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LAYER BY LAYER ASSEMBLY ON THE SURFACE OF NANOPARTICLE FOR CHEMOTHERAPEUTIC DRUG LOADING

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Keywords:

Polystyrene beads, Poly diallyl dimethyl ammonium chloride, Poly (sodium 4-styrene sulfonate), Doxorubicin, Polyelectrolytes

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ABSTRACT: In the present investigation, fluorescently labelled sulfated polystyrene beads were coated with oppositely charged polyelectrolytes {*i.e.* Poly diallyl dimethyl ammonium chloride, Poly (sodium 4-styrene sulfonate)} through an electrostatic interaction by using layer by layer assembly method. Doxorubicin is a model anticancer drug with protonatable amine group that acquires net positive charge in an acidic environment. The positively charged doxorubicin was electrostatically bound onto the surface of polystyrene beads coated with Poly (sodium 4-styrene sulfonate) as an outer layer. This model drug loaded nanocarrier was characterized by optical spectroscopy. In addition, gel electrophoresis principle was used to track the polyelectrolyte coating on the surface of the negatively charged polystyrene beads. The layer by layer method of coating drug molecule on the surface of polyelectrolyte coated nanoparticles was also confirmed with size and zeta potential measurements by using dynamic light scattering. The correlation between the movement of polyelectrolytes coated nanoparticles in gel electrophoresis and size of the nanoparticles measured by dynamic light scattering were also studied. The results suggest that layer by layer method can be used as a versatile method to load drug molecule on the nanoparticles surface.

INTRODUCTION: Nanoparticles (NPs) in the size range of 1 - 100 nm have unique properties that are distinct from that of individual atoms and bulk materials [1]. As a result of this, NPs have found application in a wide variety of fields ranging from drug delivery [2, 3], catalysis [4], solar energy [5], electronics [6], diagnostics [7, 3] and combination of therapy and diagnostics *i.e.* theranostics [8].

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Among the various applications, the use of NPs for drug delivery is an exciting area of research and has found a niche in cancer therapy [9]. Considerable research in this area has explored the use of nanocarriers to selectively deliver cancer drugs [10], especially hydrophobic varieties, to tumors for effective cancer therapy.

There are several types of nanocarriers such as liposomes [11], micelles [12], nanoemulsion [13], polymeric NPs [14], dendrimers [15], solid -lipid NPs [16], inorganic NPs [17] and mesoporous silica [18] to name a few that have been explored. Each of these nanocarriers has its own advantages and disadvantages. One of the characteristics of any NPs is the coating of surfactants / stabilizers on its

surface. The major role of this is to provide stability, functionality, and solubility [19]. There are several methods used to incorporate the surfactants on the NP surface. This could be introduced during the synthesis / fabrication or as a post-synthesis, surface modification technique [20]. Depending on the type of nanocarrier, either of these methods could be used. Among the several methods that have been used for post-synthesis surface modification, Layer-by-layer (LbL) technique is one of the versatile and simple methods [21]. In this technique, polyelectrolytes (PE) both positively charged and negatively charged are sequentially coated on NPs surface. This technique allows for nanometer thick coating on the NPs surface and the thickness of the coating can control the overall size of the NPs [22].

Here, we present the work on PE coating on polystyrene (PS) beads by the LbL technique. The positively charged PE or polycation is poly diallyl dimethyl ammonium chloride (PDDA) and negatively charged PE or polyanion is poly (sodium 4-styrene sulfonate (PSS). The PS beads are fabricated with sulfate group on the surface resulting in an inherent negative charge to the PS beads surface. Hence for the surface modification of layer by layer technique, the PS beads are first coated with PDDA and subsequently with PSS. This process is continued until the desired thickness of the coating is achieved. In this paper, we also demonstrate the use of gel electrophoresis as a simple and effective method to monitor the change in surface charge due to PE coatings. The LbL technique also offers an elegant and simple system to load drug molecules on the surface of NPs by trapping them between the PE bilayers.

In this paper, model drug, doxorubicin (DOX) was used for coating / loading on PS beads using LbL technique. DOX in acidic pH gets protonated and develops a net positive charge and tends to bind on the negatively charged surface. PS beads coated with PSS can bind DOX on its surface and subsequently protected by a layer of PDDA. This method ensures the trapping of DOX between the 2 layer of PE *i.e.* PSS and PDDA. The release of drug from these nanocarriers is by diffusion through the bilayer and / erosion of bilayer depending on the biological environment it is introduced [23].

MATERIALS AND METHODS:

Materials: Sulfate modified fluorescent polystyrene (PS) beads 2.5 wt% (Sigma-Aldrich), Poly diallyl dimethyl ammonium chloride (PDDA) average MW 100000 - 200000 (Sigma-Aldrich), Poly (sodium 4-styrene sulfonate (PSS) average MW ~70000 (Sigma-Aldrich), Sodium chloride (Merck), Agarose (MBL-013, DNA Amplification Kit, Aristogene Biosciences), tris acetate EDTA buffer (MBL-013, DNA Amplification Kit, Aristogene Biosciences), Doxorubicin hydrochloride (Gift sample from Natco Pharma Limited, Hyderabad, India).

