

Research Article

Studies on Nanoengineered Multilayer Capsules for Drug DeliveryFATEN Z MOHAMED¹, MOHAMED A SHEMIS², MONA N MOHAREB², HEBA A HOSINY¹¹Biochemistry Department, Faculty of Science, Zagazig University, Egypt²Biochemistry and Molecular Biology Department, Theodor Bilharz Research Institute, Giza, Egypt

ARTICLE DETAILS	ABSTRACT
<p><i>Article history:</i> Received on 22 September 2014 Modified on 5 February 2015 Accepted on 15 February 2015</p> <hr/> <p><i>Keywords:</i> LbL, Polyelectrolyte microcapsules, MTT, MDBK cell line</p>	<p>Polymeric multilayer capsules are now being engineered to encapsulate various classes of drug molecules, by using polymers that are biodegradable or that can respond and release their payload in response to well-defined stimuli and are produced by stepwise adsorption of oppositely charged polymers onto the surface of colloidal particles followed by core dissolution, which is called Layer by Layer technique. The present study focuses on synthesis and characterization of polyelectrolyte multilayer microcapsules which was loaded with Bovine Serum Albumin and the cytotoxic effect of the microcapsules on the living cells were studied. The microcapsules were fabricated by using Poly(allylamine) Hydrochloride (PAH) and Poly(styrenesulfonate) (PSS) as the counter charge polyelectrolytes and biocompatible calcium carbonate as the template which is highly promising to produce capsules for biological applications and loaded with bovine serum albumin (BSA) as a model drug, BSA labeled with fluorescein isothiocyanate (BSA-FITC), Calcium carbonate particles and the microcapsules were characterized by SEM and TEM, The effect of microcapsules on Madin Darby Bovine Kidney (MDBK) cell line were studied by using The 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide MTT assay. CaCO₃ microparticles are very convenient template for the preparation of polyelectrolyte microcapsules 1–4 μm, preloading method is more effective than post-loading method, Our results confirmed that this synthetic CaCO₃ microcapsules are cytocompatible. We thus have shown that the very easily prepared, stable, prototypical microcapsules composed of PSS/PAH are useful drug delivery agents for proof-of-principle drug delivery studies.</p>

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INTRODUCTION

Drug-delivery science is driven by the need to develop systems that can deliver precise quantities of a therapeutic payload at a specific target site or tissue at tailored release rate and a specific trigger [1, 2]. This technique is of special interest for medicine, particularly for treatment of cancer diseases which demands rather toxic and expensive medical substances [3]. Several drug molecules cannot be formulated or administered by conventional techniques as they exhibit poor water solubility or suffer from limited stability in a complex environment such as the human body. A beautiful example of a novel system that has recently emerged from cross-disciplinary scientific symbiosis is polymeric multilayer capsules (PMLCs) [4].

PMLCs have been extensively explored for their physicochemical properties since their advent in the late 1990s [5], and more recently they have attracted attention for drug-delivery applications [6]. Polyelectrolyte microcapsules are produced by stepwise adsorption of oppositely charged polymers onto the surface of colloidal particles followed by core dissolution, which is called (Layer by Layer) technique [7, 8]. Using this approach, a variety of materials, including charged and uncharged species, have been successfully assembled. Commercially available synthetic polyelectrolytes such as PSS, PAH, PDADMAC, Particularly, some naturally occurring polyelectrolytes, such as polysaccharides are eye-catching [9]. The sacrificing of the encapsulated template is a key step for capsule fabrication. Several templates have been used for the capsule preparation, such as weakly cross-linked melamine-formaldehyde lattices, silica particles, polystyrene lattices and

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biological templates [10-12]. However, the dissolution of many polymer cores such as melamine-formaldehyde and polystyrene requires strong acids or organic solvents, and in some cases template materials cannot be completely removed from the capsules because of the interaction between templates and capsule walls. Among these cores only carbonate particles can be decomposed under rather mild conditions [13]. By varying the intermixing speed, time, pH value and ratio of initial ingredients during precipitation such CaCO_3 templates are produced [14]. Some proteins, such as bovine serum albumin and peroxidase, were incorporated into hollow polyelectrolyte capsules by regulating permeability of the multilayer shell for macromolecules [15]. To evaluate the potential of PMLCs for drug delivery it is important to understand their interactions with living cells. One of the most important parameters is toxicity. Several research groups have assessed this topic by performing in vitro cell-viability assays such as the MTT test [16, 17]. The present study focuses on the microcapsules based on Poly(allylamine) (PAH) and Poly(styrenesulfonate) (PSS) as the counter charge polyelectrolytes and biocompatible calcium carbonate as the template which is highly promising to produce capsules for biological applications, as it shows low cytotoxic and can be easily removed with EDTA. Bovine serum albumin (BSA) was chosen as the model protein [18].

In this paper we have focused on loading of BSA-FITC into the microcapsules by preloading and post-loading method, studying the effect of pH on the synthetic microcapsules for loading and release of bovine serum albumin, characterization of the capsules by optical microscope and electron microscope and the cytotoxic effect of this microcapsules on Madin Darby Bovine Kidney (MDBK) cell line by 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide MTT assay. Good cytocompatibility of microcapsules consist of synthetic polyelectrolytes was confirmed. Their application as drug micro/nano-transporters can be envisaged and will be the subject of future investigations.

