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Surface Modification of Gentamicin-loaded Polylactic Acid (PLA) Microsphere Using Double Emulsion and Solvent Evaporation: Effect on Protein Adsorption and Drug Release Behaviour

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ABSTRACT: *Polylactic acid (PLA) microsphere as a drug carrier has been extensively investigated for drug delivery systems. However, due to its limitation of surface hydrophobicity, surface modifications have been studied to improve its utilisation in tissue engineering applications. In the present study, PLA microsphere loaded with gentamicin (GENMS) was modified to enhance its hydrophilicity by surface treatment with additional of ethanol. Ethanol was applied as a co-treating medium in alkaline hydrolysis of NaOH to assist the hydroxide nucleophilic attack on the ester bond of PLA. Alkaline concentrations of NaOH and NaOH/ethanol was set at 0.15 M, 0.25 M and 0.35 M. After surface treatment, hydrophilicity of GENMS surface were improved significantly whereby contact angle was reduced for about 23.1% and 26.8% for modification using NaOH and NaOH/ethanol, respectively, compared with the neat GENMS. Obvious surface roughness presented by NaOH/ethanol modification improved hydrophilicity of GENMS. As a result, protein adsorption on the GENMS surface treated by NaOH/ethanol were reduced than NaOH modification. Moreover, the highest encapsulation efficiency by NaOH/ethanol modification provided an advantage of co-treating by ethanol and has a greater drug release compared with NaOH modification.*

Keywords: Polylactic acid, microsphere, ethanol, hydrophilicity, modification

1. INTRODUCTION

Local antibiotic release is aimed to prevent implant-associated infections by reducing the bacteria adherence at the implantation site. The utilisation of carriers for local antibiotic release is essential to control drug release at predetermined amount of drug in a predictable manner over a specified time. Microspheres have been extensively studied for the past few decades as a targeted drug delivery device in tissue engineering applications. The use of biocompatible and biodegradable polymers as microspheres have been widely used in drug delivery systems.[1–3] The most commonly used biodegradable polymers were polylactic acid (PLA) and poly(D, L-lactide-co-glycolide) (PLGA) and poly(e-caprolactone) (PCL).[4,5]

Many studies indicate that PLA formulations containing therapeutic agents exhibit no adverse tissue reaction, either locally or systemically when used in therapeutic applications.[6,7] Generally, biodegradable polymeric carriers can be degraded via chemical hydrolysis and easily resorbed or eliminated. PLA is an aliphatic polyester. According to Da Silva et al., PLA is considered biocompatible since there was no toxic or carcinogenic substances release to biological environment during their bulk degradation.[8]

However, the consideration on its surface biocompatibility is very important since the microspheres deal with the interfaces between implanted biomaterial and host environment. It is well known that the surface properties of PLA are relatively hydrophobic resulted to ineffective to interact specifically with cells.[4] It also does not possess any functional groups for the attachment of biologically active molecules. Thus, these shortcomings restricted the application of PLA in bone tissue engineering.

Hydrophobic surfaces are having higher adsorption of proteins and denaturation of proteins at the surface. This leads to exposure of new epitopes which are believed to be a cause of immune reactions towards hydrophobic materials.[9] On the other hand, a highly hydrophilic surface may expel any protein molecules and inhibit protein adsorption. Hydrophilic surfaces are therefore preferred for microspheres aiming on cell interaction in the host implantation.

Many surface modification techniques, such as silanisation, radiation and photo-grafting techniques, and alkali hydrolysis treatment have been developed for improving the cell affinity of polymers.[10–12] Among them, alkali hydrolysis treatment is a feasible and convenient method. After surface hydrolysis of aliphatic polyester, the hydrophilic carboxyl and hydroxyl could be produced with cleavage of the ester bonds. However, strong alkali treatment is accompanied by extended

bulk degradation of the polyester and it was shown that a mild alkali treatment at concentration 0.5 M and above could not break the ester bonds effectively in a short time.[13] Previous study reported that a mixture of sodium hydroxide (NaOH) and acetonitrile can be applied to modify the surface properties of poly(ethylene terephthalate) films and membranes in which acetonitrile was used as a co-treating medium.[14] However, acetonitrile is expensive, toxic and pollution of the environment cannot be neglected. Study by Yang et al. showed that the hydrophilicity of poly (L-lactic acid) or PLLA was improved by indicating of contact angle that lowered about 39° after treating with additional of ethanol in NaOH.[13] In addition, changes in the bulk and surface of microsphere caused by hydrolysis will not only affect the bulk physical properties of the microsphere, but also release the encapsulated drug in the microsphere via diffusion.[7]