Methods:

Surface Modification of Negatively Charged Fluorescent Sulfated Polystyrene Beads with PE using LbL Technique: Surface modification of negatively charged fluorescent sulfated PS beads with PE was done by using LbL method with slight modification [24]. 25 µl of PS beads (0.625 mg) from 2.5 wt% stock solution was dispersed in 750 µl of distilled water (pH 6). 1 ml of 1mg/ml concentrated PDDA solution prepared in 0.5M NaCl (pH 6) was added and allowed to react with gentle agitation for 15 min for adsorption of PE on NPs surface. The sample was centrifuged at 14000 rpm for 1hour at 15 °C. The pellet obtained was washed with pH adjusted distilled water (pH = 6) to remove the unadsorbed PE from the surface of NPs and subjected to the same centrifugal conditions.

PDDA coated PS beads pellet was dispersed in 750µl of distilled water (pH = 6) and to this, 1ml of negatively charged PSS (1mg/ml) prepared in 0.5M NaCl (pH = 6) was added and was allowed to react for 15 min for adsorption of PE with gentle shaking. After adsorption, it was subjected to the same centrifugal conditions as mentioned above. The same above steps were repeated for multiple layers of PE on PS beads.

Drug Loading onto PE Coated PS Beads: The drug loading was done onto PE coated PS beads based on the procedure developed by Zhao *et al*., with slight modification [25]. 490 µg and 90 µg of DOX (1 mg/ml) were made up to 2 ml using 0.25M NaCl (pH 5.5). Before loading of DOX onto NPs, the concentration of DOX was determined by using spectrofluorometer (Elico SL174). 2 ml of DOX in 0.25M NaCl pH5.5 was added to NPs pellet [*i.e.* PS

beads / $(PDDA/PSS)_2$] with PSS as the outer layer and transferred to 15 ml Falcon tube. The PE-coated NPs and DOX suspension were subjected to gentle agitation at room temperature for 30 min which allows adsorption of DOX on to PE-coated NPs. After incubation, 2 ml of the above suspension was centrifuged (Remi C-24BL ultra cooling) at 14000 rpm, 15 ºC for 1 hour. After centrifugation, the supernatant was collected. The DOX concentration in the supernatant was determined using spectrofluorometer.

In order to cover DOX-loaded on PS beads, a protective layer of polycation *i.e.* PDDA was added. The PE was allowed to react for 15mins for adsorption and then centrifuged at 14000 rpm, 15 ºC for 1 hour. The supernatant was collected and unadsorbed DOX from NPs surface present with unadsorbed PDDA was calculated using spectrofluorometer. The fluorescence of DOX was measured at excitation wavelength - 485 nm, emission wavelength - 587, excitation bandpass - 10 nm, and emission bandpass - 10 nm.

Based on subtraction method, the following equation was used for calculating the concentration of drug loaded onto PE modified PS beads.

Concentration of drug loaded onto NPs = Mass of drug before loading - Mass of drug after loading - Mass of drug in the unadsorbed PE (PDDA) solution.

Characterization of Surface Coating of PE on PS Beads by Agarose Gel Electrophoresis: 0.5% and 1% of agarose gels were prepared by dissolving 0.4 gm and 0.8 gm of agarose in 80 ml of 0.5X tris acetate EDTA (TAE) buffer (pH 8) and it was heated until agarose dissolved in the buffer. The gel holding boat was taped at the edge of the boat to avoid leakage of the gelling solution. The hot gelling solution was allowed to cool and then transferred into gel holding the boat, placed with 8 wells comb in the middle of the boat.

It was allowed to settle for 30 min without disturbing to complete the solidification of gel. 250 ml of 0.5X TAE buffer (pH = 8) was added to the electrophoretic tank. 25 µl of the NPs and PE coated NPs were loaded into the well with and without bromophenol blue for 1% and 0.5% gels.

The gels were run in a horizontal electrophoresis system for 30 mins at 150 volts. Photos captured in gel box, with 302 nm excitation, using a digital camera.

Characterization of Surface Modification of PS Beads and Drug Loading on Surface Modified PS Beads using Fluorescence Measurements: The PE coated PS beads and DOX loading on PE coated PS beads were characterized using fluorescence spectrometer. The NPs and drug-loaded NPs were dispersed in 2 ml of distilled water (pH = 6). The fluorescence spectrum of DOX was taken at excitation wavelength - 485nm, emission wavelength - 587 nm, excitation bandpass - 10 nm, and emission bandpass - 10 nm.

Characterization of Drug Loading on the Surface Modified PS Beads using UV Spectrophotometer: The PE coated PS beads loaded with DOX was investigated using UV-spectrophotometer (UV-1800 Shimadzu). The drug loaded nanocarrier pellet was dispersed in 2 ml of distilled water (pH 6). The UV spectra were taken in the range 200 nm to 700 nm.

Determination of Particle Size and Surface Charge of Polyelectrolyte-Coated PS Beads and Drug Loaded PS Beads using Dynamic Light Scattering: The particle size and zeta potential of PE coated PS beads and DOX-loaded PS beads were characterized by using dynamic light scattering (Malvern Nano ZS 90 instrument, UK). The pellets of NPs were dispersed in 1ml of pH 6 adjusted distilled water. About 20 µl of NPs and PE coated NPs were diluted to 1 ml with distilled water (pH = 6). The size of the particle was measured at 90º and temperature at 25 ºC. The average count rate was represented in kCPS. The size distribution of the NPs and PE coated NPs were measured by calculating polydispersity index.