MATERIALS AND METHODS

MATERIALS

PSS, PAH, EDTA 99+%, MTT isomer I and fluorescein isothiocyanate (FITC) calcium chloride dehydrate 99,99%, sodium carbonate

99,99% were purchased from Sigma-Aldrich; Sodium chloride 99,5% from Biostain; Bovine serum albumin > 98.0% From BIO BASIC INC; Sodium carbonate buffer(0.1M, PH 9), MEM Earle's medium with L-Glutamine, HEPES, Antibiotics PEN-STREP contain 10,000U pencillin/ml, 10,000µg Streptomycin/ml and 25µg Amphotericin B/ml were purchased from Lonza- swiss company; 5% heat-inactivated fetal bovine serum, L-Glutamine, Dulbecco's Phosphate Buffered Saline (PBS) without magnesium and calcium, Trypsin-ethylenediaminetetraacetic acid 1X in PBS were purchased from Biowest. All chemicals were of high quality and purity. MDBK cells were provided by tissue culture laboratory of VACSERA were prepared from a stock stored in liquid nitrogen, at Theodore bilharz research institute.

METHODS

Fabrication of Polyelectrolyte Microcapsules

Polyelectrolyte microcapsules were prepared using CaCO_3 microparticles as a sacrificial template. CaCO_3 microparticles were synthesized according to Volodkin *et al* [19] by mixing CaCl_2 and Na_2CO_3 solutions (0.33 M) with vigorous stirring for 30 s followed by extensive washing with pure water to remove un reacted reagents. Spherically shaped CaCO_3 microparticles with an average diameter of 1 to 4µm were obtained. For synthesis of CaCO_3 (PSS) particles Firstly, PSS (4g/L) was mixed CaCl_2 (0.33 M) under magnetic agitation (1000 rpm), Then an equal molar of Na_2CO_3 solutions was rapidly poured into a flask together with CaCl_2 solution contained PSS. The CaCO_3 particles were coated using the layer-by-layer technique by dispersing them in PSS solution (2mg/ml 0.5 M NaCl). After 10 min shaking the microparticles were collected by centrifugation, and residual PSS was removed by washing twice with pure water. Thereafter the microparticles were suspended in PAH solution (2mg/ml 0.5 M NaCl) and shaken for 10 min, followed by centrifugation and two washing steps. This procedure was repeated until the desired number of layers was deposited (five bilayers in this study). Hollow capsules were obtained by removing the CaCO_3 core by incubating the coated microparticles for 10 min in 1 ml of 0.2 M EDTA solution (pH 5.5) to dissolve the CaCO_3 . The dissolved ions were then removed by three centrifugation and washings steps. Finally the capsules were resuspended in 1 ml PBS. The capsule concentration was determined by haemocytometry.

Labeling of Bovine Serum Albumin with Fluorescein Isothiocyanate (FITC)

BSA was labeled with FITC for loading it into the microcapsules by preloading and post-loading method and detecting its loading by fluorescence microscope. BSA was labeled with FITC by Amicon Ultra-15 Centrifugal Filters (5KDa) according to [20]. 5 mg/ml of BSA that was dissolved in (0.1 M sodium carbonate buffer, pH 9) was mixed with FITC solution (3 mg in 1ml 0.1M sodium carbonate buffer PH 9) then was shaken orbital overnight at room temperature protected from light. after the end of incubation of BSA-FITC were moved into 50 ml amicon ultra-15 (5KDa) filters and the volume was completed with deionized water to 15 ml and centrifuged at 3500 rpm for 25 min then the downstream are trashed and upstream was completed with water and repeat till get clear downstream which when checked on UV are not flure anymore. The upstream should be yellow in colour then the absorbance of BSA-FITC was measured at 280 nm for BSA and 495 nm for FITC. Then Calculate protein concentration according to the Beer-Lambert law as follows:

$$\text{Protein concentration (M)} = \frac{A_{280} - (A_{\max} \times CF)}{\epsilon_{\text{protein}}} \times \text{dilution factor}$$

Where $\epsilon_{\text{protein}}$ is, protein molar extinction coefficient (e.g, the molar extinction coefficient of BSA is $\sim 43824 \text{ M}^{-1}\text{cm}^{-1}$)

$$\text{Correction Factor, CF} = \frac{A_{280}}{A_{\max}} = 0.3$$

Determination of Encapsulation Efficiency of BSA into the Microcapsules

Preloading Method

Effectiveness of protein entrapping (immobilization) was determined by a difference between optical density of the initial solution and a supernatant obtained after protein adsorption into the microparticles [21]. CaCO_3 particles with BSA -FITC adsorbed on its surface was obtained by mixing of 770 μl labeled BSA (50 μM), 615 μl CaCl_2 , 615 μl Na_2CO_3 then centrifuged then measure absorbance of BSA and FITC supernatant using UV-VIS spectrophotometer then the amount of BSA adsorbed on the particles was detected by subtracting the concentration of BSA in supernatant from original concentration (50 μM).

$$\text{Encapsulation efficiency} = \frac{\text{Concentration of encapsulated protein}}{\text{total protein concentration}} \times 100$$

Post-Loading Method

The effect of Temperature, pH on the loading of BSA -FITC into the microcapsules was studied.

A-Temperature Effect

A solution of 70 kDa BSA that labeled with FITC in a concentration of 50 μM was added to the microcapsules and was left one to two hours for incubation at room temperature. During this time the mixed solution was wrapped in aluminum foil in order to protect it from light. In the meanwhile a water bath was heated to 68 $^{\circ}\text{C}$ in which the tubes were placed subsequently for the heating. They were left for 30min in thermal treatment for the shrinking process protected from light. Afterwards the suspensions were allowed to cool down to room temperature for 5min and washed twice to remove the non-encapsulated FITC-BSA. Then absorbance of BSA, FITC was measured to determine the encapsulation efficiency.