Considering mild concentration of aqueous NaOH solution used in previous study, present study is aimed to use low concentration and mixture of aqueous NaOH and ethanol to modify the surface properties of gentamicin GEN-loaded PLA microsphere (GENMS). Here, non-toxic and cheap ethanol was used as co-treating medium. GEN was used in the study due to its most common antibiotics for bone replacement and provides the wide antibacterial spectrum. PLA microspheres were fabricated using single emulsion and solvent evaporation (ESE) technique. The GENMS were produced by double ESE method since this technique can produce microspheres with controlled-release profile using different biocompatible water-insoluble polymers.[15] The changes of surface properties and morphology were investigated by scanning electron microscopy (SEM), water contact angle and surface energy, protein adsorption and drug release profile.

2. EXPERIMENTAL

2.1 Materials

In fabrication of drug-loaded microsphere, PLA microspheres was fabricated by PLA pellet, purchased from Nature Works. Dichloromethane (DCM) was purchased from Merck Millipore and poly(vinyl) alcohol (PVA, 80% hydrolysis) was acquired from Sigma Aldrich. Ethanol (95%), sodium hydroxide (NaOH) and hydrochloric acid (HCl, 37%, fuming acid) from Sigma Aldrich were used to modify the surface of fabricated drug-loaded PLA microspheres. Gentamicin (GEN) reagent solution (10 mg ml^{-1}) as encapsulated drug was purchased from Gibco, Life Technologies. Distilled water was used as liquid medium. Bovine serum albumin (BSA, A2058-1G, 40 mg ml^{-1} water soluble) for protein adsorption test was purchased from Sigma Aldrich.

2.2 Fabrication of Gentamicin-loaded PLA Microsphere

PLA microspheres were fabricated using single emulsion and solvent evaporation (ESE) technique while for GENMS, double ESE was used. In this study, the dispersed phase volume ratio of 1:3 (PLA: PVA) was constructed in fabricating PLA microsphere. Firstly, 2.7 g of PLA pellets was dissolved in 30 ml DCM and followed by dispersion of 1 ml GEN solution (with concentration of 10 mg ml^{-1} or 10000 ppm). This solution is subjected to vigorous homogenisation to yield the primary emulsion. Then the primary emulsion was immediately emulsified into 90 ml PVA solution. The mixtures were stirred at ~1250 rpm for 3 min to form secondary emulsion at room temperature. Then, the speed of the stirrer was decreased to ~250 rpm for overnight to allow the evaporation of DCM. The particles of the PLA were formed at the bottom of the flask and was washed, filtered and dried overnight at room temperature before the fabricated microspheres were collected. Figure 1 shows double ESE process to produce GENMS.

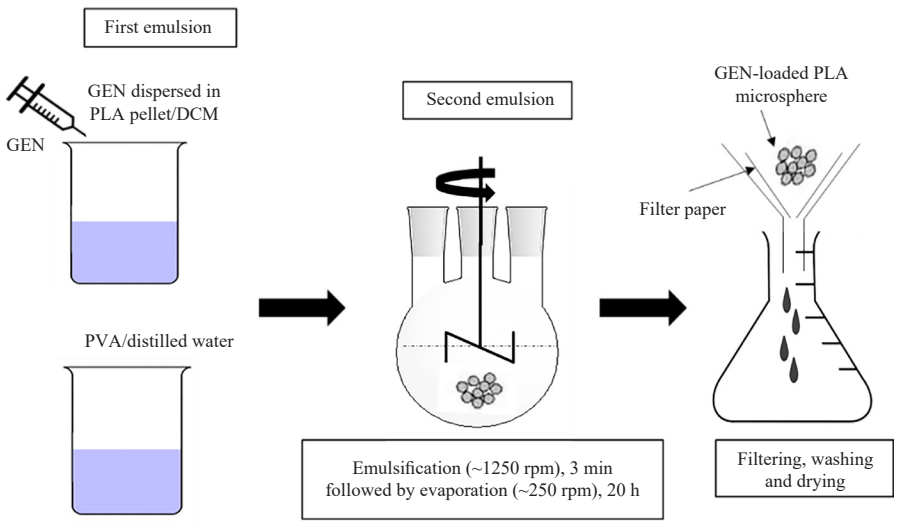

Figure 1: Schematic of double ESE process to produce GENMS.