The stability of the NPs and PE coated NPs were measured by surface charge of the NPs based on the electrostatic repulsion. The samples which were used for particle size determination were also used for zeta potential measurements. The sample was added to the cuvette that was connected to the electrode. The electrophoretic mobility of NPs was measured by the instrument and then converted to zeta potential values.

RESULTS AND DISCUSSION:

Characterization of Surface Modification of Fluorescent Sulfated PS Beads Coated with PE by Agarose Gel Electrophoresis: The PS beads are coated with sulfate groups and results in an inherent –ve charge on the surface of fluorescently labeled PS beads. Using LbL technique oppositely charged polycation followed by polyanion can be sequentially added. Agarose gel electrophoresis method was used to characterize the PE coatings.

The surface modified PS beads were subjected to electrophoresis on 1%, 0.75% and 0.5% agarose gel. In 1% agarose gel, PS beads, and PE coated PS beads was unable to move from the sample well. In 0.75% agarose gel, the samples were unable to migrate as similarly to 1% gel. This is due to the smaller pore size with a higher percentage of agarose. The PS beads and PE coated PS beads in the sample well was able to move towards the electrodes in 0.5% agarose gel, due to the larger pore size with the lower percentage of the gel. But the 0.5% gel was flimsy compared to 0.75% and 1%.

The fluorescent NPs bands were visualized under UV transilluminator at 302 nm. The samples for agarose gel electrophoresis were prepared with and without tracking dye *i.e.* bromophenol blue for loading into wells of the gel. Bromophenol blue is negatively charged in nature. The PS beads coated with PDDA as positive charge outer surface was bound to negatively charged dye, resulted in fluorescence quenching of PS beads when observed under UV transilluminator. The fluorescent PS beads and PE coated PS beads with outer negative charge did not react with the dye due to negative charge repulsion. Based on the results obtained, the NPs samples were run in 0.5% agarose gel without the tracking dye, bromophenol blue.

The gel electrophoresis to track the surface coating is shown in **Fig. 1**. PS beads and PE coated PS beads were run on 0.5% agarose gel for 30 min at 150V. Sulfated PS beads are –vely charged and hence move towards +vely charged electrode (lane 1). When these PS beads are coated with +vely charged PDDA as an outer layer, this resulted in net +ve surface charge and hence moved towards the –ve electrode (lane 3). Subsequently, when the PDDA coated PS beads are coated with PSS, this again results in a net –ve charge on the surface resulting in its migration towards +ve electrode (lane 5). The following results confirmed that the surface of PS beads was modified with oppositely charged PE by LbL assembly technique.

As seen in lane 1, the –ve charged PS beads are small in size and moves the greatest distance and as expected moved towards the +ve electrode. When this is coated with the first layer of polycation it develops a net +ve charge and hence moved towards the –ve electrode. As the PE is coated on PS beads, it results in an increase in particle size and hence distance of movement in the gel is relatively less when compared to blank NPs. Similarly, when the subsequent polyanion is added the nanoparticles with net –ve charge moved towards the +ve charge electrode and due to the compacting of PE bilayers, the particle size decreases and the distance of NPs movement is longer.

The surface charge of the NPs is controlled by the outermost PE layer and is correspondingly attracted to the electrode of the opposite charge. Thus the sequential deposition of PE layers can be monitored using the agarose gel electrophoresis method. Hence, we have demonstrated the use of agarose gel electrophoresis as a simple, low-cost and reliable technique to monitor the surface modification process by LbL technique.

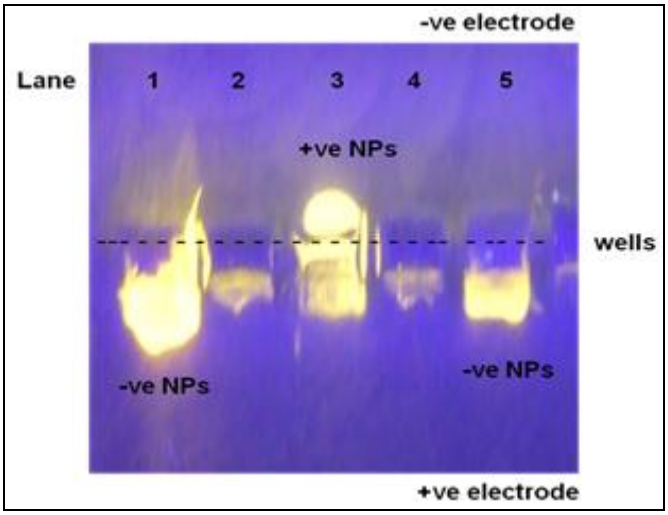

FIG. 1: AGAROSE GEL ELECTROPHORESIS OF PE MODIFIED PS BEADS. THE DASHED LINE IN THE MIDDLE INDICATES THE POSITION OF THE GEL WELLS. THE THREE LANES (1, 3 AND 5), LOADED WITH PS BEADS, PS BEADS+[(PDDA/PSS)$_3$PDDA] AND PS BEADS+(PDDA/PSS)$_3$ RESPECTIVELY

