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Synthesis of semi-biodegradable crosslinked microspheres for the delivery of 1,25 dihydroxyvitamin D_3 for the treatment of hepatocellular carcinoma

T.L. Uyen Nguyen [a], Siew Y. Tey [a], Mohammad H. Pourgholami [b], David L. Morris [b], Thomas P. Davis [a], Christopher Barner-Kowollik [a], Martina H. Stenzel [a,*]

[a] *Centre for Advanced Macromolecular Design (CAMD), School of Chemical Sciences and Engineering, University of New South Wales, Sydney, NSW 2052, Australia*
[b] *Cancer Research Laboratories, Department of Surgery, University of New South Wales, Sydney, NSW 2052, Australia*

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Abstract

It has been demonstrated that 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2VD_3$) can inhibit the proliferation of cancer cells, including colorectal and hepatocellular cells which are mainly responsible for liver cancer. However, the use of $1,25(OH)_2VD_3$ is hampered due to the development of hypercalcaemia. We hereby report a promising technique in liver cancer treatment by utilizing crosslinked microspheres prepared by polymerization as a carrier to control the release of $1,25(OH)_2VD_3$ or hydrophobic drug in general at targeted sites over a long period. Microspheres in the size range of 35 μm were prepared and the drug was loaded to these poly(vinyl neodecanoate-crosslinked-ethyleneglycol dimethacrylate) microspheres after polymerization. The release study has shown that up to 1% of the drug was released after 40 days. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and direct contact cytotoxicity assay using HT-20 and L929 confirm the non-toxicity of these spheres.

Keywords: Microspheres; Suspension polymerization; Liver cancer; 1,25 Dihydroxyvitamin D_3

1. Introduction

Liver cancer is the fifth most common type of cancer worldwide with more incidences occurring in Asia. There are various treatment methods, of which operation is considered the most effective way. However, only about 15% of liver cancer patients qualify for operation [1]. Alternative methods of treatment such as chemotherapy, chemoembolization, radiation, hepatic arterial infusion can only control the symptom of the disease but cannot cure it. In fact, these treatments could only prolong the life of patients in order of months [2]. Thus, a

* Corresponding author. Tel.: +61 2 9385 4344; fax: +61 2 9385 6250.
E-mail addresses: camd@unsw.edu.au, M.Stenzel@unsw.edu.au (M.H. Stenzel).

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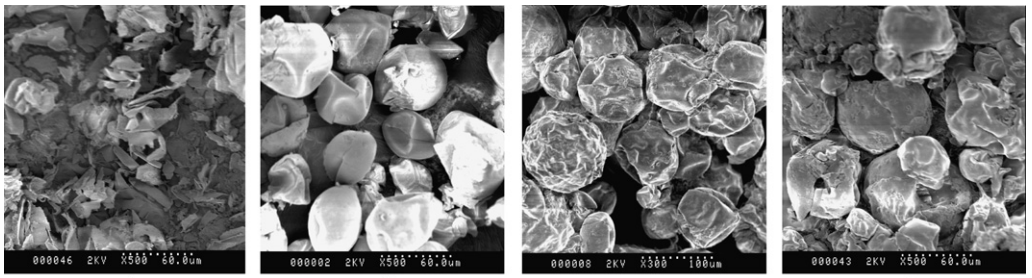


Fig. 1. Effect of temperature on the formation of spheres in the suspension polymerization of vinylneodecanoate VND/ethyleneglycol dimethacrylate EGDMA employing 5 wt% of monomer mixture and 0.025 wt% of AIBN after a reaction time of 20 h. A heating rate of 50 K per hour was applied for the initial stage of the polymerization.

new approach for the treatment of inoperable liver cancer is desirable. We have previously shown that 1,25 dihydroxyvitamin D_3 (the chemical structure is shown in Fig. 1) inhibits the proliferation of a number of cancers, including liver cancer [3–5]. $1,25(OH)_2VD_3$ targets cells through both the genomic (via binding with vitamin D receptors found in various cell types) and non-genomic (fast response via membrane receptors) [6]. However, the clinical use of 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2VD_3$) has been hampered due to the development of hypercalcaemia. In our lab, we have previously reported a significant hepatic first-pass effect for $1,25(OH)_2VD_3$ in treatment of liver cancer [7]. The results revealed that local treatment through hepatic injection of $1,25(OH)_2VD_3$ solution prevents the development of hypercalcaemia. In order to increase the drug uptake of the tumor, $1,25(OH)_2VD_3$ was dissolved in lipiodol solution and doses up to 10 μg/day can be administered safely by hepatic arterial administration [8]. The drug, however, was cleared within a short time window. The concentration of the drug within the therapeutic window for an extended period of time, preferably several weeks, is one of the most desirable aims since the drug administration via hepatic arterial infusion is invasive and repeated administration should be avoided. It is hypothesized that a polymeric drug carrier system with a suitable size – which can be retained at the tumorous site during the required treatment period while sparing most of the healthy cells – would be highly useful in the treatment of liver cancer. Depending on the design of the carrier a slow release formulation of the drug may be produced.