B- pH effect :

The effect of pH on BSA- FITC loading was studied. The pH of BSA- FITC studied was in the range of 3.4 to 5.0. After mixing 2×10^8 microcapsules with 200 μL (1 mg/mL BSA- FITC) , the mixture was incubated for 12 hours at ambient temperature , followed by centrifugation at 3500 rpm for 5 minutes. FITC, BSA was quantified, using a UV-vis spectrophotometer, according to its absorbance peak at 280,495nm [22]. The post-loading of BSA- FITC was also confirmed using afluorescence microscope.

Determination of the Amount of Released BSA from the Microcapsules

The BSA- FITC concentration was measured using spectrophotometer. The amount of unloaded protein and overall loss during capsules washing was deducted from the initial amount of BSA- FITC added to the system to give the total amount of encapsulated BSA- FITC. To investigate the release of BSA from the capsules, the BSA- FITC -loaded microcapsules were mixed with 1 mL phosphate-buffered saline (PBS; pH 7.4) or 1mL HCl solution (pH 5.0 or 1.0) and incubated at ambient temperature under gentle shaking. A 200- μL aliquot of supernatant was taken out at a given time and supplemented

with 200 μ L fresh PBS or HCl solution. Each 200 μ L of supernatant was diluted with PBS or HCl solution, the absorbance of FITC, BSA was recorded, and the cumulative release of FITC-BSA was calculated. The results were analyzed by excel program.

Characterization of Polyelectrolyte Microcapsule

Optical Microscopy

The zeiss model microscope, equipped with 10x, 40x and 100x lenses and a Coolsnap Color camera were used for bright field inspection and recording^[23].

Electrophoretic Mobility

The electrophoretic mobility of the polyelectrolyte microcapsules was measured in deionized water at room temperature using a Malvern Zetasizer 2000 (Malvern, UK). The zeta-potential (ζ -potential) was calculated from the electrophoretic mobility (μ) using the Smoluchowski function $\zeta = \mu * \eta / \epsilon$ where η and ϵ are the viscosity and permittivity of the solvent, respectively.

Scanning Electron Microscopy

A drop of capsule suspension was placed on a silicon wafer (a metal bead) and dried under a nitrogen stream, and then coated with gold. SEM images were recorded with LEO 1550 VP scanning electron microscope (SEM, Inspect S, FEI Company, JAPAN) operated at 5 kV.

Transmission Electron Microscopy

The hollow and filled structures of the microcapsules were observed by transmission electron microscopy (TEM) (EM 208 S Philips, Japan). Nickel grids sputtered with carbon films were used to support the microcapsules. Ten microlitre of the microcapsule suspension was put on the Nickel grids and air-dried before measurement.

Fluorescence Microscope

The FITC labelled microcapsules were imaged with fluorescence microscope.

Viability Assay

MTT assays were used to measure the viability of MDBK cells cultured in 2D in the presence of microcapsules^[24]. The cells were cultured in MEM Earle's medium containing 10% fetal bovine serum, 1% antibiotic (penicillin-streptomycin- Amphotericin B 100x), 1% L-glutamine, 1% HEPES in a humidified incubator at 37 °C, with 5% CO₂ and 95% relative humidity. For determination of cytotoxicity of

the microcapsules on MDBK cells, 100 μ L of cell suspension at density of 10⁴ cells was added to plate wells and were incubated for 12h to allow cells to attach onto the surface. Then microcapsules were added serially at 1 \times 10⁸, 1 \times 10⁷, 1 \times 10⁶, 1 \times 10⁵, 1 \times 10⁴, 1 \times 10³/ml, the microcapsules was diluted using MEM Earle's medium. Four samples (in four wells on the same plate) were prepared at each microcapsule concentration. Four control wells with cells (10⁴ but no microcapsules were also prepared. well of only media was prepared for background measurement and two well for blank solvent, also wells contain only low and high concentration of microcapsules. The plate was placed in an incubator at 37°C for 24h. Then 20 μ L of MTT reagent was added to each well, Incubate the plate for approximately 2 - 4 hours at 37° C in CO₂ incubator. The plate was examined periodically for the appearance of punctate, intracellular precipitate (formazane crystals) using an inverted microscope. When purple precipitate is clearly visible under the microscope the culture medium was aspirated from each well carefully to prevent disruption or the precipitate. The resulting MTT formazane crystals were dissolved with 100 μ L acidified isopropanol solution, The plates were ready within 1 hour after adding acidified isopropanol solution.

After the end of incubation time, pipetting up and down was required to completely dissolve the MTT formazane crystals. Absorbance at wavelength of 570 nm was spectrophotometrically measured. Cell viability is expressed as:

$$\text{Viable cells (\% Control)} = \frac{A_{\text{treated}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100$$

Where A_{treated} is the average absorbance in wells containing cells cultured with a particular concentration of microcapsules, A_{blank} is the absorbance of the MTT solvent, and A_{control} is the average absorbance in wells containing cells but no microcapsules. The results of MTT assay were analyzed by excel program.

RESULTS

Encapsulation Efficiency of BSA-FITC into the Microcapsules

Preloading method

Protein concentration was calculated according to the Beer-Lambert law. The encapsulation efficiency was found to be 86%.