Drug Loading onto PE Coated PS Beads: The model drug, DOX has a protonatable amine group in the sugar moiety of its structure that gets protonated and acquires a net positive charge at mild acidic environment by converting NH_2 to NH_3^+. DOX was loaded on to the outermost PSS layer of the PE modified PS beads through an electrostatic interaction. The electrostatic interaction was generated between sulfate group of PSS and NH_3^+ group of DOX to facilitate drug loading on the outermost PSS layer of the PS beads. The schematic representation of drug loading is shown in **Fig. 2.** DOX being positively charged would bind to a negatively charged PE surface. As shown in **Fig. 2** the negatively charged PS beads were coated with two bilayers of PDDA/PSS PE coatings. To the outermost PSS layer DOX is reached. The sample is centrifuged and unreacted DOX in supernatant is collected. The pellet is then reacted with PDDA to ensure the trapping of DOX between the PSS and PDDA layer. This sample is centrifuged and any unreacted DOX in the supernatant is collected. Hence the actual loading of DOX onto the PS beads is calculated based on the subtraction method that is detailed in materials and methods. The amount of DOX-loaded onto PE coated PS beads was found to be 48.7 µg and 38.43 µg when reacted with 490 µl (1mg/ml) and 90 µl (1 mg/ml) respectively. The percentage of drug loading efficiency based on the subtraction method was found to be 9.93% for 490 µl and 42.7% for 90 µl. It is interesting to observe that when higher concentration of drug solution is reacted with the NPs, the percent loading is significantly lower when compared to reacting with lower concentration of DOX solution. Therefore for efficient drug loading lower concentration of drug is suggested to improve drug loading efficiency. **Fig. 2** explains the drug loading mechanism onto PE coated NPs and also describes the mechanism to evaluate the actual concentration of drug loading.

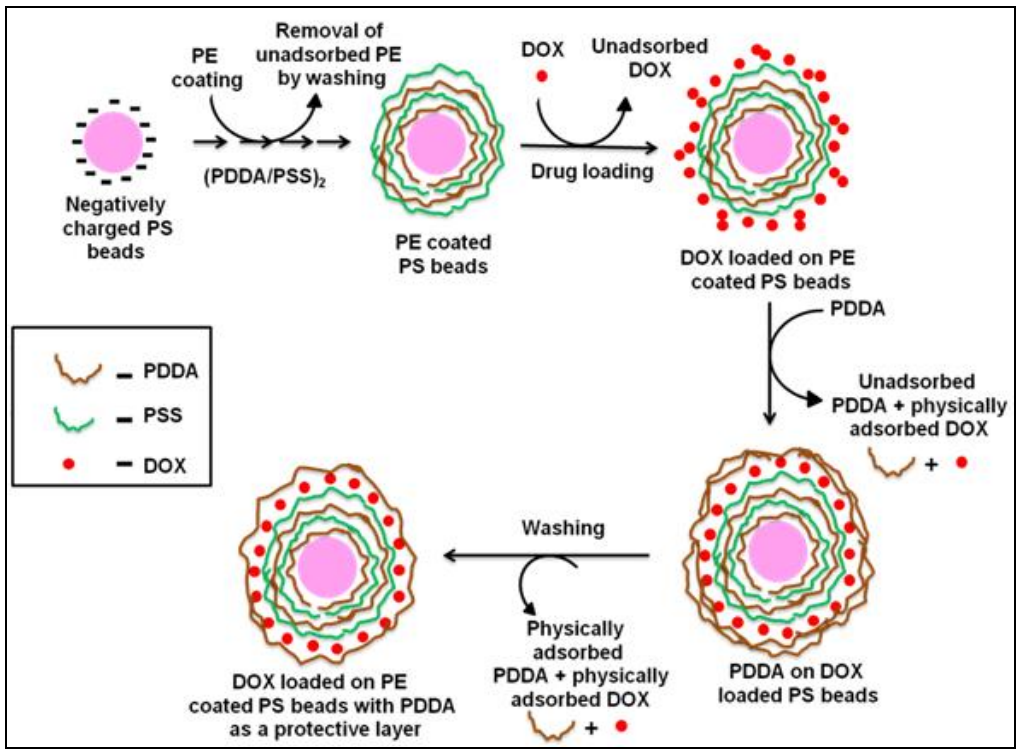

FIG. 2: SCHEMATIC REPRESENTATION OF DOX LOADING ONTO PE COATED PS BEADS

Characterization of Surface Modification of PS Beads by Fluorescence Measurements: The surface modification of fluorescence sulfated PS beads with PE was also investigated by fluorescence spectrometer. The PS beads have a fluorophore in the core which can be excited at λex = 585 nm with an emission peak at λem = 635 nm and another small peak at 732 nm. When PE is coated onto the NPs surface the fluorescence quenching should be affected. The fluorescence intensity of free PS beads at λem = 635nm was 7353.2 mV and it was reduced to 812.8 mV after modifying the surface of PS beads with 2 bilayers of PDDA/PSS.