The idea of using microspheres for the treatment of liver cancer was first mentioned in 1965 ([9]). Most studies have utilized microspheres incorporated with radioactive material and administered through the hepatic artery. At present, there are two commercial sources of microspheres including glass spheres (TheraSphere) and resin spheres (SIR-Sphere) having diameters of 25 ± 10 μm and 32 ± 10 μm, respectively. It has been recommended that spheres in the range of 20–40 μm are suitable for application in the treatment of liver cancer since these are large enough to be lodged in the terminal arterioles of the tumor while not passing through smaller vessels that enter the venous circulation [10]. Micronsized spheres have found extensive applications in the medical field in recent years due to improved efficacy of treatment and the relative ease of administration. They can be used to deliver proteins and peptides as they provide protection for these medications in vivo, thus retarding their degradation [11]. The spheres can also be used to deliver other therapeutic agents such as magnetic or radioactive particles.

Most of these spheres do remain within the liver due to their non-biodegradability. Some biodegradable monomers have been tested, showing, however, only a limited stability against erosion or degradation resulting in a faster release of the drug [12]. Prerequisites for a suitable drug carrier for liver cancer treatment via hepatic arterial infusion are a high loading capacity of the carrier while releasing the drug over several weeks or even months. In addition, no degradation or significant surface erosion should occur while the carrier releases the drug. However, degradation of the carrier after the usage would be advantageous.

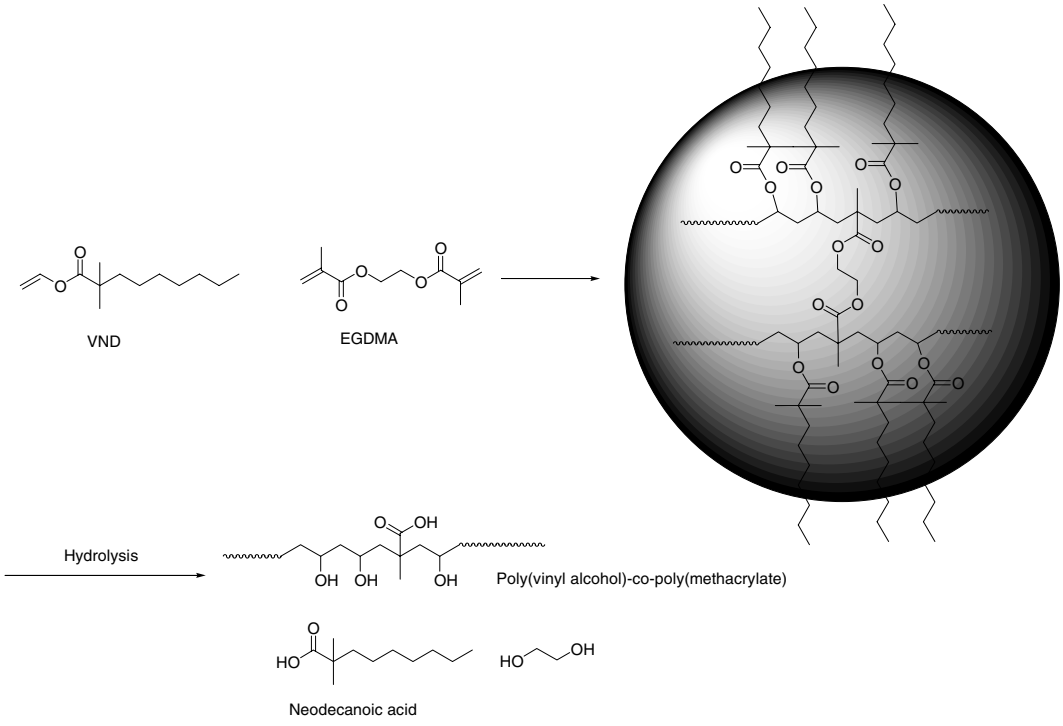
There are various approaches to generate spheres including preparation via polymer synthesis or the processing of exciting polymers into spheres (fabrication method). Microspheres prepared via suspension have sizes ranging from 20 μm to 2 mm; while particles produced by emulsion, precipitation and

dispersion polymerization are usually smaller than 10 μm [13]. There are three different procedures for the fabrication of spheres: hot melt, solvent removal and recently spray drying technique [14,15]. Hot melt could potentially destroy the drug or protein molecules due to high temperature. The other two methods are the most common ones that have been studied well in the literature. The solvent evaporation method utilizes the oil-in water emulsion technique to form particles. Berkland et al. [16,17] studied in depth the fabrication method using acoustic excitation combined with a carrier stream producing uniform spheres with average diameters from 5 to 500 μm. The most common polymers used in these fabrication techniques are polyesters with the most frequent one being PLGA.