2.3 Surface Modification by Alkaline Hydrolysis

After preparation of PLA microspheres, surface hydrolysis treatment was performed to modify surface and introduce functionality on PLA microsphere. The microspheres were immersed in NaOH or NaOH/ethanol solutions for 24 h with 0.15 M, 0.25 M and 0.35 M concentration each. After immersion in NaOH or NaOH/ethanol, neutralisation was done by immersing treated microspheres in HCl for 2 h followed by repeated washing with 500 ml distilled water before being

dried for overnight. In this study unmodified PLA microsphere was denoted as neat GENMS while modified PLA microsphere was denoted as modified GENMS (with NaOH or NaOH/ethanol).

2.4 Protein Adsorption on Neat GENMS and Modified GENMS using BSA

The protein solutions were prepared by directly dissolving BSA into deionised water with pH 7.4. The prepared concentration of BSA was 0.5 mg ml^{-1}. Adsorption analysis were carried out by contacting 0.08 g of microspheres (neat GENMS and modified GENMS) with a 10 ml solution of 0.5 mg ml^{-1} BSA concentration in glass vial. After 40 min whereby, the microspheres were pulled down at the bottom of bottle, $C_{initial}$ (C_i) of each sample was measured using UV-VIS at 279 nm of BSA absorbance intensity. After that, the mixtures were left for 24 h for $C_{equilibrium}$ (C_e). The readings of concentration were based on standard curve plotted as shown in Figure 2.

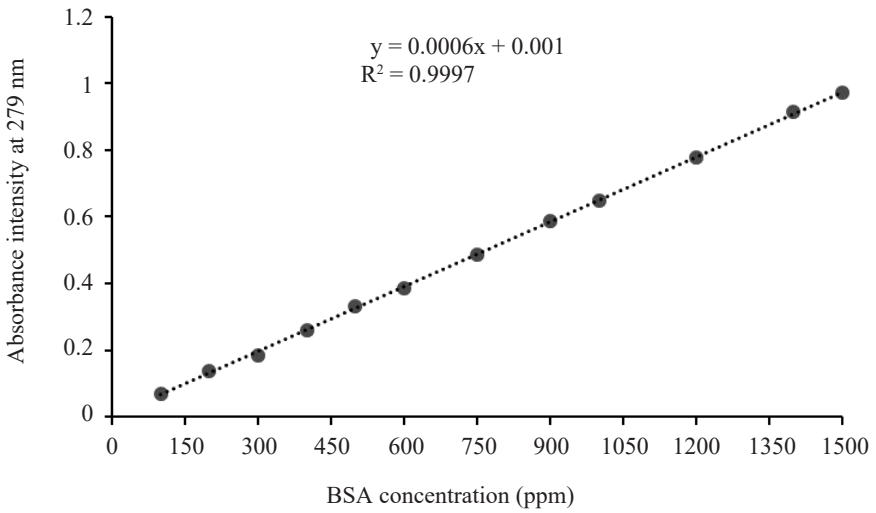

Figure 2: Standard curve of BSA from absorbance intensity at 279 nm and BSA concentration of 100–1500 ppm.

The adsorption amount (q, mg g^{-1}) was calculated based on Equation 1:[16]

$$q = \frac{(C_i - C_e)V}{W} \quad (1)$$

where C_i and C_e (mg ml^{-1}) are the initial concentration of protein and the concentration of protein at adsorption equilibrium, respectively, V (ml) is the volume of protein solution and W (g) is the weight of microspheres.

2.5 Percentage of Encapsulation Efficiency and Drug Loading of Neat GENMS and Modified GENMS

In order to determine the encapsulation efficiency, PLA microspheres loaded with gentamicin at weight 40 mg were fully degraded in 5 ml of 1 M NaOH solution. The samples were left overnight until it was fully degraded and ultraviolet (UV) visible spectroscopy was conducted to determine the percentage of encapsulation efficiency and drug loading of the GEN. Through a scanning of absorbance intensity of GEN at 195 nm wavelength, a standard curve was plotted with known concentration of 10–400 ppm (Figure 3). The calculation of EE% and DL% of the GEN were calculated using Equations 2, 3 and 4.

