การพัฒนาไฮโดรเจลชนิดเมตาคริเลทจากกรดไฮยาลูโรนิกแบบฉีดที่มีแมนนิทอลและบีเอสเอเพื่อใช้ทำเป็นระบบนำส่งยา

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บทคัดย่อ

วัตถุประสงค์: เพื่อพัฒนาไฮโดรเจลรูปแบบฉีดจากกรดไฮยาลูโรนิกโดยนำมาดัดแปลงด้วยเมตาคริลิกแอนไฮไดรด์ (Methacrylic anhydride) และครอสลิงค์ด้วยไดไทโอทรีไอทอล (Dithiothreitol; DTT) เกิดเป็นไฮโดรเจลแบบฉีดชนิดเมตาคริเลทเตทจากกรดไฮยาลูโรนิก (Methacrylated Hyaluronic acid injectable Hydrogels; MeHA injectable hydrogels) โดยมีแมนนิทอล (Mannitol) เป็นสารเพิ่มปริมาณยาและใช้บีเอสเอ (Bovine serum albumin; BSA) เป็นตัวแทนโปรตีน

วัสดุและวิธีการ: สังเคราะห์ไฮโดรเจลแบบฉีดชนิดเมตาคริเลทเตทจากกรดไฮยาลูโรนิก และนำไปผสมรวมกับสารละลายที่มีส่วนผสมของแมนนิทอล และบีเอสเอ ในอัตราส่วน 1:1, 2:1, 4:1 และ 100:1 ตามลำดับ และนำไปศึกษาลักษณะโครงสร้างพื้นผิวของไฮโดรเจลภายใต้กล้องจุลทรรศน์อิเลคตรอนแบบส่องกราด ระยะเวลาในการก่อตัวเป็นเจล คุณสมบัติการพองตัว และคุณสมบัติในการปลดปล่อยโปรตีนของไฮโดรเจล

ผลการศึกษา: พบว่าบีเอสเอ สามารถผสมเข้ากับแมนนิทอลในไฮโดรเจลแบบฉีดชนิดเมตาคริเลทเตทจากกรดไฮยาลูโรนิกได้เป็นอย่างดี และแมนนิทอลเกิดการตกผลึกอย่างเห็นได้ชัดภายใต้กล้องจุลทรรศน์อิเลคตรอนแบบส่องกราด โดยพบว่าระยะเวลาในการก่อตัวเป็นเจล ของทุกอัตราส่วนรวมถึงกลุ่มควบคุม (ได้แก่ ไฮโดรเจลชนิดเมตาคริเลทเตทจากกรดไฮยาลูโรนิกที่ไม่มีส่วนผสมของแมนนิทอล และบีเอสเอ) อยู่ในช่วงระยะเวลาไม่เกิน 30 นาที ในส่วนของคุณสมบัติในการพองตัวของไฮโดรเจล พบว่าส่วนผสมทุกอัตราส่วน มีความสามารถในการกักเก็บน้ำไว้ในตัวเองได้สูงสุดประมาณร้อยละ 90 นอกจากนี้จากคุณสมบัติในการปลดปล่อยโปรตีนพบว่าในทุกอัตราส่วนสามารถปลดปล่อยโปรตีนได้อย่างช้าๆ ภายในระยะเวลา 24 ชั่วโมง

สรุป: แมนนิทอลและบีเอสเอสามารถผสมเข้าได้ดีกับไฮโดรเจลแบบฉีดชนิดเมตาคริเลทเตทจากกรดไฮยาลูโรนิก โดยยังคงคุณสมบัติทางกายภาพที่ดีไว้ โดยไม่เกิดการรบกวนต่อคุณสมบัติทางกายภาพของไฮโดรเจล นอกจากนี้ยังสามารถปรับอัตราส่วนของแมนนิทอลต่อบีเอสเอให้เพิ่มขึ้นหรือลดลงได้ ในกรณีที่ต้องการปรับปริมาณยาหรือโปรตีนตามความเหมาะสมเฉพาะบุคคล

คำสำคัญ: ไฮโดรเจลแบบฉีด แมนนิทอล กรดไฮยาลูโรนิก ระบบการขนส่งยา

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The Development of Mannitol/BSA Methacrylated Hyaluronic Acid Hydrogels as an Injectable Drug Delivery Platform

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Abstract

Objective: The aim of this study was to develop an injectable hyaluronic acid modified with methacrylic anhydride (MeHA) crosslinked with Dithiothreitol (DTT). Mannitol was used as a drug diluent and Bovine Serum Albumin (BSA) was used to represent active proteins.