Suspension polymerization is a convenient avenue to generate microspheres of varying sizes. The size of the sphere is commonly determined by reactor design, but also by other parameters such as the properties of water and oil phase [13]. The average size of the particles can be controlled by the empirical relationship:

$$d = k\frac{D_v R v_d \varepsilon}{D_s N v_m C_s} \quad (1)$$

with d = average particle size, k = parameters representing reactor design, type of stirrer, self stabilization, etc. …, D_v = diameter of vessel, D_s = diameter of stirrer, R = volume ratio of the droplet phase to the suspension medium, N = stirring speed, v_d = viscosity of the droplet phase, v_m = viscosity of the suspension medium, ε = interfacial tension between the two immiscible phases, C_s = stabilizer concentration. Suspension polymerization is commonly employed using styrene and methylmethacrylate as monomers while other monomers do only play a minor role. The advantage of suspension polymerization over fabrication methods lies in the possibility of in situ crosslinking by employing bifunctional vinyl compounds. Surface erosion and dissolution of particles are commonly absent in crosslinked particles. Due to the hydrophobic nature of $1,25(OH)_2VD_3$, a hydrophobic polymer matrix is essential to ensure a high drug loading capacity. Poly(vinyl neodecanoate) can be identified as a suitable polymer for the drug carrying polymer matrix since it is highly hydrophobic, monomer and polymer are non-toxic and the polymer can potentially degrade via (enzymatic) hydrolysis into neodecanoic acid and water soluble poly(vinyl alcohol). Stabilization can be achieved by addition of a



Scheme 1.

crosslinker, here ethyleneglycol dimethacrylate (EGDMA) (Scheme 1).

In this work, we attempt a suitable design for a drug carrier for $1,25(OH)_2VD_3$, but also other hydrophobic drugs. Such microspheres are ultimately intended for the loco-regional treatment of primary or secondary liver tumors. The purpose of this study is the creation of a hydrophobic and biocompatible drug carrier in the size range of approximately 35 μm. These microspheres should show a high drug loading capacity while the drug is released over an extended period of time such as several weeks.

2. Materials and methods

2.1. Materials

Ethyleneglycol dimethacrylate (EGDMA) and vinyl neodecanoate (VND) obtained from Aldrich were passed through a basic aluminum oxide column to remove the inhibitor. Azobisisobutyronitrile (AIBN) was crystallized twice with methanol before use. Poly(*N*-vinyl pyrrolidone)PVP ($M_n = 40{,}000$ g mol^{-1}) (Science), poly(vinyl alcohol)PVA (Mowiol® 4-98), Sudan II and vitamin D_3(VD_3) (all Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Promega) were used without further purification. RPMI and Fetal Bovine Serum (FBS) were obtained from Invitrogen. Fibroblast Earle's L cells-NCTC Clone 929 (Murine) was obtained from the Biomedical Lab, UNSW.

2.2. Suspension polymerization of VND crosslinked EGDMA

The reactor is a 250 ml wide mouthed flask modified to include four 10 mm radial baffles with a removable five necked lid. It is equipped with an overhead stirrer, two four-bladed 40 mm turbine impeller, a condenser and an oil bath. A mixture of 1.05 g PVP and 199.5 g water was added to the reactor and purged with nitrogen with the aid of a sonifier. After the degassing and sonification step, a mixture of monomer (10.0 g, 4.7 wt%) and initiator (0.2 g, 0.095 wt%, 2 wt% of oilphase) was introduced to the reaction flask and degassing was continued with slow stirring at 200 rpm for another 30 min. The reaction was started by ramping the temperature (20 °C) from room temperature to 70 °C in 1 h. After a reaction time of 20 h, the microspheres were filtered off and washed with water and acetone.

The following samples were prepared for cell test experiments:

S1: aqueous phase: 199.5 g deionized water, 1.05 g PVP; oil phase: 8.0 g VND, 2.0 g EGDMA, 0.2 g AIBN, reaction time 20 h;
S2: aqueous phase: 199.5 g deionized water, 1.05 g PVP; oil phase: 7.0 g VND, 3.0 g EGDMA, 0.2 g AIBN, reaction time 20 h;
S3: aqueous phase: 199.5 g deionized water, 0.57 g PVP, 0.57 g PVA; oil phase: 8.0 g VND, 2.0 g EGDMA, 0.2 g AIBN, reaction time 20 h.