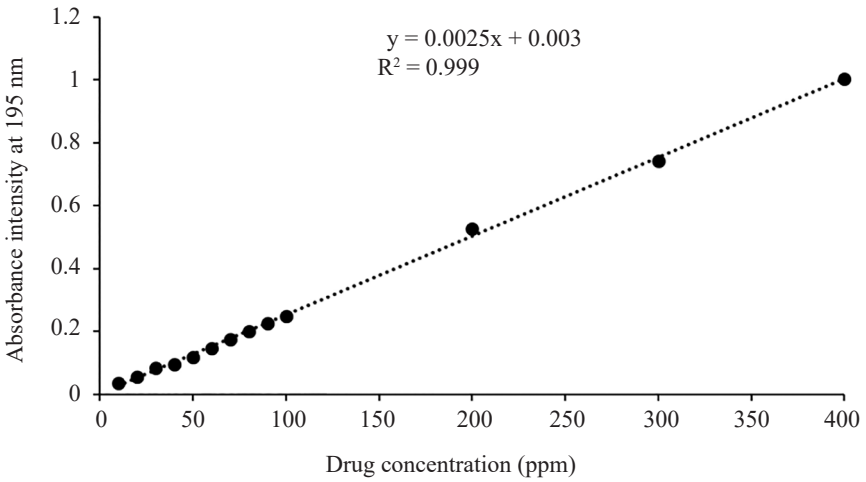

Figure 3: Standard curve of GEN from absorbance intensity at 195 nm and GEN concentration of 10–400 ppm.

$$\text{Encapsulated drug mass (mg)} = \frac{\text{Concentration from data (ppm)} \times 1\text{ ml}}{1000} \quad (2)$$

$$\text{Encapsulated Efficiency (\%)} = \frac{\text{Encapsulated drug mass (mg)} \times 100}{\text{Initial drug mass}} \quad (3)$$

$$\text{Drug loading (\%)} = \frac{\text{Encapsulated drug mass (mg)} \times 100}{\text{Microsphere (g)} \times 1000} \quad (4)$$

2.6 Drug Release Assessment

The drug release of neat and modified GENMS was measured by dispersion of 30 mg PLA microsphere in 0.1 M of 10 ml PBS in glass vial. Then, the glass vials were placed in shaker with 60 rpm at constant temperature of 37°C.

At pre-determined time intervals, aliquots of 3 ml from each sample were extracted and replenished with fresh PBS solution. This is to maintain the total volume of 10 ml. The level of GEN in the elution was detected by UV-Vis spectrophotometer at a wavelength of 195 nm.

2.7 Surface Characterisation

2.7.1 Morphology

Before observation, samples were coated with gold (Au). Surface morphology of PLA microspheres before and after surface modification were evaluated using scanning electron microscope (SEM, Zeiss Supra 55VP, Germany).

2.7.2 Contact angle measurement

Contact angle, θ, is a quantitative measure of wetting of a solid by a liquid. This test is used to determine hydrophobicity or hydrophilicity of the neat GENMS and modified GENMS by using ramé-hart instrument co. with DROPimage software. Distilled water was used as a contact medium.

3. RESULTS AND DISCUSSION

Figure 4 shows the bulk shape morphology of neat GENMS fabricated by double ESE technique. The images show that the method is able to produce almost perfect spherical microspheres with a uniform surface morphology.

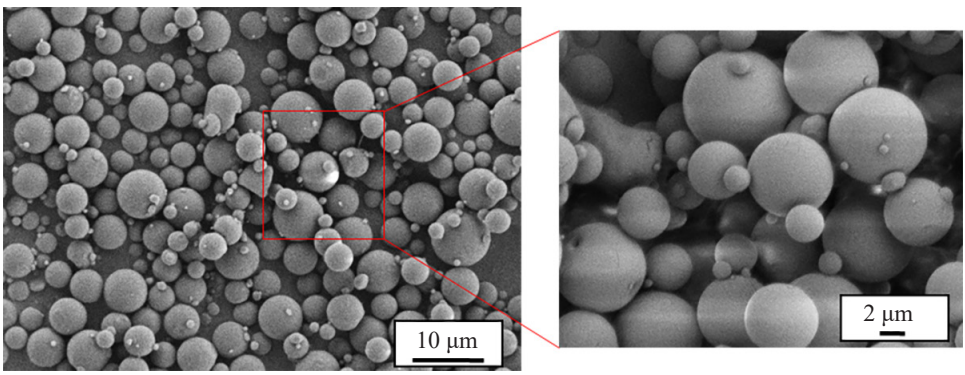

Figure 4: Size of neat GENMS fabricated through double emulsion ESE technique. Images were observed at 1500X (left) and 3000X (right).