Materials and Methods: The MeHA injectable hydrogels were synthesized and mixed with mannitol/BSA which were prepared in 4 different preparations (1:1, 2:1, 4:1 and 100:1 in ratio). The surface morphology under Scanning Electron Microscopy (SEM), gelation time, swelling properties and protein releasing profiles were determined.

Results: The BSA was successfully assembled in mannitol/BSA-MeHA hydrogels. All 4 preparations of mannitol/BSA-MeHA hydrogels 1:1, 2:1, 4:1 and 100:1 were more heterogeneous in appearance with larger crystal structures under SEM. Also, with a higher proportion of BSA, the crystal shape demonstrated rhomboidal in shape. The gelation time of both control (MeHA hydrogels alone) and the four study groups were within 30 minutes. From the swelling properties, all preparations were able to absorb the water for approximately 90%. In addition, from the protein releasing profile, all samples released BSA sustainably for over 24 hours which the mannitol/BSA-MeHA hydrogels of 100 : 1 showed the best rate of BSA release (80% ~ 33.08 µg) from total loading (40 µg).

Conclusion: Mannitol/BSA was successfully added to the MeHA injectable hydrogels with promising physical properties. Adjusting the ratio of mannitol and BSA will lead to ultimate properties of the hydrogels which can be customized for individual patients in a controlled drug delivery manner.

Keywords: Injectable hydrogels, mannitol, hyaluronic acid, drug delivery system

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Introduction

In the field of Dentistry, dental implant has been considered the most innovative and superior treatment. It is used for a variety of cases as it provides good outcome and gains patient satisfaction. Successful dental implant requires adequate bone volume in 3 dimensions (1). Since the advancement in pharmaceutical and medical preparations, scientists have found the benefits of using peptides and proteins as therapeutic substances for treating many diseases (2-4) In Implant Dentistry, many specific growth factors with vascular properties (such as vascular endothelial growth factor; VEGF and basic fibroblast growth factor; bFGF), and growth factors with potential osteoinductive properties (such as bone morphogenetic protein; BMP and transforming growth factor-b; TGF-b), platelet-derived growth factor (PDGF), and insulin-like growth factor-1 (IGF-1), have been reported to improve bone repair and outcomes in healthy and medically compromised patients (5). Unlike traditional small-molecule drugs, these proteins and growth factors are large, structurally complicated and generally unstable *in vivo.* Moreover, they could easily be digested and destroyed in the gastro-intestinal tract leading to an unacceptably low bioavailability. For this reason, these proteins cannot simply be administered orally. Regarding the instability and short half-lives of the proteins, many therapeutic proteins had been administered systemically by frequent injections (6-8). However, this method was not a proper solution for giving protein drug into human body. Administering large quantities of proteins repeatedly in order to achieve a therapeutic concentration would simultaneously increase the risk of side effects from protein overdose. It also required high cost of treatment. For this respect, a well-controlled drug delivery system would be an appropriate choice for protein administration (9).

Injectable hydrogels is one type of drug delivery system that have been widely used and investigated to deliver pharmaceutical compounds in a controlled manner for a prolonged time period, aiming to achieve the optimal therapeutic effects of the delivered pharmaceuticals (6-7). There are many starting materials of hydrogels. Hyaluronic acid (HA) is of particular interest because of its biocompatibility, biodegradability and the fact that it is commonly found in human body. Thus, HA hydrogels have been extensively studied and developed to be formed *in situ,* under physiological conditions (10-11). Interestingly, the structure of HA can be chemically modified through its hydroxyl or carboxyl group to further improve its property.

Maturavongsadit *et al.* (12-13) developed injectable hyaluronic acid hydrogels modified with methacrylic anhydride and form metharylated hyaluronic acid (MeHA). Dithiothreitol (DTT) was used as a crosslinking agent since it presented appropriated pore sizes and gelation times. Also, the stiffness of the hydrogels could be adjusted by increasing or decreasing the ratio of DTT (14-15). Bone marrow stem cell was found to remain intact and maintain its properties within the MeHA hydrogels scaffold. It also showed sustained release of bovine serum albumin (BSA) profile (12-13). Taken together, MeHA crosslinked with DTT seems potentially an interesting candidate for drug delivery platform apart from the scaffolding ability.

Mannitol is a hexahydric alcohol related to mannose. It occurs as a white, odorless, crystalline powder, or free flowing granules and has a sweet

taste (16). There are many types of mannitol, but D-Mannitol is widely used in pharmaceutical formulations and food products. In pharmaceutical preparations, it is primarily used as a diluent (10–90% w/w) (17-18), drug stabilizer (19) and pore former (20). Besides, mannitol is a good candidate for drug diluent since it has many desire physicochemical properties of an ideal drug diluent: high physiological tolerability with low toxicological concerns, inertness towards other excipients and the active pharmaceutical ingredients (API), good taste, high compactibility and flowability, low hygroscopicity and the most importance is it can also be used instead of lactose for those with lactose intolerance (21).