This change in fluorescent intensity is shown in **Fig. 3**. The decrease in the fluorescent intensity of PS beads after coating with PE revealed that the fluorescence of PS beads was quenched by PE. It is important to point out that in spite of two bilayers of PE, the coating was thin enough to ensure that the fluorescence from the core of PS beads is still detectable. This indicates that the LbL coating on NPs surface results in the formation of a very thin coating on the surface and would not contribute to an overall increase in the size of the NP.

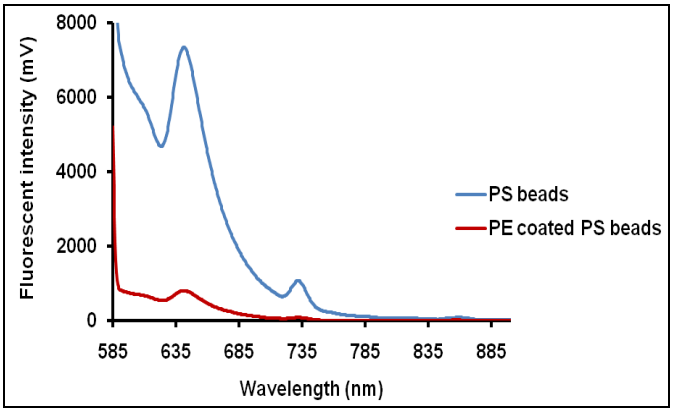

FIG. 3: FLUORESCENCE MEASUREMENTS OF PS BEADS AND POLYELECTROLYTE COATED PS BEADS

Confirmation of DOX Loading onto PE Modified PS Beads by using Spectro-fluorometer: The DOX loading on PE coated PS beads was also characterized by using the fluorescent spectrometer. The fluorescent spectra of DOX-loaded PE coated PS beads was compared with the spectra of free DOX, PE coated PS beads and DOX-loaded PS beads with PDDA as an outer layer. Free DOX has three peaks a doublet at 558nm and 587 nm and a short peak at 642 nm. The PS beads have a fluorescence signal at 635 nm and 732 nm. Therefore when DOX is loaded onto PE coated PS beads the peaks of DOX at 587 nm and PS beads at 635 nm should be seen. As seen in **Fig. 4,** for the sample DOX loaded PS beads a very intense peak from DOX at a 560.5 nm is seen and it is also observed that the doublet hump of DOX is merged into a single peak.

When a PDDA layer is coated onto this layer, the fluorescent intensity drops indicating the quenching of DOX spectrum by outer PDDA layer. The peak from PS beads overlaps with the second peak of DOX at 644 nm. Apart from spectral changes in DOX, the fluorescent spectra of DOX-loaded PE coated PS shown the maximum intensity of 9017.5 mV and it was reduced to 4229.05 mV after coating of PDDA could be observed **Fig. 4**. It is important to point out that the amount of DOX that was reacted with PE coated PS beads was 490 µg and the spectrum was measured immediately after the loading. Hence it also included the freely adsorbed and unreacted DOX on the surface of PE coated PS beads. Therefore, the intensity of this peak was greater than the free DOX spectrum shown for 100 µg sample in **Fig. 4**. This revealed that the fluorescence of DOX was quenched by PDDA. The above results demonstrated that the DOX was loaded onto PE coated PS beads.

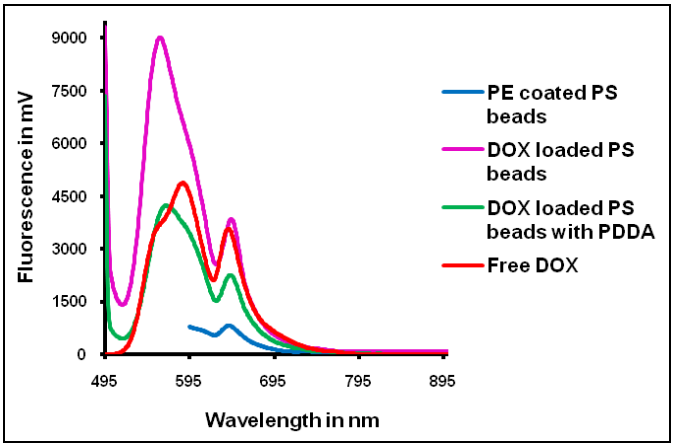

FIG. 4: FLUORESCENCE MEASUREMENTS OF POLYELECTROLYTES COATED PS BEADS, DOX-LOADED PS BEADS AND DOX-LOADED PS BEADS WITH PDDA

Confirmation of DOX Loading onto PE Modified PS Beads by UV Measurements: The DOX loading on to PE coated PS beads was also investigated using UV- spectrophotometer. The UV spectra of DOX-loaded PS beads was compared with PE coated PS beads *i.e.* PS beads with three bilayers of PDDA/PSS, pure PS beads, pure PSS, pure PDDA and free DOX. As shown in the **Fig. 5** PDDA and PSS have characteristic peaks at 214nm and 232 nm respectively. PS beads have absorption maxima at 234 nm shown broad peak and a shoulder in the right side at 293 nm. DOX has a characteristic peak at 480 nm. As expected, no peak was noted for PS beads, PSS, PDDA, and PE coated PS beads at 480 nm.