Figure 5 shows SEM images of surface morphology of modified GENMS with NaOH/ethanol (b, d and f) were observed much rougher with small pores existed on their surface than those of modified GENMS with NaOH, shown in Figure 4 (a, c and e). As can be seen, at higher alkaline concentration of 0.35 M NaOH and NaOH/ethanol, surface of the microsphere changed to rougher and ripple morphology compared to those 0.15 M and 0.25 M concentrations. In addition, the hydrophilic polar hydroxide and carboxyl groups originating from the cleavage of the surface ester bonds during hydrolysis increased the surface roughness. Swamy et al. reported that the pores on microsphere surface could help in drug release by diffusion mechanism.[15] Furthermore, the roughness of the material surface greatly influence the cell attachment and cell growth on the material.[13]

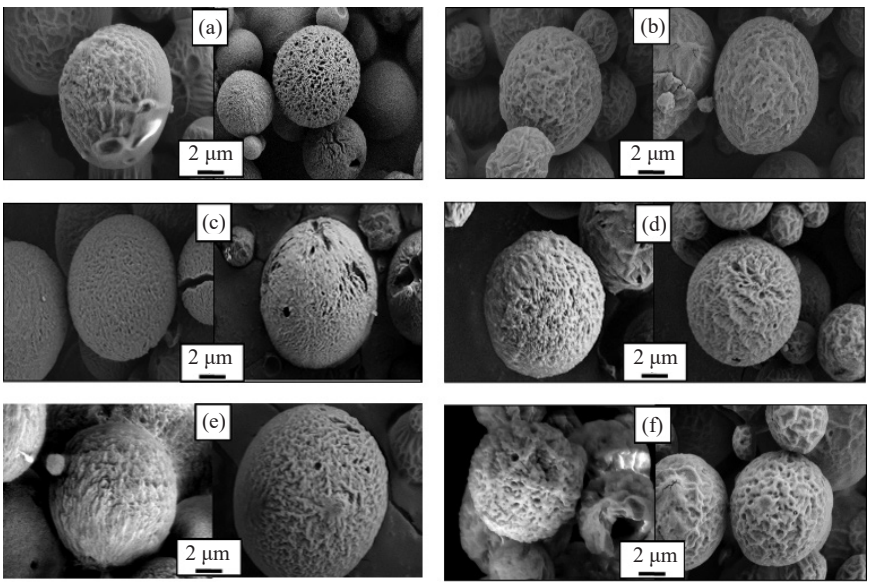

Figure 5: SEM images of modified GENMS with (a) 0.15 M NaOH, (b) 0.15 M NaOH/ethanol (c) 0.25 M NaOH, (d) 0.25 M NaOH/ethanol, (e) 0.35 M NaOH and (f) 0.35 M NaOH/ethanol.

The wettability of a solid surface is usually expressed by the contact angle and surface energy and it is closely related to the surface morphology.[17] Water contact angle measured the hydrophobicity or hydrophilicity of neat GENMS and modified GENMS, with more hydrophilic GENMS having smaller water contact angles. Figure 6 shows the drop profiles of water on the surfaces of modified GENMS with NaOH and NaOH/ethanol while neat GENMS is used as a reference. Table 1 represents the contact angle measurement and surface energy of modified GENMS. Modified GENMS with NaOH/ethanol indicated that contact angle was lower (more hydrophilic) than that of NaOH.

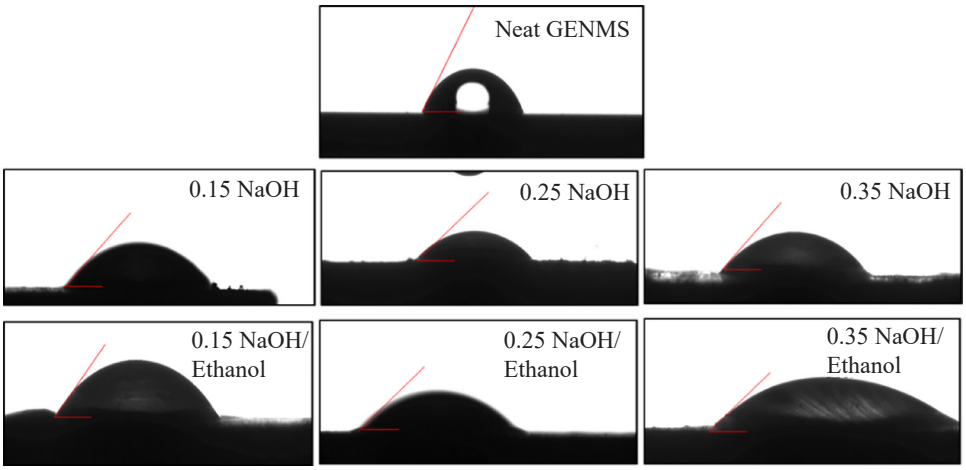

Figure 6: Optical images of drop profiles of neat GENMS and modified GENMS.

Table 1: Contact angle and surface energy of modified GENMS.