In this study we aimed to develop MeHA hydrogels for using as a drug delivery platform combined with mannitol, as a drug diluent. BSA was used to represent future therapeutic peptide/protein. Physical properties related to clinical used were assessed.

Materials and Methods

Modification of HA

MeHA was synthesized following previous protocol (13). Briefly, HA of molecular weight 47 kDa (Liuzhou Shengqiang Biotech Co., Ltd., Guangxi province, P.R.China) was dissolved at 1 wt% in potassium phosphate buffer, pH 8, and methacrylic anhydride (Sigma-Aldrich Pte. Ltd., Singapore) of molar ratio 1:10 was added dropwise to the solution at 0 °C. The pH of the two-phase reaction mixture was adjusted to 8.0 with 5 M NaOH aq., and the reaction continued for 24 hours at 4 °C with frequent readjustment of the solution pH. The product was dialyzed against ultrapure water for at least 48 hours, followed by centrifugation at 10,000 rpm, 4 °C for 10 minutes to remove the precipitate, then the liquid part was flash frozen and lyophilized (LABCOCON), resulting in powder that was analyzed by degree of modification by Proton nuclear magnetic resonance. (^{1}H NMR spectroscopy)

Mixture of Mannitol and BSA

The whole mass of mannitol and BSA is 1% w/v of hydrogels. Mannitol (Sigma-Aldrich Pte. Ltd., Singapore) and BSA (HiMedia Laboratories, Pvt. Ltd., Mumbai, India) were prepared in 4 different ratios as list in Table1. Then dissolved mannitol and BSA of each ratio with 100 µL of PBS and mixed with 3% MeHA in PBS for 400 µL which approximate 20-40% degree of modification. The mixture was adjusted to pH 8.0 with 2 M NaOH aq. before add the DTT as a crosslinker to form a gel.

Table 1. The 4 different preparations of mannitol and BSA for each 500 µL of hydrogels used in this study.

Mannitol: BSA	Mannitol (mg)	BSA (mg)
1:1	2.5	2.5
2:1	3.3	1.7
4:1	4	1
100:1	4.95	0.05

Hydrogels formation

To form the thiol-ene "click" HA hydrogels, the mixture in 2.2. were added with DTT (Sigma-Aldrich Pte. Ltd., Singapore) in molar ratio of thiol: ene of 1:2

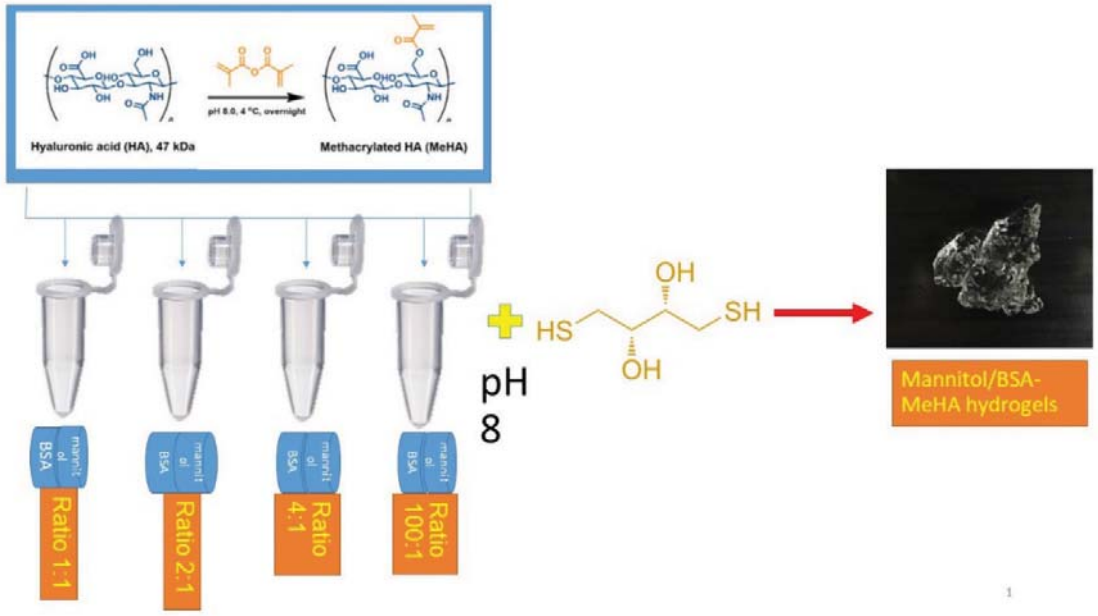

Fig 1. The Mixture of mannitol and BSA and the hydrogels formation process.