Post-loading method

In the case of temperature effect: the encapsulated amount of protein is $7\mu\text{M}$ due to The capsules gradually shrink with increasing temperature The shrinkage process is mainly powered by decreasing the area of the water/polyelectrolyte interface. The encapsulation efficiency was found to be 14%

In the case of pH effect: Encapsulation Efficiency = 78.8%

Our results showed high encapsulation efficiency in the case of preloading method due to the high porosity of calcium carbonate particles, followed by pH effect due to any changes in pH effect on the charge balance of polyelectrolyte layers of the microcapsules and permeability to the proteins with high molecular weight (BSA) but in the case of temperature effect, by increasing the temperature, the microcapsules was shrunk and the wall thickness of the polyelectrolytes increased that lead to permeability to the proteins with high molecular weight decreased as shown in Fig. 1.

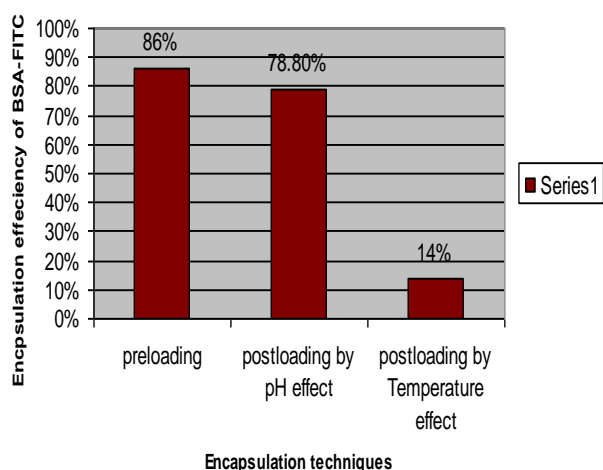


Figure 1: Encapsulation efficiency of BSA-FITC into the microcapsules by different techniques.

Cumulative release of BSA-FITC from PMLCs under different pH values:

After loading of BSA-FITC into the microcapsules at PH 3.8, $39.4\mu\text{M}$ was encapsulated, the results show that the amount of protein released in the case of phosphate-buffered saline (PBS; pH 7.4) more than HCl solution (pH 5.0 or 1.0) as in Fig 2.

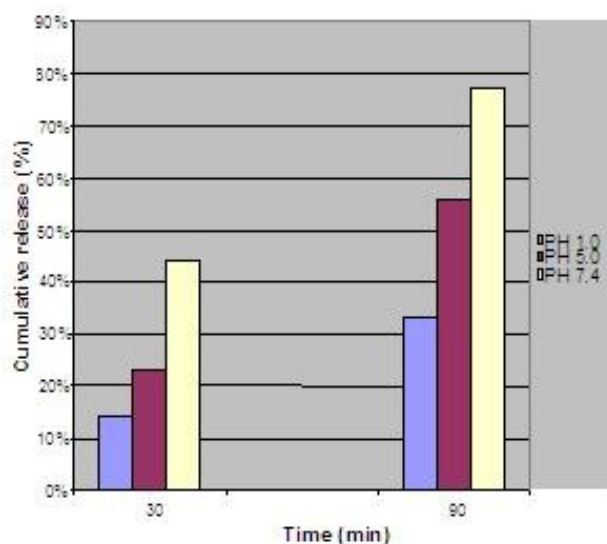
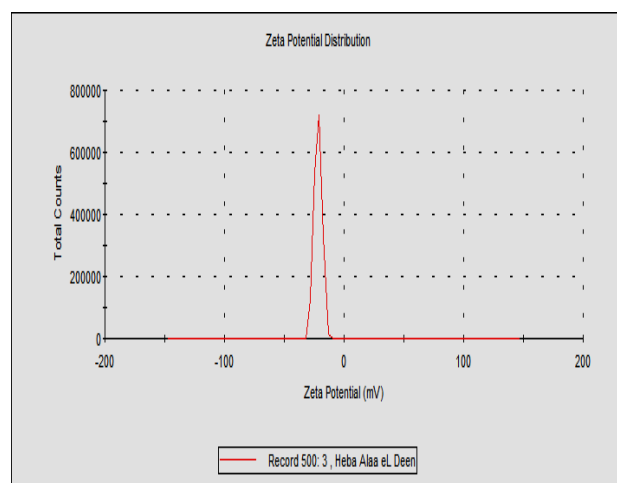
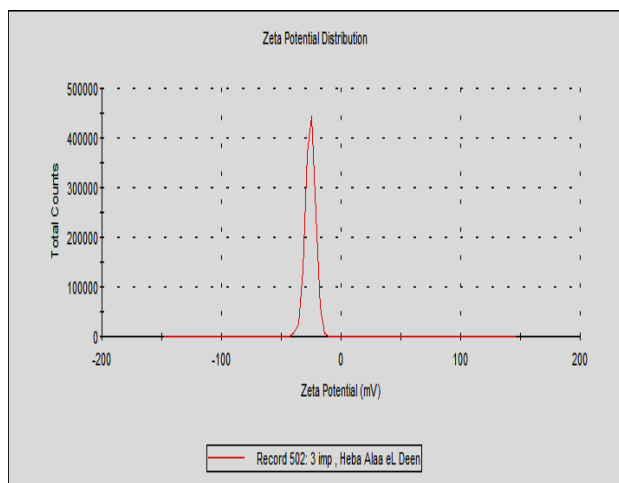


Figure 2: FITC-BSA cumulative release from microcapsules containing PSS versus incubation time at 30 and 90 minutes. Release was significantly different between the pH values tested. Release was conducted at 37°C in pH 7.4, 5.0, and 1.0 solution.