In the case of DOX-loaded PE coated PS beads, spectral changes in DOX peak maxima was observed. The λ_{max} of DOX-loaded nanocarriers appeared at 513 nm, and a small bump at this range

was observed suggesting that DOX-loaded onto nanocarriers induced a red shift of 33 nm. The choice of polyelectrolytes for the surface coating of nanoparticles was done based on the non interference with the peak of the drug. As observed that the characteristic peaks of PDDA and PSS will not interfere with the DOX peak at 480 nm. Due to this, the loading of DOX onto PE coated PS beads is distinctly visible. It can be hypothesized that if the number of layers of DOX increases, the absorbance will also increase. These results suggest that the drugs having absorbance maxima beyond 350 nm can be used for loading onto this LbL nanocarrier design and observed using UV spectrophotometer.

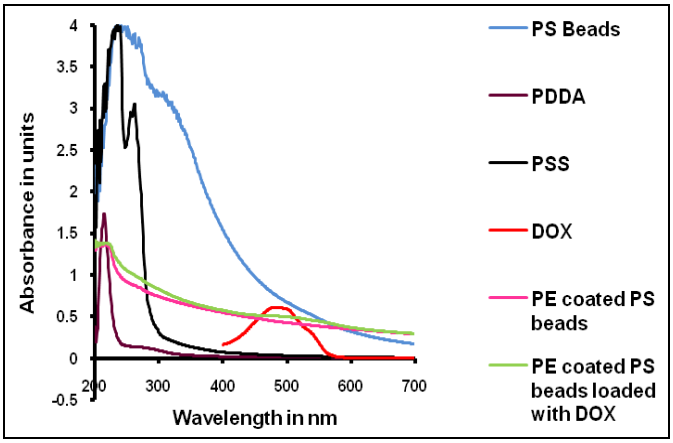

FIG. 5: UV SPECTRA OF DOX-LOADED POLY-ELECTROLYTES COATED PS BEADS, PS BEADS, FREE PDDA, FREE PSS, FREE DOX, AND POLYELECTROLYTES COATED PS BEADS

Characterization of PE Coated PS Beads and DOX-Loaded PS Beads using Dynamic Light Scattering:

Particle Size Measurements: The hydrodynamic size of PE coated PS beads and DOX-loaded PS beads were determined by using dynamic light scattering. The hydrodynamic size of sulfate modified fluorescent PS beads were 67.02 ± 0.98 nm and it increased to 165.35 ± 13.36 nm after coating with the first layer of PE *i.e.* PDDA. The PS beads / PDDA coated with PSS as an outermost layer shown the hydrodynamic size of 110.65 ± 13.93 nm. For the second pair of PE, PDDA/PSS coated on PS beads / $(PDDA / PSS)_1$ shown the particle size of 303.00 ± 21.77 nm and 191.05 ± 7.99 nm for PDDA and PSS layer respectively.

The size of the particles was found to be increased from 191.05 ± 7.99 nm to 339.85 ± 40.9 nm for DOX-loaded PS beads. The increased particle size reveals that the DOX was loaded onto PE coated PS beads. After DOX loading, another layer of PE *i.e.* PDDA was coated to cover DOX layer on PS beads showed particle size of 470.5 ± 21.9 nm **Fig. 6.** The polydispersity index of PS beads and PS beads coated with oppositely charged PE was less than 1. This reveals that the size distribution of the PS beads coated with PE is not in the broad range. It is important to point out that there is a fluctuation in the particle size with the addition of each layer of PE. When the first layer of PDDA is coated on the –vely charged PS beads surface, as expected, the size increase from 67 - 165 nm.

However, when the next anionic PE layer of PSS is added, the decrease in size to 110 nm is due to compacting of the bilayer. This trend is noticed again when the next layer of PDDA is added followed by the PSS layer.

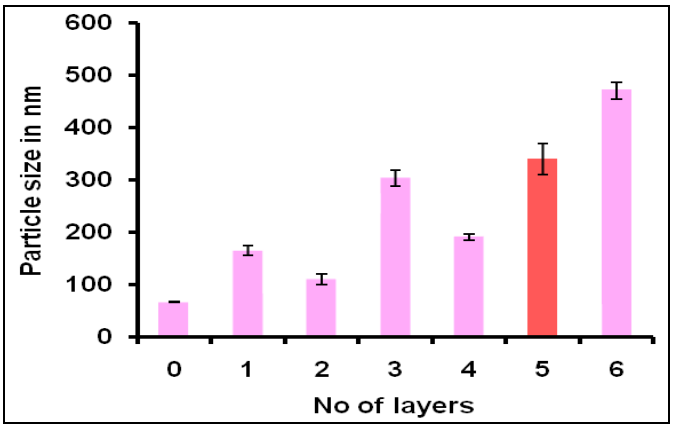

FIG. 6: PARTICLE SIZE MEASUREMENTS OF PS BEADS COATED WITH OPPOSITELY CHARGED POLYELECTROLYTES AND DOX-LOADED PSS BEADS

NOTE:
0 Layer: PS beads;
1 Layer: PS beads/PDDA;
2 Layer: PS beads/PDDA/PSS;
3 Layer: PS beads/PDDA/PSS/PDDA;
4 Layer: PS beads/PDDA/PSS/PDDA/PSS;
5 Layer: PS beads/PDDA/PSS/PDDA/PSS/DOX;
6 Layer: PS beads/PDDA/PSS/PDDA/PSS/DOX/PDDA

Zeta Potential Measurements: The zeta potential measurements of PS beads, PS beads coated with oppositely charged PE and drug loaded PS beads was taken. The sequential coating of PDDA and PSS onto negatively charged fluorescent sulfated PS beads was done using a LbL assembly technique.