Microstructure analysis

Microstructure of the hydrogels were characterized by Scanning Electron Microscope (SEM). The hydrogels samples were lyophilized and mounted on metal stubs with carbon tape. The samples were sputtered coated with gold/palladium for 30 seconds on a Denton Desk II sputter coater with global rotation and tilted. The surfaces of the gold/palladium coated hydrogels samples were observed at 5-8 random locations per sample (n = 3) on a Zeiss Ultraplus Thermal Field Emission SEM at 10 kV (12).

Gelation time

Oscillatory shear rheology was performed using a HAAKE rheostress1 (themoscientific) (see in supporting information Fig.S1) with parallel-plate geometry diameter 35 mm. (P35 Ti L) temperature-controlled Peltier plate. Mannitol/BSA-MeHA hydrogels were crosslinked in situ between the rheometer plates in a close chamber, protecting hydrogels from dehydration. The measurement in oscillatory was in time sweep mode and conditions were fixed at 25 °C, 1 Hertz of frequency and auto strain amplitude, 10 rad/second, immediately started after the freshly prepared prehydrogels mixture was loaded on the Peltier plate (13).

Swelling properties

The hydrogels (250 μL) were prepared previously described by using PBS as the medium. After gelation, the hydrogels were swollen in PBS for 2 days and were then blotted to remove

excess water and weighed to obtain the swollen hydrogels mass (Ms). The dry mass of these hydrogels (Md) were then determined after drying by lyophilization method. Water content were calculated based on the equations of

$$\frac{(Ms - Md)x100}{Ms}$$

The mass based swelling ratio, Q_m, was calculated by dividing the hydrogels mass after swelling, Ms, by the mass after the hydrogels has dried, Md. The volumetric based swelling ratio, Q_v, was then calculated from QM according to Eq.

$$1 + \frac{\rho p}{\rho s}(Qm - 1)$$

Where ρ_p is the density of the dry polymer (Hyaluronic acid in this study) and ρ_s is the density of the solvent (1 g/cm^3 for PBS) (22).

Protein release profile

The experimental procedure was adapted from Maturavongsadit et al. (12). Briefly, all 4 preparations of mannitol/BSA-MeHA hydrogels were prepared in 1.5 mL vial and then 200 µL of PBS was added. After that, all of the supernatant was collected and replaced by fresh PBS at 5, 10, 15, 90 minutes and 24 hours. A Coomassie (Bradford) protein assay (Thermo- scientific) was used to quantify the amount of released BSA in the supernatant.

Results

Synthesis of HA hydrogels

From previous study (13), it was found that HA macromers at 47 kDa was an optimal molecular weight to be modified with methacrylates to form hydrogels. In this study, we synthesized 8 batches of the MeHA hydrogels with 1:10 molar ratio of HA monomer and methacrylic anhydride. The degree of modification was then determined by1H NMR spectroscopy and MestReNova6 software. The result showed that 1:10 molar ratio was able to generate 21.8-41.38% degree of modification of HA backbones in all 8 batches of MeHA hydrogels.

Microstructure

The morphology of freeze-dried mannitol/BSA-MeHA hydrogels were determined by SEM as shown in Fig. 1. MeHA hydrogels without mannitol and BSA (A, F) demonstrated homogeneous appearance. All 4 preparations of mannitol/BSA-MeHA hydrogels 1:1 (B, G), 2:1 (C, H), 4:1 (D, I) and 100:1 (E, J) were more heterogeneous in appearance with larger crystal structures. Also, with a higher proportion of BSA, the crystal shape demonstrated rhomboidal in shape.