Characterization of the prepared Polyelectrolyte Microcapsules
Electrophoretic mobility of CaCO_3 microparticles and microcapsules: Zeta analyzer measurements show that CaCO_3 microparticles containing BSA adsorbed on it had negative charge -21.7mV and the microcapsules with (PAH/PSS)5 exhibited a value of -25.7mV as shown in (Fig. 3).



(A)



(B)

Figure 3: (A) Zeta potential of CaCO_3 microparticles containing PSS, (B) Zeta potential of microcapsules with $(\text{PAH}/\text{PSS})_5$.

Optical Microscope: Light microscopy was utilized to demonstrate the external shape of microcapsules, the micro photo that were taken show microcapsules are spherical, monodisperse and non-aggregated as shown in (Fig 4).

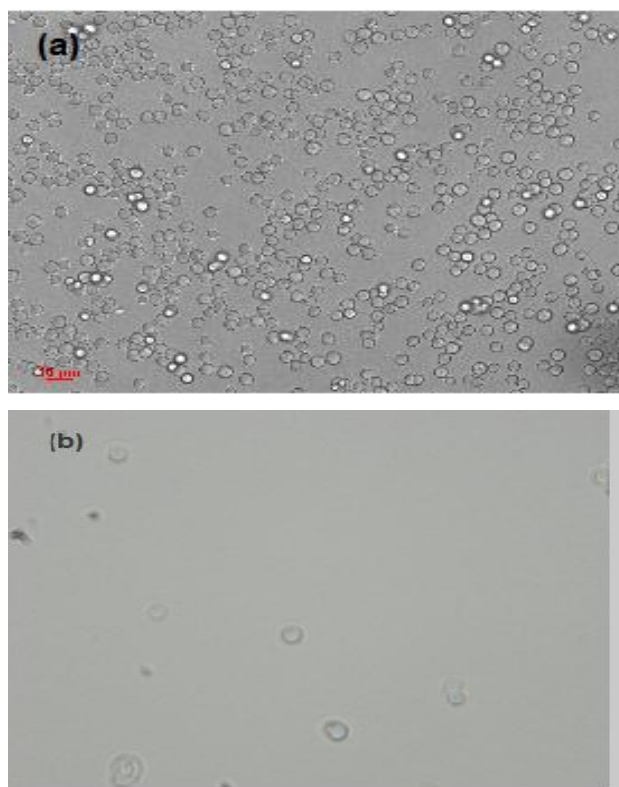


Figure 4: Photos of CaCO_3 microparticles (a) and the microparticle-based polyelectrolyte $(\text{PSS}/\text{PAH})_5$ (b). Optical microscopy, 100 X magnification.

Scanning Electron Microscopy: SEM was utilized to demonstrate the morphology difference between CaCO_3 core and microcapsules, size of them and precipitation of BSA on the capsules as shown in (Fig 5).