2.3. Drug loading experiment

VD_3 (0.15 g) were dissolved in 10 ml of acetone followed by the dispersion of 0.75 g spheres (**S1**) in this VD_3/acetone solution. The drug uptake was aided by gentle shaking of the mixture. After predetermined times, drug incorporated spheres were recovered by filtration and quickly washed with acetone and deionised water. The loading efficiency was determined by weight gain.

2.4. Sudan loading experiment

Sudan II was used as a model drug for the loading experiment. An amount of 30 mg of Sudan was dissolved in 2 ml of ethanol. An amount of 100 mg of polymer (**S1**) was then suspended to this solution. The particles were taken at 20 min time intervals and examined by fluorescence microscope for the distribution of Sudan II.

2.5. Drug release experiment

Drug release studies were carried out in deionized water (containing 1% ethanol) at a temperature of 37 °C using an orbital shaker with 40 rpm. An amount of 50 mg of drug loaded microspheres (**S1**) was suspended in 10 ml of solution for a predetermined time. The samples containing microspheres were then filtered and replaced with fresh media. The filtered solutions were then measured by UV–Vis using the absorption of vitamin D_3 at 265 nm. The amount of drug released was back calculated from the standard curve prepared at different concentrations of vitamin D_3 in water/ethanol.

2.6. Cell growth inhibition test

Polymer samples (**S1**, **S2**, **S3**) (0.75 g) were measured into 5 ml sample vials and sterilized by steam for 15 min at 121 °C and dried for 5 min. MEM (Eagle's minimum essential medium) solution (3.75 ml) with 10% FBS (fetal bovine serum) was added to each vial. This solution was extracted after 24 h in incubation at 37 °C and 5% CO_2. To each plate containing cell line 929 was added 0.8 ml of extracted solution, ethanolic solutions (4, 5 and 7.5 vol.%) as control samples and null samples. Each sample was done in quadruplicate. The cells were incubated for another 24 h and counted by a Coulter Counter.

2.7. MTT assay

A cell suspension containing HT-29 cells (75 ml) was vortexed and incubated (37 °C, 5% CO_2, 95% humidity). After an incubation period of 72 h, 5 ml of Trypsin was added to the flask and incubated for another 5 min. The supernatant was removed and 10 ml of media was used employing a mixer. An amount of 20 μl of this mixture was used together with 20 μl of 0.4% Trypan blue solution to determine the cell number. The number of cells for this assay was determined to be 1.1×10^6 cell ml^{-1}. A 24-well plate was prepared using a cell concentration of 5000 cells per well in 500 μl of media. The well plate was incubated for 24 h. (Note: all media used in the previous steps were RPMI + 10% PBS + antibiotics). The medium was removed and 500 ml of RPMI and 5% PBS were added to each well. An amount of 10 mg of microspheres was mixed with the cells and incubated for 72 h. After 72 h the media and particles were poured out and new 5% PBS media and 100 μl of MTT were added to each well. After 4 h incubation, 400 ml of DMSO was added and shaken for 20 min. The solution was transferred into a 96-well plate, each well containing 100 μl of solution. Optical density was then read at 562 nm using a fully automated plate reader equipped with KC4 software.

2.8. Direct contact cytotoxicity assay

HT-29 cells were seeded at a concentration of 100,000 cells/35 mm plate. A layer of agar was added on top of the cell surface. Polymer microspheres (**S1**) were placed on top of this agar layer. Following 24 h of incubation, the plates were examined under the microscope. Cell viability was performed using Trypan blue. A zone and lysis grading system was used to estimate the effect of testing materials on cells.

2.9. Scanning electron microscope (SEM)

The Hitachi S900 Field Emission SEM was utilized to determine the size and observe the surface morphology of microspheres. Stubs were prepared by suspending the microspheres in ethanol with ultrasonics (for as brief a period as possible) and dropping the solution onto the stub. Once dry, a chromium sputter coat was applied.

2.10. Transmission electron microscope

Polymer particles were dispersed and imbedded in LR-White resin at 60 °C for 24 h. Thin section of the sample (~90 nm) was cut by ultra-microtome and stained with OsO_4. The samples were examined under a Hitachi H-7000 TEM.

3. Results and discussions

3.1. Suspension polymerization

Since its introduction in 1909, suspension polymerization has been used widely in industrial processes. It is usually utilized to synthesize large-sized particles with diameter in the range of 20 μm to 2 mm [13]. The major drawback of this technique is the high polydispersity of the final product. In suspension polymerization, the formation of particles is based on the balance of break-up and coalescence of droplet under the effect of agitation [18]. A range of parameters affect the outcome of the suspension polymerization since droplet break-up and coalescence are affected by experimental conditions such as the viscosity and the interfacial tension, which are a function of concentrations and temperature. The size as well as the dispersity of particles is determined by both the design of the reactor and the physical properties of the reaction mixture. These parameters have been well studied for monomers such as styrene and methacrylates [19–21], however, applications in the biomedical sector require the exploration of new monomers, which are non-toxic and degradable to a certain extent. The initial step for the synthesis of vinyl neodecanoate microspheres is therefore the investigation of all

processing parameters to ensure the synthesis of particles with particle sizes of 35 μm while having a narrow polydispersity. A range of temperatures, monomers and stabilizer concentrations as well as varying stirring speeds were used to optimize the final product. A significant influence on the water droplets can be expected from the stabilizing polymer in the aqueous face affecting both the viscosity and the interfacial tension [22].