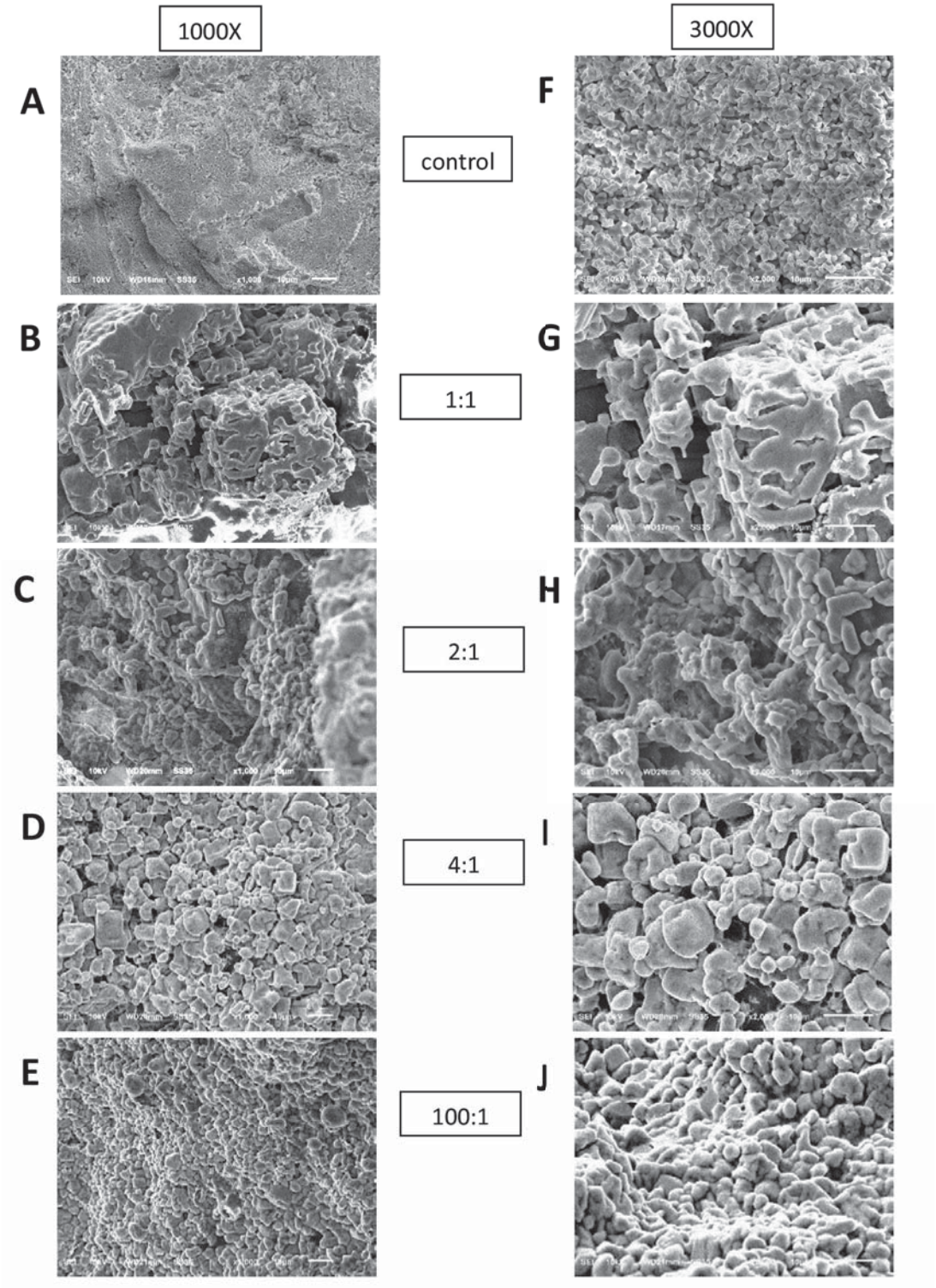

Fig 2. SEM demonstrated the external surface of MeHA hydrogels. A, F: MeHA hydrogels without mannitol. B, G; Mannitol/BSA-MeHA hydrogels: BSA of 1:1. C, H: Mannitol/BSA-MeHA hydrogels: BSA of 2:1. D, I: Mannitol/BSA-MeHA hydrogels: BSA of 4:1. E, J: Mannitol/ BSA-MeHA hydrogels: BSA of 100:1. (A-E: 1000X magnification, F-J: 3000X magnification).

Gelation time

The gelation time of the hydrogels was investigated by the result from HAAKE rheostress 1 (Fig. 2). The gelation time is the beginning time point of G' line and G" line crossovered that means the hydrogels is starting to change from sol phase to gel phase. The gelation time of controlled hydrogels and the ratio of 1:1, 2:1, 4:1 and 100:1 are 25 minutes 28 seconds, 25 minutes 34 seconds, 26 minutes 59 seconds, 17 minutes 58 seconds and 27 minutes 29 seconds, respectively.