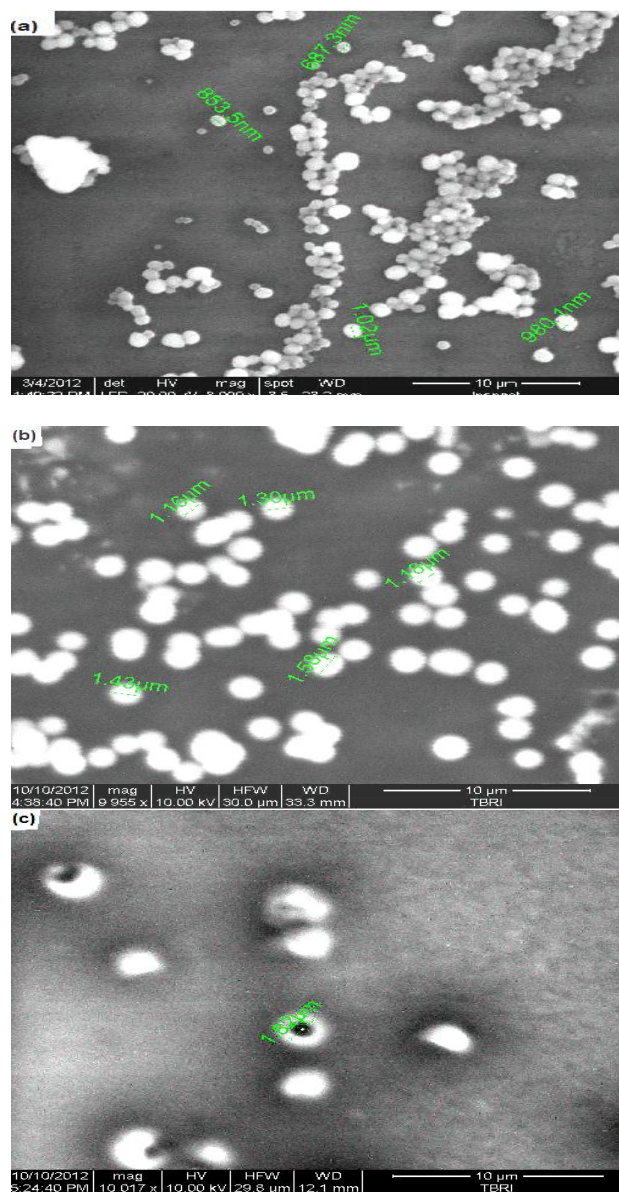
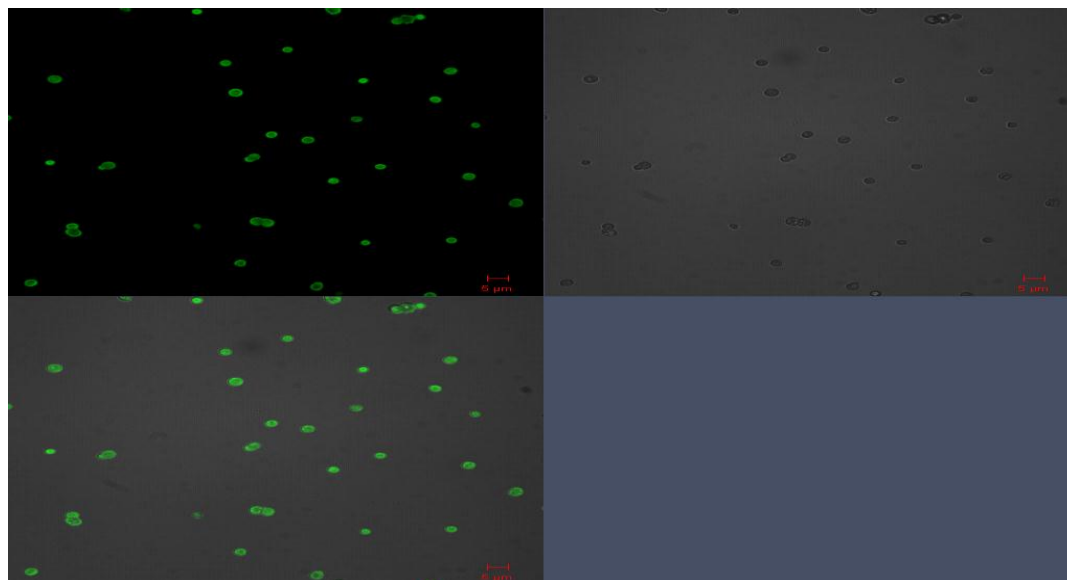
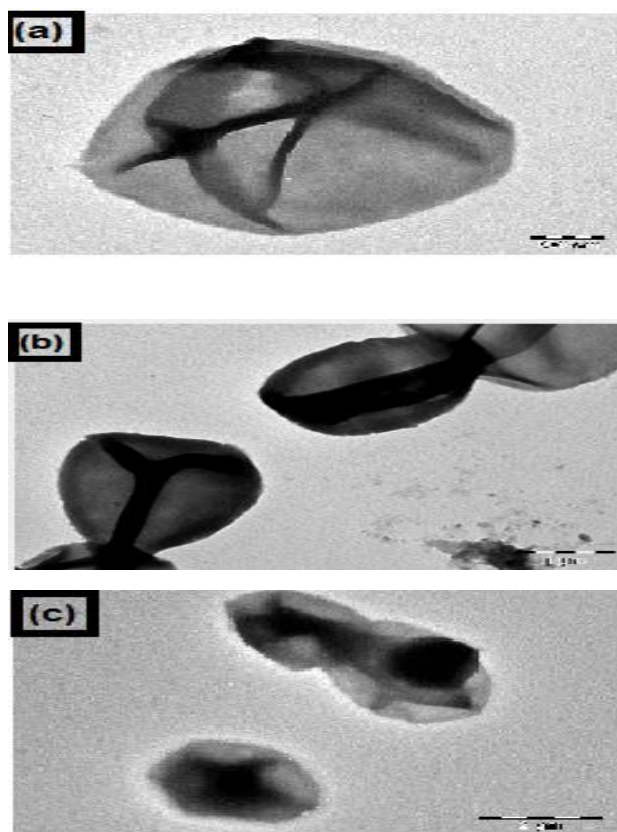


Figure 5: photos of CaCO_3 microparticles (a) aggregated with small size, (b) non-aggregated and CaCO_3 microcapsules (c) obtained by scanning electron microscope. Those differences (shape and size) between (a) and (b) are due to the different conditions used during the synthesis of the microparticles including stirring speed and room temperature.

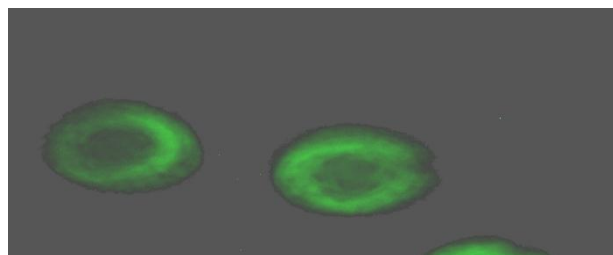
Table 1: Cumulative release of BSA-FITC from PMLCs under different pH values

PH	Initial concentration of BSA-FITC	Concentration of encapsulated protein	Amount of protein released after 30min	Amount of protein released after 90min
PBS (7.4)	50 μ M	39.4 μ M	44%	77%
HCL(5)	50 μ M	39.4 μ M	23%	56%
HCL(1)	50 μ M	39.4 μ M	14%	33%

**Figure (8):** fluorescence images typical CaCO_3 capsules with encapsulated BSA, green colour in the image is due BSA which was fluorescently labelled with FITC adsorbed on cores.**Figure (6):** TEM images of CaCO_3 microcapsules with collapsed layers (A) The hollow capsules

are collapsed completely due to the absence of templated cores and the evaporation of water (B,C) The dark shadows in the capsule centers undoubtedly depict the existence of BSA that deposited between the polyelectrolyte layers after dissolution of CaCO_3 particles .

