ag particles already formed in the presence of losartan during the preparation with homogenous distribution and average size of 1.15 nm (as represented in figure 18) in the range of the nanocluster AgNCs. It is clear that the
size of the Ag particles decreased comparing with that of the free losartan microgels, which indicates that presence of the losartan predominant nucleation over growth.

Conclusion

In this article, we investigated the using of the electrochemical route for synthesis of AgNPs@CS/PL microgel particles, the effect of the in-situ formation of AgNPs during the electro-microgelation process on the morphology of the polymeric system and finally, the entrapment efficacy of losartan as a model drug within the microparticles in-situ during the preparation. At appropriate conditions, a polymeric microsphere gel was successfully synthesized. The TEM, UV-vis, FTIR, ICP and zeta potential results explored that under constant applied voltage (1.5 V), the scarified Ag$^{+}$ ions in-situ reduced within the synthetic system during the electro-collapsing of CS/PL chains from coil to globule structure, which in turn form dense microspheres including the AgNPs as represented by TEM images. The size distribution curves worked out from TEM explored a bimodal behavior with average size 151.4 and 43.5 nm. Besides, AgNPs with average size 5.4 nm were uniformly distributed within the microgel particles. UV-vis confirmed the formation of the AgNPs. In addition, studying the aging effect explored that a little Ag$^{+}$ ions slowly reduced to metallic particles within the microparticles preferring the nucleation behavior than growth. This behavior assigned to the dense interconnection between the chain’s segments of the CS/PL network under the umbrella of the applied potential and the release of the Ag$^{+}$ ions which promote the chance of the chains interlink and hence increase the probability of the Ag$^{+}$ ions reduction with the opportunity of the nucleation process. Also, the result revealed that incorporation of LOS during the formation of the microgel spheres was effectively worked well. It is worth stating that the Es LOS@microgel spheres had small particle size and optimum positive surface charge.

Reference


Declarations

- **Availability of data and materials**
  The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request

- **Conflict of Interest**
  The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

- **Authors' contributions**

  **Conceptualization:** Fikry, Mostafa and Ayman, **Methodology:** Mostafa, Ayman and Fikry, **investigation:** Mostafa, Ayman and Fikry, **Analysis:** Mostafa, Ayman and Fikry; **writing original draft:** Mostafa, Ayman and Fikry; **Writing review and editing:** Mostafa, Ayman and Mohamed. All authors have read and agreed to the published version of the manuscript.

- **Acknowledgments**
  We thank Professor Fikry M. Reicha for supporting this research and to make this work for his generous soul, sincere greetings from his dear students. This paper and the research behind it would not have been possible without the exceptional support of him.
  Last, we would like to pay our gratitude and our respects to our co-author and colleague Prof. Mohamed M. Gabr, he is taking the responsibility of our laboratory (Biological Advanced Materials laboratory) after prof. Fikry passed away.
  The work was supported in part by a grant from Mansoura University (Grant No. MU-SCI-16-11).
Title
Electro-microgellation of CS/PL spheres self-embedded with in situ AgNPs formation as a losartan delivery system

Affiliation
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