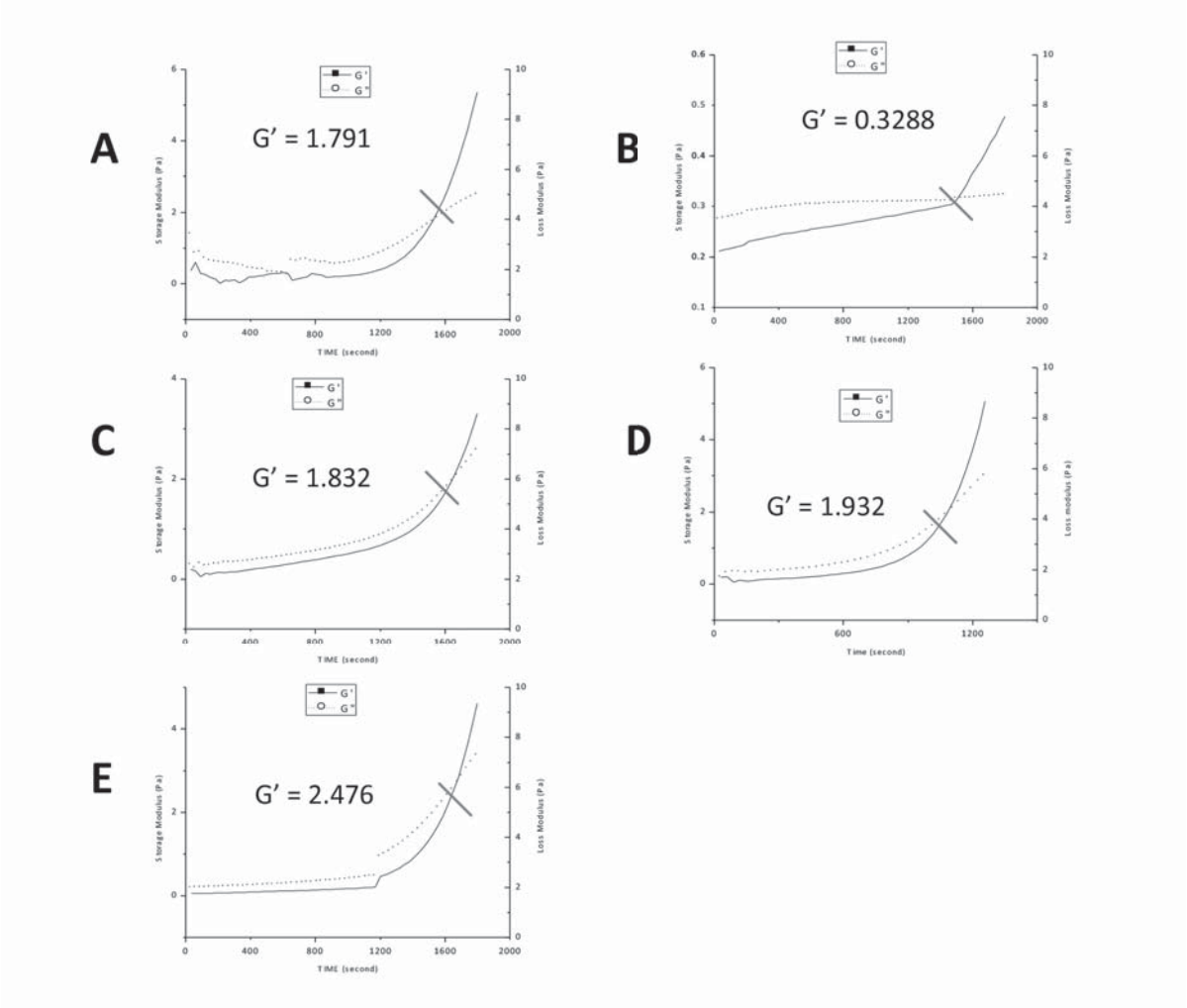

Fig 3. The geltation time of hydrogels. A: The gelation time of MeHA hydrogels. B: Mannitol/ BSA-MeHA hydrogels of 1:1. C: Mannitol/BSA-MeHA hydrogels of 2:1. D: Mannitol/BSA-MeHA hydrogels of 4:1. E: Mannitol/BSA-MeHA hydrogels of 100:1.

Swelling properties

After gelation, the weight of hydrogels gradually increased and then started decreasing after 24 hours (see in supporting information Fig. S2). The water content and swelling ratios of the hydrogels were shown in Fig. 3. The water content of mannitol/BSA-MeHA hydrogels at the ratio of 1:1, 2:1, 4:1 and 100:1 are 88.56%, 90.07%, 91.07% and 90.64%, respectively. The swelling ratios of mannitol/BSA-MeHA hydrogels of 4:1 showed the highest score followed by 2:1, 100:1 and 100:1, respectively.