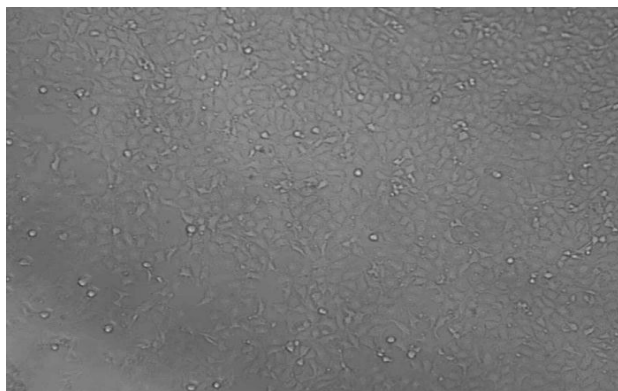
Transmission electron microscopy: TEM was utilized to demonstrate the precipitation of BSA within the microcapsules. The morphology changes between cores and the microcapsules as shown in (Fig 6).

**Figure (7):** Image of CaCO_3 microparticles by fluorescence microscope after incubation with FITC- BSA.

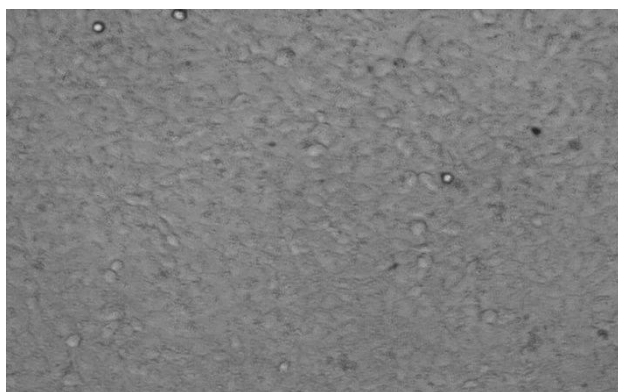
Fluorescence microscopy: was used to determine the loading of BSA-FITC in to the CaCO_3 particle and microcapsules.

Effect of the synthetic microcapsules on cell viability:

The effects of the PSS/PAH microcapsules and PAH/PSS microcapsules on the viability of MDBK cell line were examined by the MTT assay. While PSS/PAH microcapsules are inexpensive and straight forward to prepare as well as exceptionally stable, (Figure. 9a) shows normal MDBK cell line, (figure. 9b) shows image of 10^5 /ml cells incubated with 10^7 /ml 1-4 μm microcapsules, indicate little decrease in cell viability after incubation with the microcapsules (1-4 μM), the present results indicate that MDBK cells cultured with 1-4 μm PAH/PSS microcapsules have viability percentage ranging between 83% to 94% while cells cultured with 1-4 μm PSS/PAH microcapsules have viability percentage ranging between 79% to 92% as shown in (figure 9c). With increasing the microcapsules concentration, cell viability decreased due to decreasing the conversion of MTT in to the formazane crystals by mitochondrial reductase enzyme producing by viable cells.



(a)



(b)

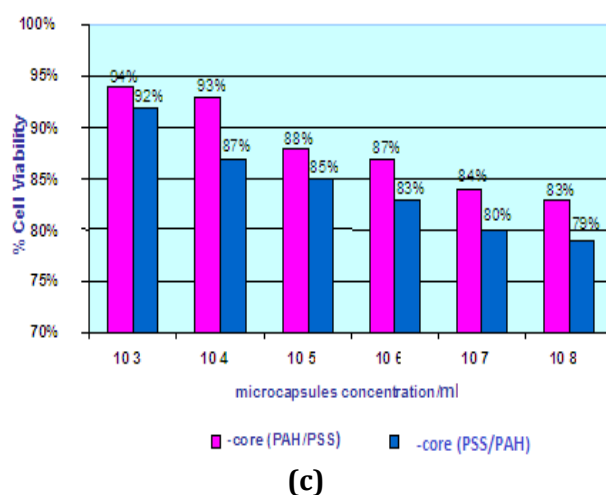


Figure (9): (a) show MDBK cell sheet after incubation 24 h with MEM Earle's medium, (b) MDBK cell line incubated with concentration 10^8 of the microcapsules by inverted microscope (c) Number of viable cells relative to cells cultured in the absence of microcapsules as measured by MTT assay as a function of microcapsules with different concentration.

DISCUSSION:

CaCO_3 microparticles were used as templates for the synthesis of hollow microcapsules using the LbL technique [25]. The quality of the resultant microparticles was found to be strongly dependent on the experimental conditions such as type of salts used, their concentration, pH values of (CaCl_2 , Na_2CO_3 , PSS, PAH), room temperature, rate of solution mixing and intensity agitation of the reaction mixture [26,27]. These microcapsules were obtained by first adsorbing positively and negatively charged polyelectrolytes namely the PSS/PAH pair onto a CaCO_3 core. After coating, the CaCO_3 cores were dissolved in EDTA solution. EDTA sequestered Ca^{2+} ions, leading to the production of hollow microcapsules made of five bilayered (PSS/PAH) loaded with BSA with diameter similar to that of the starting CaCO_3 cores (1-4 μm) as shown in (figure 4a,b). BSA as protein model was loaded into CaCO_3 particles during mixing CaCl_2 and Na_2CO_3 solutions by coprecipitation method (preloading method) [28]. The CaCO_3 microparticles obtained by this simple route are uniform and homogeneously sized non-aggregated and highly porous spheres (Fig. 4 a).