Concentration (M)		Contact angle	Surface energy
Neat GENMS		70.65 ± 0.02	41.31 ± 0.01
0.15	NaOH	58.36 ± 0.06	48.82 ± 0.04
	NaOH/ethanol	55.03 ± 0.02	50.81 ± 0.01
0.25	NaOH	52.25 ± 0.36	52.45 ± 0.21
	NaOH/ethanol	51.07 ± 0.60	57.47 ± 0.03
0.25	NaOH	52.49 ± 0.33	52.31 ± 0.19
	NaOH/ethanol	49.88 ± 0.24	53.83 ± 0.14

The contact angles were reduced by 26.8% and 23.1% after treated using NaOH and NaOH/ethanol, respectively comparing to neat GENMS. It indicated that the hydrophilicity of GENMS was enhanced by treatment with NaOH/ethanol mixture due to modification of the surface by increasing the roughness and introducing pores. This is because ethanol was found to assist the hydroxide nucleophilic attack on PLA's ester bonds.[13] A lower water contact angle of modified GENM with NaOH/ethanol was also supported by its high surface energy (51–58 mJ m^{-2}) than those with NaOH (49–52 mJ m^{-2}). The surface energy of all modified GENMS increased in range of 18.2% to 39.2%compared to the neat GENMS. The water contact angle of modified GENMS with NaOH/ethanol and higher concentration for both NaOH and NaOH/ethanol (from 0.15 to 0.35), show lower contact angle owing to the enriched hydrophilic polar of hydroxyl (OH) and carboxylic acid (COOH) terminal groups. In addition, the improvement of surface hydrophilicity

and surface energy may be attributed to the increase of the surface roughness as discussed in morphology part. Thus, it was noted that additional ethanol in alkaline hydrolysis treatment improved the hydrophilicity of the GENMS surfaces.

Figure 7 shows the results of protein adsorption on neat GENMS and modified GENMS. It could be observed that modified GENMS with NaOH showed higher ability to bind BSA molecules than modified GENMS with NaOH/ethanol while neat GENMS has the highest protein adsorption. Less hydrophilicity of modified GENMS with NaOH played a major role in more protein adsorption at the interface. It is generally understood that hydrophilic surfaces are more resistant to proteins compared to hydrophobic surfaces.[9,18] Therefore, more hydrophilicity presented by GENMS with NaOH/ethanol proved that the existing functional group repelled the protein adsorption and consequently a low degree of denaturation obtained.

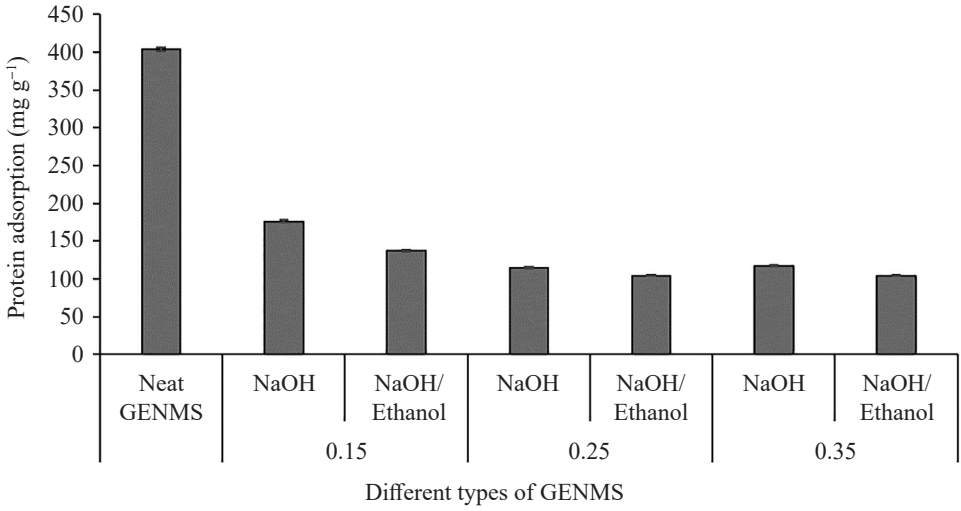

Figure 7: BSA adsorption on neat GENMS and modified GENMS with NaOH and NaOH/ethanol at different alkali concentrations of 0.15 M, 0.25 M and 0.35 M.