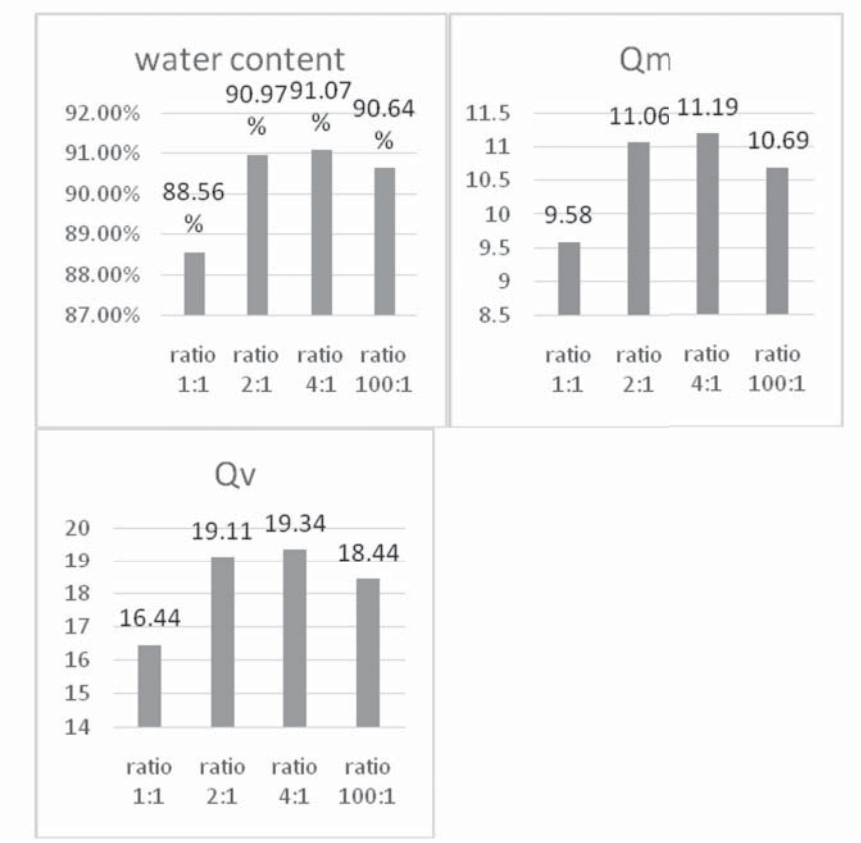

Fig 4. The water content and swelling ratio of hydrogels.

Protein release profile

The amount of BSA released from mannitol/ BSA-MeHA hydrogels was shown in Fig. 4. All preparations of mannitol/BSA-MeHA hydrogels gradually released BSA, which was not complete even after 24 hours of observation. In quantitative assessment, the 1:1 mannitol/BSA- MeHA hydrogels showed the highest protein released at all time point of up to 24 hours followed by 2:1, 4:1 and 100:1 mannitol/BSA respectively. These were clearly due to the higher loading of BSA into the hydrogels. However, when the percentage of the released BSA was calculated back and compared to the initial BSA loading, the 100:1 mannitol BSA preparation had the highest released percentage of 82.71 followed by 4:1, 2:1 and 1:1 preparations (7.8%, 5.21% and 6.4%) respectively.

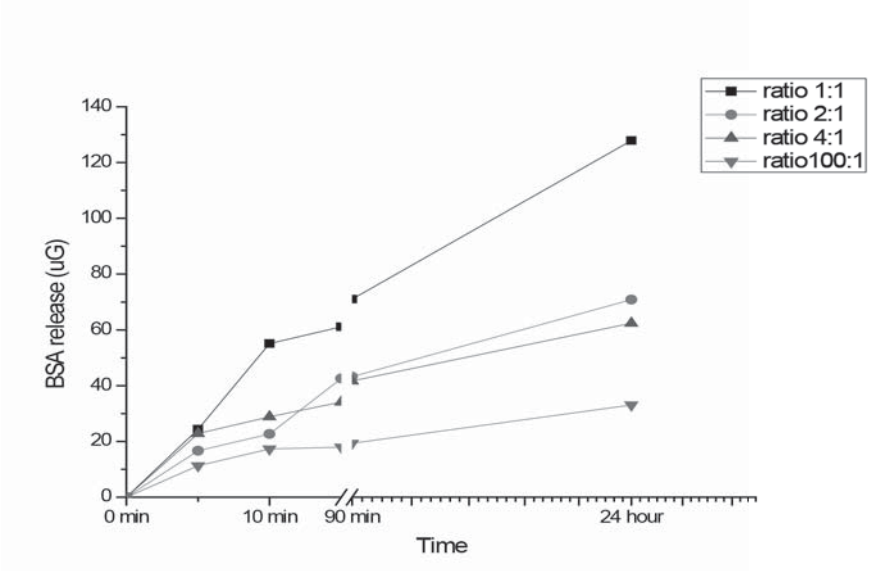

Fig 5. The BSA release profile from the hydrogels to supernatant for 24 hours by Bradford protein assay.