Since the synthesis of microspheres with a high stability against erosion was targeted, a crosslinker was introduced into the monomer mixture to generate a network. Network formation not only affects the mechanical stability of the microsphere, but also the drug release kinetics and the internal structure of the microsphere. Preliminary studies regarding the copolymerization of both monomers – VND and EGDMA – are therefore essential since no literature values are available. Vinyl neodecanoate (VND) was therefore copolymerized in bulk with varying amounts of methyl methacrylate (as a model compound) to conversions below 5% (1 h reaction time at 70 °C) and the resulting polymer was analyzed using ^{1}H NMR in $CDCl_3$. The reactivity ratios obtained were $r_1 = 6.54$ and $r_2 = 0.43$, respectively, indicating a strong preference for the methylmethacrylate monomer to be built into the polymer. The favored consumption of methacrylate prior to the polymerization of VND will have a profound effect on the structure of the microsphere. Most of the methacrylate crosslinker will have reacted before any significant amount of VND has been polymerized. Consequently, the insoluble polymer composed mainly from the crosslinker will precipitate along the surface of the monomer droplet along the interface between water and oil phase. As a result, the synthesized particles will have a core-shell structure with a crosslinked surface layer and an only lightly crosslinked PVND core [23]. However, it should be noted here that the reactivity ratios of two monomers in suspension polymerization can slightly deviate from the bulk polymerization [24].

After preliminary studies regarding the copolymerization were carried out, the conditions for the suspension polymerization were systematically investigated and optimized.

3.1.1. Effect of temperature

The reaction temperature will not only affect the rate of polymerization, but even more the physical properties of the reaction mixture such as viscosity and interfacial tension. Not only was the reaction temperature observed to have a significant influence on the reaction, but also a more significant influence was noticed by the rate of heating in the initial state of the reaction. It was found to be essential to heat the suspension polymerization slowly to the final temperature. A rate of 50 K per hour was suitable to obtain well-defined particles. In contrast, the immediate heating of the mixture to the reaction temperature did result in broad particle size distributions. This effect can be understood by the slowly occurring polymerization in the monomer droplet below the final reaction temperature was reached. The small amount of polymer formed in the oil phase increases the viscosity plus the interfacial tension, hence, stabilizing the delicate droplet coalescence-break-up mechanism. In addition to the initial heating rate, the final temperature influences the rate of polymerization and the physical properties of the solution as mentioned above. Therefore, suspension polymerizations were carried out at varying final temperatures ranging from 50 °C to 90 °C. However, particle formation using 10% of crosslinker has only been observed in the range of 65–75 °C (Fig. 1), while agglomeration was obtained when polymerization was carried out outside this range. Within this range, the temperature did not significantly affect the particle size distribution.

3.1.2. Polymer concentration

After a reaction temperature of 70 °C has been identified as optimum, further parameters such as the monomer concentration were investigated. Suspension polymerizations using up to 50% volume of monomer have been reported earlier [25]. However, in this study, only monomer concentrations of up to 8 wt% could lead to appropriate particle formation. Attempts to polymerize at concentrations higher than 8 wt% resulted in agglomeration. It is well established that the formation of droplets in suspension polymerization is the balance between break-up and coalescence. Due to the low viscosity of the water phase in this system, the break-up rate would be slower than the coalescence rate, thus resulting in agglomeration at high monomer concentration. An optimum result was achieved with a crosslinker concentration of 5 wt% resulting in particles with an average particle size of 40 μm while showing the smallest particle size distribution. With increasing monomer concentration the particle size increases dramatically with 6 wt% leading to an

average particle size of 80 μm while 7 wt% results in particles of 120 μm. It is interesting to note here that the particles in suspension polymerization are sufficiently large for conventional free radical kinetics to be applied. The conversion of the polymerization was therefore found to be independent within errors of the monomer fraction in the mixture and is only determined by the AIBN concentration and the reaction temperature as expected for polymerizations in bulk. The monomer conversion after 20 h was in all cases around 80%.