Our results are in agreement with Volodkin *et al* who reported that more than 80% of just-prepared porous CaCO_3 microparticles without dispersants recrystallized after storage overnight in water [29]. PSS possess functional groups on its

long hydrophilic chains, which can complex with Ca^{2+} and disperse them to avoid agglomerating when calcium carbonate particles growing. In the presence of PSS, the polydispersity of the calcium carbonate particles was decreased and more homogeneous calcium carbonate particles with the size around $4\mu\text{m}$ were obtained [30].

During crystallization and formation of CaCO_3 particles, BSA-FITC ($50\mu\text{M}$) solution was added to salt mixing solutions. The resultant particles were spherical. One observed capture and spontaneous accumulation of the labeled macromolecules within the particle interior as shown in (Fig. 7) and by calculating the concentration of non-adsorbed BSA ($6.9\mu\text{M}$) in the supernatant, we determined the concentration of Adsorbed BSA on the particles which equal to $43.1\mu\text{M}$. This result mean high encapsulation efficiency of BSA-FITC in the preloading method as shown in (Fig. 1). Our results are in line with Volodkin *et al* who observed that a significantly large adsorption of protein on the surface of CaCO_3 particles even when both species possess the same sign of charge. Under PH effect, concentration of post-loaded protein is $39.4\mu\text{M}$, this result main that changing PH effect on charge balance of the polyelectrolytes and causing disassembly of capsules layers leading to high loading of protein in to the capsules. Our results are in line with Zhao and Li who found that „more FITC-BSA was loaded into the microcapsules at a lower pH [31] and Petrov *et al* who found that polyelectrolyte capsules composed of weak polyelectrolytes are responsive to the pH of the environment [32].

The release rate of BSA was higher at a higher pH. 77% of BSA was released at 90 min at pH 7.4, and by contrast, only 56% and 33% of BSA were released at pH 5.0 and 1.0, respectively. The release (e.g., at 30 and 90 min) was significantly different between pH values 7.4, 5.0, and 1.0 (Fig. 2). Our results are in line with many authors, Sui and Schlenoff who found that multilayers composed of weak polyelectrolytes can be affected and eventually destroyed at extreme pH conditions. This phenomenon is due to the pH-induced imbalance of charges, and they ultimately lead to the permeability change of the capsule walls [33], Tian *et al.*, who found that the synergetic controlled release of BSA-FITC can be triggered very well by bio-stimulus – DNA nuclease recognition capsule decomposition, as well as pH value and salt concentration [34]. Zeta analyzer measurements showed that CaCO_3 microparticles containing BSA adsorbed on it

had negative charge -21.7mV and the microcapsules with $(\text{PAH}/\text{PSS})_5$ exhibited a value of -25.7mV as shown in (Fig. 3).

SEM was utilized to demonstrate the morphology difference between CaCO_3 core and microcapsules, size of them and precipitation of BSA on the capsules, (Fig. 5) shows microphotos of CaCO_3 microparticles (a) aggregated with small size, (b) non-aggregated obtained, those differences between a and b are due to the different conditions used during the synthesis of the microparticles including stirring speed and room temperature and (Fig. 5c) shows microphotos of CaCO_3 microcapsules obtained by scanning electron microscope. TEM was utilized to demonstrate the precipitation of BSA within the microcapsules. The hollow nature of capsules is revealed in Fig. 6a [35]. The dark shadows in the capsule centers undoubtedly depict the existence of BSA as shown in Fig. 6 b and c. Fig. 8 presents typical CaCO_3 capsules with encapsulated BSA-FITC, our results are in accordance with Stein *et al.*, who found that in the case of CaCO_3 capsules the most of encapsulated material is condensed on inner side of the capsule matrix wall, meaning that BSA is not homogeneously distributed in the capsule interior [36].

MTT assay was used for determination of the effect of polyelectrolyte microcapsules that end with PSS or PAH on viability of MDBK cell line, MTT system measures the activity of living cells via mitochondrial dehydrogenase activity. Several studies have determined the effect of the PMLCS on different types of cell lines but MDBK cell line was not used before. The viability was determined from the equation for the different concentration of microcapsules, Fig. 9a show MDBK cell line after incubation 24 h with MEM Earle's medium, Fig. 9b indicate little decrease in cell viability after incubation with microcapsules, Fig. 9c shows cell viability as a function of different concentration for 1-4 μm PSS/PAH microcapsules and PAH/PSS microcapsules. With increasing the microcapsules concentration, cell viability decreased due to decreasing the conversion of MTT in to the formazane crystals by mitochondrial reductase enzyme producing by viable cells, this mean that capsules ended with PSS as outermost layer have less effect on the viability of this cells due to PSS is negatively charged so that the capsules remain on the top of cell line and decrease in viability occur due to mechanical stress for competition with cells for surface area but in the case of PSS/PAH capsules

viability decrease due to PAH is positively charged and penetrate inside the cells and caused decreasing in cell proliferation.

Our results do not agree with De Koker *et al.*, who found that PSS/PAH microcapsules are inexpensive and straight forward to prepare as well as exceptionally stable, their use in *in-vitro* studies has been limited by concerns about cytotoxicity, particularly due to the presence of the high concentration of sulfonic [37]. While we are in accordance with many authors including Tryoen-Toth *et al.*, who found that PSS did not affect cell health when used as the outermost layer of a planar multilayer film, plating cells on a film of a particular material may not induce the same effects as culturing cells with microcapsules fabricated of identical material [38].

CONCLUSION

It could be concluded that the very easily prepared, stable, prototypical microcapsules composed of PSS/PAH are useful drug delivery agents for proof of principle drug delivery studies.

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