Discussion

Recently, injectable hydrogels have emerged as a promising biomaterial for therapeutic delivery of cells and bioactive molecules for tissue regeneration in Dentistry and Medicine. The reasons were due to their biomimetic properties, controllability of degradation and release behavior, adaptability in a clinical setting for minimally-invasive surgical procedures, and ability to conform to the three-dimensional (3-D) defect upon gelling (23) However, most of the active ingredients such as drugs, growth factors, have a very lowtherapeutic dose (24-25). For this reason, we need drug diluent for practical clinical drug administration. In this study, we use mannitol as a drug diluent and BSA as a rerepresentative for future peptides/ proteins used. We found the 8 batches of 3% MeHA hydrogels modified with methacylated anhydried in ratio of 1:10 showed appropiated degree of modification (21.8% – 41.38%) and they were easily crosslink with DTT to form hydrogels.

As seen by SEM, assembled of mannitol/ BSA resulted in a more heterogeneous surface structure with larger crystal size of the hydrogels. Orther previous studies also reported effect of mannitol on a crystal size of a lyophilized drugs (26-28). The crystal size and shape could possibly due to the BSA added. Yao et al. in 2009 (29) found the increased of BSA concenteration resulted in a more rhomboidal shape of a crystal which similar to what we observed in this study. The crystallization of mannitol is resulted from temperature changing during freeze-dried process and it can form up in different sizes and shapes. In general, they can be divided into 2 groups; anhydrous mannitol and mannitol hemihydrate (MHH). The type of mannitol played the importance role in the product instability. MHH has some water remaining in the crystal though it has been lyophilized and the existence of water within the crystal can cause undesirable physical and chemical changes of API (30). Further study is required for clarification of the crystal structure,

which can investigated by methods such as Fourier-transform infrared spectroscopy (FTIR) and X-ray Powder Diffraction (XRD).

Gelation time is the time that the hydrogels solution transforms into solid phase and important for determining clinical working time. The appropriate gelation time depends on the purposes of clinical appication (31). However, the ideal gelation time should be long enough to allow proper working time but should not be too long to delay the operation. In the present study, the gelation time of mannitol/BSA- MeHA hydrogels was between 17 minutes 58 seconds to 27 minutes 29 seconds. This time period is shorter in comparison to the previous studies (12-13) of MeHA hydrogels crosslinked with DTT of 1 - 2 hours gelation. The difference was probably due to thiol:ene ratios. In our opinion, within 30 minutes is appropriate for many dental surgery procedure. Nevetheless, adjustment of the DTT ratio can be performed to fasten or delay the gelation time (12-13).

For the swelling properties, all of mannitol/BSA- MeHA hydrogels had water content between 88.56% – 91.07%. which is the basic properties of hydrogels that it is capable of absorbing large amount of water (9,32,33). Previous study of MeHA hydrogels reported the water content of more than 90% (12). In contrast, the mass based ratio and volumetric based ration in this study was less than those hydrogels; Qm ~9-11 and Qv ~16-18 while the MeHA hydrogels without mannitol has Qm ~40 and Qv ~50. Again, this might be from the different of thiol:ene ratio. Another factor is the present of mannitol in hydogel since mannitol is able to absorb water in its crystal which cannot be easily to eliminated through lyophilization (34). Future attemps to completely eliminate water from mannitol is required since remaining water might have some effects on the added therapeutic peptides/proteins. Also, the dry weigth of mannitol/BSA-MeHA hydrogels will be precisedly estimated.

The observation of the protein release profile showed that BSA was sustained released from the hydrogels for over 24 hours. The mannitol/BSA-MeHA hydrogels of 100:1 showed the best rate of BSA release (80%) from total loading. The study by Jaipal et al. in 2015 (20) reported the presence of mannitol in drug delivery system affected on drug releasing rate. Increased levels of mannitol resulted in faster rate of drug release and rapid uptake of water in vitro. These caused from the formation of channels in the matrix that correlated to the swelling properties of hydrogels. Moreover, the ratio of mannitol/BSA can be individually adjusted to make the appropriate drug dosage use for each patients.

Conclusion

The mannitol/BSA-MeHA hydrogels has been developed with an acceptable physical properties. Addition of mannitol as a drug dilent and BSA as a representative protein did not interfere the hydrogels properties. Furthur study to investigate biocompatibility of our mannitol/BSA-MeHA hydrogels as well as addition of a osteoinductive peptides/proteins is on going in our laboratory in hope for developing a promising drug delivery system.

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