TABLE 1: PARTICLE SIZE, ZETA POTENTIAL AND PDI MEASUREMENTS OF PS BEADS, PS BEADS COATED WITH OPPOSITELY CHARGED PES, DOX-LOADED PS BEADS AND DOX-LOADED PS BEADS WITH OUTER LAYER OF POSITIVELY CHARGED PE

S. No.	PS beads coated with Polyelectrolytes	Size (nm)	Zeta potential (mV)	Polydispersity Index (PDI)
1	PS beads	67.02±0.98	-24.4±0.56	0.141±0.02
2	PS beads/PDDA	165.35±13.36	+22.75±0.91	0.378±0.05
3	PS beads/PDDA/PSS	110.65±13.93	-29.55±0.35	0.517±0.09
4	PS beads/PDDA/PSS/PDDA	303.00±21.77	+21.75±0.35	0.602±0.01
5	PS beads/ PDDA/ PSS/ PDDA/PSS	191.05±7.99	-29.15±0.07	0.457±0.15
6	PS beads/ PDDA/PSS/ PDDA/PSS/ DOX	339.85±40.9	-24.9±0.56	0.56±0.21
7	PS beads/ PDDA/PSS/ PDDA/PSS/ DOX/ PDDA	470.5±21.9	17.25±2.33	0.28±0.04

The LbL assembly was associated with the electrostatic interactions between sulfate group of sulfated PS beads, NH_3^+ of PDDA and sulfate group of PSS. PS beads are -vely charged and when coated with +vely charged PDDA, resulted in net +ve charge and similarly coating with –vely charged PSS on the NP surface acquires net -ve charge as mentioned above. The zeta potential of PS beads was -24.4 ± 0.56 mV. The zeta potential of the first pair of PE (PDDA/PSS) coated on PS beads was +22.75 ± 0.91 mV for PDDA and -29.55 ± 0.35 mV for PSS.

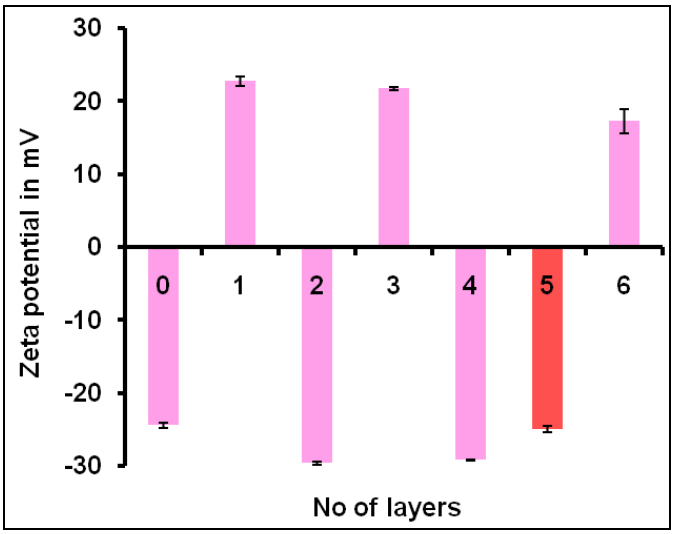

FIG. 7: ZETA POTENTIAL MEASUREMENTS OF PS BEADS COATED WITH OPPOSITELY CHARGED POLYELECTROLYTES AND DOX-LOADED PSS BEADS

NOTE:
0 Layer: PS beads;
1 Layer: PS beads/PDDA;
2 Layer: PS beads/PDDA/PSS;
3 Layer: PS beads/PDDA/PSS/PDDA;
4 Layer: PS beads/PDDA/PSS/PDDA/PSS;
5 Layer: PS beads/PDDA/PSS/PDDA/PSS/DOX;
6 Layer: PS beads/PDDA/PSS/PDDA/PSS/DOX/PDDA

Similarly, the adsorption of the second pair of PE (PDDA/PSS) shows the zeta potential of +21.75 ± 0.35 mV and -29.15 ± 0.07 mV for PDDA and PSS respectively. The zeta potential of DOX-loaded PS beads / $(PDDA/PSS)_2$ was -24.9 ± 0.56 mV. This decrease in the zeta potential confirmed the presence of +vely charged DOX, which was loaded based on electrostatic interaction. The zeta potential acquires 17.25 ± 2.33 mV when it was coated with positively charged PDDA as an outer layer to pack DOX-loaded onto nanocarrier **Fig. 7. Table 1** is a compilation of the particle size, zeta potential, and polydispersity index of bare PS beads, PE coated PS beads and DOX loaded PE coated PS beads.