3.1.3. Crosslinker concentration

Preliminary experiments on the reactivity ratios revealed that the crosslinker will preferably be consumed during early stages of the reaction resulting in the formation of networks in the monomer droplet. The amount of network can easily be correlated with the amount of crosslinker employed in the suspension polymerization. Increasing network or gel formation has a significant influence on the viscosity of the monomer droplet, hence, droplet break-ups are prevented or delayed. Consequently, with the increase of crosslinker, the particles appeared to be more stable. In addition, a noticeable increase in particles size was observed with increasing crosslinker concentration – another indicator for the delayed droplet break-up (Fig. 2).

3.1.4. Polymer conversion

Kinetics in suspension polymerization is found to be in good agreement with polymerizations in bulk [26,27]. It can be argued that the amount of initiator in each droplet is sufficiently high to sustain typical bulk kinetics.

Five different initiator concentrations have been investigated ranging from 0.5 wt% to 3 wt% (of monomer weight) using a 80/20 wt% mixture of VND and EGDMA. The conversion was found to increase linearly with the square-root of the initiator concentration ranging between 25% and 85% after 20 h at a temperature of 70 °C. The evolution of the conversion with time was investigated employing 2 wt% (in regards to monomer weight) of AIBN at 70°C. Conversion during the first 8 h of reaction was steady and slow but increased rapidly afterwards (Fig. 3). An increased rate of polymerization with conversion can possibly indicate an occurring gel-effect. However, it has to be considered that during this copolymerization the methacrylate crosslinker EGDMA is consumed at the early stage of the polymerization leaving VND to almost homopolymerize at an advanced phase. The monomer composition drifts substantially during the course of the reaction, altering the overall propagating rate constant. While methyl methacrylate (as a model compound for the crosslinker) has a propagating rate constant of $1060\,l\,mol^{-1}\,s^{-1}$ at 70 °C [28], vinyl neodecanoate shows a substantially higher reaction rate with a propagating rate constant of $8520\,l\,mol^{-1}\,s^{-1}$ at the same temperature [29].

The reaction time does not influence the size of the particle but does substantially determine the stability of the particle (Fig. 3). At conversions below

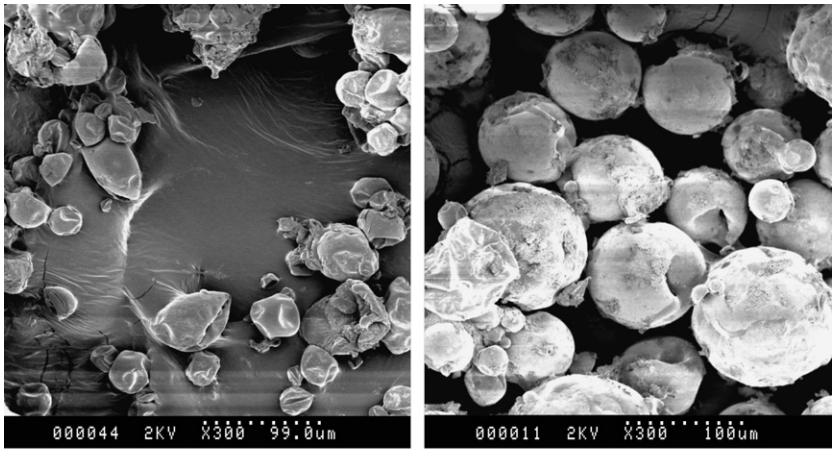


Fig. 2. The effect of crosslinker concentration on particles formation in the suspension polymerization of vinylneodecanoate VND/ethyleneglycol dimethacrylate EGDMA at 70 °C employing 5 wt% of monomer mixture and 0.025 wt% of AIBN after a reaction time of 20 h.

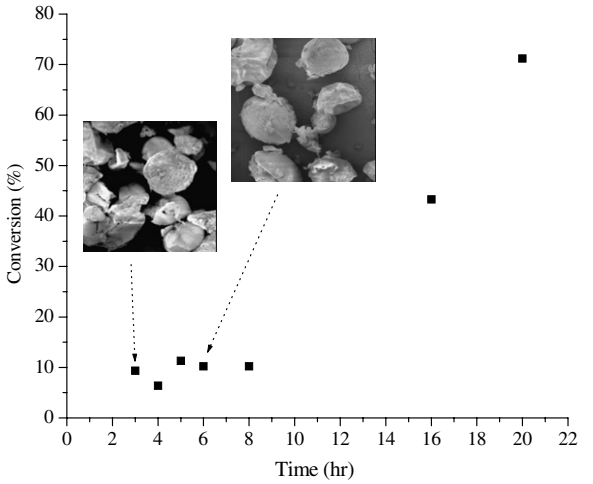


Fig. 3. Conversion of suspension polymerization of vinylneodecanoate VND/ethyleneglycol dimethacrylate EGDMA (80/20 wt%) at 70 °C employing 5 wt% of monomer mixture and 0.025 wt% of AIBN after varying reaction times.