The percentage of encapsulation efficiency and drug loading of GEN in GENMS was determined based on standard curve of gentamicin concentration (10–400 ppm) as shown in Figure 3. Table 2 presents the encapsulation efficiency (%) and drug loading (%) of neat GENMS and modified GENMS. From the calculation, the average encapsulation efficiency and drug loading of GENMS were 13.970% ± 0.311 and 0.028 % ± 0.001, respectively. Encapsulation efficiency can be defined as the percentage of the ratio of mass drug encapsulated to the mass of drug loaded in the emulsion. The drug loading is related to the drug contained

in certain mass of microspheres. Since GEN is a very hydrophilic drug, it tends to come out into water phase when microsphere are fabricated using ESE method which probably made the obtained encapsulation low.[19]

In overall, higher encapsulation efficiency and drug loading is observed for the modified GENMS with NaOH/ethanol compared to modified GENMS with NaOH. As expected, both modifications show lower encapsulation efficiency and drug loading than neat GENMS. The differences of encapsulation efficiency and drug loading between modification with NaOH/ethanol and NaOH were determined and shown by percentage value in Table 2. Interestingly, even though more hydrophilicity was created by NaOH/ethanol during hydrolysis, the encapsulation efficiencies of modified GENMS were not reduced. For example, encapsulation efficiency and drug loading of GENMS with 0.35 M NaOH/ethanol increased 3.98% and 4.37%, respectively compared to NaOH modification. This is probably due to hydrolysis with 0.15–0.35 M NaOH/ethanol that changed the GENMS by surface erosion reaction without bulk degradation. This is supported by rougher surfaces of modified GENMS with NaOH/ethanol as shown in Figure 5 which demonstrated the occurrence of surface erosion. It is well known that, bulk degradation on microspheres during alkaline hydrolysis was not preferable in surface modification because certain amount of GEN might be loss in this process.[7] NaOH/ethanol provided rougher surface of GENMS in order to increase encapsulation efficiency.

Table 2: Encapsulation efficiency and drug loading of neat GENMS and modified GENMS.

GENMS	Neat GENMS	Modified GENMS					
		0.15 M NaOH	0.15 M NaOH/ethanol	0.25 M NaOH	0.25 M NaOH/ethanol	0.35 M NaOH	0.35 M NaOH/ethanol
Encapsulation efficiency (%)	13.970	9.204	9.321 (1.27%)*	9.051	9.588 (5.93%)	8.947	9.303 (3.98%)
Drug loading (%)	0.0280	0.0236	0.0239 (1.27%)	0.0232	0.0246 (6.03%)	0.0229	0.0239 (4.37%)

( ) % is obtained from the difference between GENMS modified with NaOH/ethanol and NaOH*

In the present study, low alkaline concentrations of 0.15 M to 0.35 M can be applied to avoid severe bulk degradation beside of improving its hydrophilicity and cell affinity.[13] The degradation might be very fast at highly basic and highly acidic mediums (as compared to neutral conditions).[20] Therefore, surface modification using NaOH/ethanol brings out an advantage over modified GENMS since higher encapsulation efficiency is a desired goal for controlled drug release studies.[21]

The cumulative GEN release amount by difference encapsulation efficiency which measured by UV-Vis spectrophotometry is shown in Figure 8. The GEN release in PBS solution was measured for over 10 days, which the GEN release behaviour from neat GENMS was used as comparison. The release behaviour of GEN from neat GENMS and modified GENMS (0.15 and 0.25) indicate that all samples have an initial burst release within 7 h. The burst release was in the range of 20%–35% and 28%–58% for modification with 0.15 M and 0.25 M, respectively.

Modification with 0.15 M NaOH/ethanol shows a secondary burst release starting at 72 h while increasing release rate for modification with 0.25 M NaOH/ethanol at the same point. Secondary burst release occurred whenever the entrapped GEN was released from interior side of microsphere, which showed the lagging time from 24 h to 72 h. This phase can be useful for pulsatile immunisation applications.[22] In contrast, 0.25 M NaOH/ethanol which probably has undergone the modification entirely on the surface than low alkaline concentration of 0.15 M. Thus, GEN might be released uniformly over the time.

The initial burst release can be explained by the presence of surface associated GEN which is close to the surface and diffused into PBS phase faster as GEN is highly hydrophilic.[19] This is also supported by previous work that claimed the pores on microsphere surface could help in drug release by diffusion mechanism.[23]