Earlier in **Fig. 1**, the agarose gel electrophoresis was used as a simple method to demonstrate the process of sequential coating of PE on NPs surface. The PS beads and PS beads with outermost PSS layer moved to the +ve electrode and PDDA outermost layer moved to –ve electrode. This phenomenon of successful sequential coating with polycationic and polyanionic PE was evidenced in **Fig. 1** through gel electrophoresis method and confirmed through zeta potential measurement. Additionally, the principle of gel electrophoresis explains, based on the size, the particles moves forward in the gel. The magnitude of the movement of PS beads, PE coated PS beads in the gel was also calculated. The negatively charged PS beads moved towards the positive electrode and the magnitude of movement within the agarose gel was the maximum because it had the smallest size. When PDDA was coated on the surface of PS beads, it acquired a net positive charge and also increased in size. Hence the magnitude of movement in the gel was approximately half the distance of bare PS beads. However, when PSS layer was added to the PDDA coated PS beads, it results in compacting the bilayer resulting in the particle size.

This decrease in particle size helps to increase the magnitude of movement towards the positive electrode. The magnitude of movement is comparable to that of the bare PS beads. Hence, the electrophoresis method was used to not only to monitor the LbL coating but also to understand the effect of coating on changes in the particle size as well **Fig. 1**. The similar trend was evidently shown in particle size measurement **Table 1.**

LbL technique is a versatile technique has been extensively used for thin films and also for hydrogels, in which drugs have been loaded to facilitate drug delivery. In addition, LbL technique can be used to conform to any shape and size [23]. However, LbL assembly on NPs surface incorporating drug has not been extensively studied. In other LbL-based techniques, NPs are coated with PE and subsequently, the core is sacrificed to create a hollow core inside which drugs are loaded to form drug - loaded NPs [26]. The loading capacity of the drug in hollowed out NPs with LbL coating is controlled by the size of the core NPs. This capacity is predetermined and cannot be altered during the drug loading part. Typically for such method, large particles, usually in the micron size range is used and drug loading of 33 - 52% has been demonstrated [27]. However, in our technique, we have incorporated up to 42% drug loading with just one layer of the drug on the PE coated surface on the core PS beads.

The size of PE coated PS beads is ~350 nm and after coating with an outer layer of PDDA, it increased by about 100 nm. The LbL technique results in the formation of a thin layer of coating on the NPs surface. Fluorescently labeled PS beads were used as a model NPs with emission peak λ_{max} at 635 nm. Upon coating four layers of PE+ one layer of drug $(DOX)^{+}$ one outer layer of PE, the fluorescent signal at 635nm is still visible **Fig. 4**. Hence the LbL technique results in a very thin layer of coating which does not affect the optical property of the inner core. It is important to point out that the drug used here is DOX, which is a highly fluorescent compound with λmax at 587 nm. DOX was introduced by LbL technique and its presence does not affect the fluorescence peak at 635 nm of the core. Hence LbL technique can be used to coat drugs and also simultaneously monitor the optical property of the core.

Therefore, using this method, the concentration of the drug to be loaded can be controlled by the number of layers of drugs coating on the NPs surface and hence depending on the application the concentration of drugs can be altered irrespective of the size of the core NPs. It is important to point out that the PE coating on the surface is extremely thin and adding multiple bilayers with drug molecule sandwich between bilayer does not result in marked increase in particle size. This is evidenced by the observation of fluorescence peak of the inner core of PS beads inspire of multiple layers of PE coatings. It can be inferred that each drug bilayer will increase the size of the NP core by ~ 100 nm and within each core about 40% drug could be loaded.

Hence this is a simple, efficient and a quick method to load chemotherapeutic drug directly onto the surface of the NPs without the necessity to sacrifice the inner core. The choice of drug here was DOX which is a chemotherapeutic agent and also an excellent fluorescent compound. Therefore, this method of LbL can be used to load drugs through several of these bilayers. Inorganic NPs in the form of gold NPs, quantum dots, silver NPs or magnetic NPs have size-dependent properties which can be further exploited using LbL technique to load drugs on their surface without sacrificing the core.

CONCLUSION: In this paper, we have presented a versatile, robust and simple system to load chemotherapeutic drugs on NPs surface through surface modification using LbL technique. The chemotherapeutic drug is trapped within the PE bilayer in the LbL method. The concentration of the drug that can be loaded on the surface of the NP is determined by the number of bilayers that is coated on the NP surface. This allows for the full control of drug loading on the NP surface and can be tailored based on the application.

Furthermore, in this paper, we have demonstrated a simple gel electrophoresis method to qualitatively monitor the LbL coating method and change in particle size due to PE coating. The results from gel electrophoresis method were validated by DLS and zeta potential measurements.

Future Directions: The method of LbL technique has demonstrated an elegant method to load drug

molecules on NP surface by trapping them between the PE bilayer. The release of drug from NP is through diffusion. This release can be further controlled or manipulated by cross linking the PE layers. The frequency of cross linking layers, the extent of cross linking and number of cross-linked bilayers can be manipulated to regulate the drug release.

Among the inorganic NPs, Iron oxide NPs have several advantages as drug delivery, thermal therapy, and contrast enhancing agent. Loading the surface of these iron oxide NPs with chemotherapeutic agents to further improve their application is a new exciting area of research. This is the focus of our group and results of this will be communicated in a future publication.

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