30% the particles tend to collapse after a washing step. The removing of unreacted monomer leads to porous areas, which cannot maintain the shape of the microsphere.

3.1.5. Stabilizer

The stabilizer of the aqueous phase plays a significant influence on the particle size and particle size distribution as well as the surface smoothness. The interfacial tension between aqueous and oil phase in addition to the viscosity of the aqueous phase strongly determines the equilibrium during suspension polymerization. From Eq. (1), it becomes evident as to how these parameters affect the droplet size. An earlier detailed study using PVP polymers with varying architectures (linear and star shaped PVP polymers prepared via RAFT polymerization) shows that only PVP polymers with a medium molecular weight range are suitable to create a stable suspension [30]. It has been concluded that only a small window of viscosity and interfacial tension is suitable to obtain microbeads. In this study we replaced the commercially available PVP by varying fractions of poly(vinyl alcohol) PVA. The overall stabilizer content was kept constant. With increasing amount of PVA content the viscosity of the solution was found to increase from 0.497 cSt to 0.540 cSt. Meanwhile, the particles experience a significant improvement in particle size distribution and surface roughness (Fig. 4). However, 100% PVA as stabilizer does only result in agglomerated products.

3.2. Drug loading

The synthesized microspheres were employed as a polymer carrier for the controlled release of 1,25 dihydroxyvitamin D_3. Drugs in general can be loaded in polymer matrices using a range of approaches – either during the course of the polymerization or after the reaction. For drug loading during the polymerization process, drug molecules are usually distributed evenly in the polymer matrix due to thorough mixing before the reaction. However, this approach faces the probability of destroying the drug by heat or by harsh chemical environment. Especially VD_3 was found to be highly sensitive against heat and light. NMR studies confirmed that within a short time of being exposed to these influences, the molecule degraded substantially. Moreover, if the drug molecule is not chemically bonded to polymer, it may be washed out during the purifying process. Drug loading after polymerization would address these limitations. A popular method that has been used recently by different research groups is a solvent removal method [31] which utilized commercially available PLGA. Generally, a mixture of PLGA and the desired drug in a volatile solvent is suspended in water. Due to the effect of stirring, the polymer will form polymer droplets and the solvent will evaporate off under heat. The so-called fabrication technique was reported by Berkland et al. [16] using ultrasonic probe to break the stream into droplets containing polymer and drug molecules. These two methods form the polymeric microspheres from linear polymer. Zhu and McShane [32] have recently loaded the drug to already formed microspheres using a solvent to swell the polymer particles and stabilizer to avoid coagulation of spheres. In our loading method, there is no need to use a stabilizer since the crosslinked microspheres are very stable in solvent, thus reducing the impurity created by additional stabilizer.

To optimize the loading procedure with a range of experiments, the commercially available vitamin D_3 was utilized instead of the very expensive active form of VD_3 – 1,25 dihydroxyvitamin D_3 (1,25-$(OH)_2VD_3$). The microspheres employed were prepared using 5 wt% oilphase (80/20 wt% VND/EGDMA with 2 wt% AIBN) and a reaction time of 20 h at a temperature of 70 °C (**S1**). VD_3 was then loaded by dispersing polymeric spheres (0.75 g) into a solution containing VD_3 and acetone (0.15 g in 10 ml solvent). The diffusion of the parti-

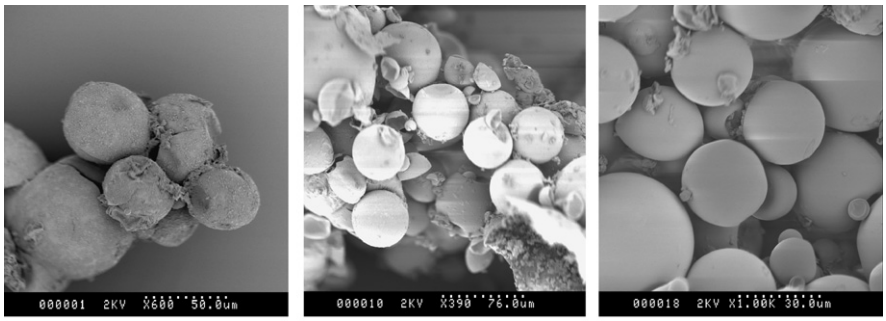


Fig. 4. Effect of stabilizer composition on the surface smoothness of microspheres. The weight percentage of stabilizer in the aqueous medium is 0.5%.

cles is facilitated by swelling of the microspheres in acetone. The flow of drug molecules into polymeric particles is then caused by a concentration gradient but also by the better compatibility of the highly hydrophobic drug with the hydrophobic polymer matrix. Several replicates were prepared and the particles were removed at certain time intervals. The remaining VD_3 in solution was determined using UV–Vis spectroscopy. After around 1 h a maximum loading was observed, but the particles were usually kept in acetone/VD_3 solution for 4 h. At this stage, a maximum loading of VD_3 in the particle of 10.5 wt% was obtained, i.e., 58 wt% VD_3 has penetrated into the polymer matrix while the remaining 42% stay in solution.