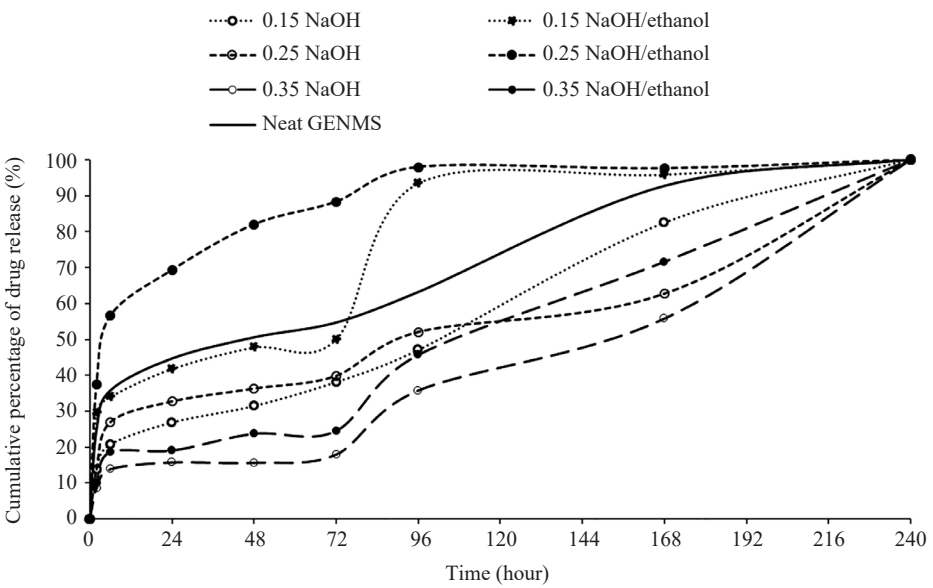

Figure 8: Cumulative GEN release from modified GENMS with 0.15 M, 0.25 M and 0.35 M concentration of NaOH and NaOH/ethanol comparing with neat GENMS after 10 days in PBS.

However, modification with higher concentration of 0.35 M NaOH and 0.35 M NaOH/ethanol presented that the lowest initial burst happened, 12%–19% within 7 h. This is due to low encapsulation efficiency, which had been lost during hydrolysis process. It might also be that the surface associated GEN was diminished using this concentration as alkaline hydrolysis treatment.

Even though initial high burst release rate may cause unfavourable roles, i.e., may lead to drug concentrations near or above the toxic level, excreted without being effectively utilised and wasted, there are still have favourable perspective.[22] Initial burst releases can provide immediate relief such as those used at the beginning of wound healing followed by prolonged release to promote gradual healing and has ability to localise delivery to the specific site of implantation.[24]

Relating to higher encapsulation efficiency by modified GENMS with NaOH/ethanol, GEN release rate increased compared with modified GENMS with NaOH. Additionally, more hydrophilicity of modified GENMS with NaOH/ethanol was also attributed to the increased in GEN release.[25] This is due to the diffusion path of the surface had been reduced which the drug molecules have to cross. However, based on modification using both 0.15 M and 0.25 M, GEN release 0.15 M NaOH/ethanol and 0.25 M NaOH/ethanol were higher than neat GENMS and those 0.15 M NaOH and 0.25 M NaOH, respectively. This is probably the presence of surface associated GEN to diffuse easily than 0.15 M NaOH and 0.25 M NaOH and assisted with their higher encapsulation efficiency. In contrast, modification with 0.35 M showed both 0.35 M NaOH/ethanol and 0.35 M NaOH have the release rate were than neat GENMS. This might be due to these modification concentrations which had diffused out the encapsulated GEN in modified GENMS during hydrolysis process.

Therefore, the alkaline hydrolysis does not necessarily lead to improve hydrophilicity, in fact co-treating by ethanol had maximised the encapsulation efficiency, thus beneficial in controlling the release profile. It can be suggested that an optimised concentration using alkaline hydrolysis is 0.25 M NaOH/ethanol for 24 h treatment.

4. CONCLUSION

The introduction of ethanol in alkaline treatment assisted the hydrolysis using NaOH. Ethanol acts as a co-treating medium in 0.15 M, 0.25 M and 0.35 M of NaOH. Therefore, the present study concludes that:

(a) Ethanol in NaOH treatment facilitated the hydroxide nucleophilic attack on the ester bonds and avoiding severe bulk degradation.

(b) Surface roughness of the modified GENMS by NaOH/ethanol led to the improvement of surface hydrophilicity by 4% reduction of contact angle compared with NaOH modification.

(c) Hydrophilicity by NaOH/ethanol contributed to the low degree of protein adsorption on the GENMS surfaces compared with NaOH modification.

(d) Encapsulated drug was not reduced even though the hydrophilicity was improved by NaOH/ethanol. The encapsulation efficiencies increased up to 6% compared to that modification done by NaOH.

(e) 0.25 M NaOH/ethanol was suggested as a suitable mixture for alkaline treatment due to the difference on encapsulation efficiency (%) and drug loading (%) comparing to 0.25 M NaOH as well as greater drug release rate.

5. ACKNOWLEDGEMENTS

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