The concentration of VD_3 in the particle follows usually an asymptotic behavior indicative of saturation. As a result, a substantial amount of drug remains in solution. This approach can be enhanced by the evaporation of solvent during loading, maintaining a high concentration gradient between solution and microsphere. Therefore, the particles were stirred in the drug/solution mixture using an open beaker, which allows the evaporation of the solvent within 1 h. After evaporation of all solvents the swollen particles start contracting and therefore encapsulate the drug. The final drug concentration in the particle is then equivalent to the amount dissolved in solution. It should however be considered that drug loading is not only a matter of concentration gradients, but also of the similarity of the polarity of the drug and the polymer (as expressed using Flory–Huggings parameter). It is therefore important to confirm that the drug is indeed loaded into the polymer matrix and is also deposited on the surface after evaporation. A washing step (ethanol) and the analysis of the washing solution using UV–Vis can verify the full encapsulation of the drug. In this example, the final drug concentration in the microsphere can be calculated to be around 17 wt%. Absorbed VD_3 on the surface was clearly absent in this case and the penetration of all available drugs into the carrier was guaranteed.

3.3. Distribution of drugs in the microsphere

The evaporation technique obviously allows the loading of drug post polymerization. This loading technique, however, cannot ensure the even distribution of the drug within the microsphere. It has been observed that the drug is accumulated within the upper layer of the sphere. Moreover, the evaporation technique may also lead to the precipitation of the drug on the surface without being physically entrapped in the polymer layer. The distribution of the drug within the microsphere was therefore studied using TEM and fluorescence microscopy using a model compound.

Transmission electron microscopy (TEM) studies allow the visibility of the drug within the microsphere. Particles with and without drug were investigated after being cut into 90 nm slices. Unloaded particles showed an even grey color with or without additional staining of OsO_4 (Fig. 5). Irregularities may be caused by the cutting technique of these slightly soft samples. In contrast, drug loaded particles showed immediate darkening, which is intensified after staining. The TEM photos taken in the center of the core and close to the surface show an even distribution of the drug with significant nano-sized agglomerates of the drug being absent (Fig. 5).

Fluorescence microscopy is a versatile way to further investigate drug loading in particles. Since VD3 does not show any fluorescence activity a

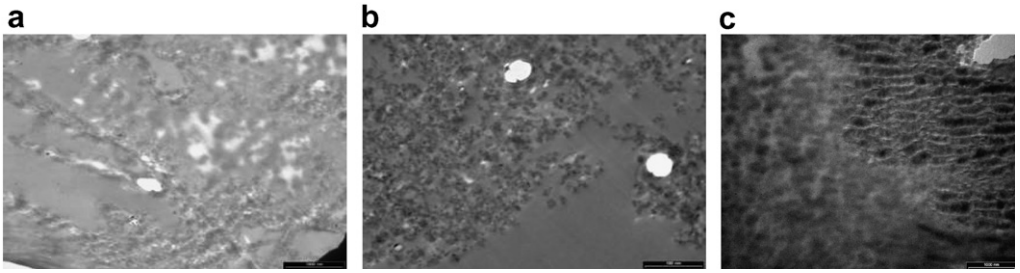


Fig. 5. TEM images of the microspheres (obtained after imbedding the particles in LR-White resin and cutting in 90 nm slices using an ultra-microtome followed by staining with OsO_4): (a) empty microspheres S1 before loading with VD3; (b) after loading with VD3 showing the center of the microsphere and (c) after loading with VD3 showing the area close to the surface of the microsphere; The bar corresponds to 1000 nm.

fluorescing model compound – Sudan II – was used instead. The particles were loaded in a similar way described above without the evaporation of the solvent. The particles were then removed from the solution at certain time intervals and the relative degree of Sudan loading was examined by using fluorescence microscope. It can be seen that Sudan II migrates rapidly to particles and the amount of Sudan absorbed increases with time until reaching saturation at about 60 min (Fig. 6). Fluorescence and the drug loading experiment are therefore in good agreement. The microspheres prepared therefore allow the fast loading of hydrophobic drugs in acetone. The drug was found to be evenly distributed, but a slight enrichment of the drug beneath the surface layer can be observed resulting in a drug concentration gradient from the outer layer to the center of the sphere.

3.4. Drug release studies

A significant prerequisite for the successful designing of a drug delivery system for hepatocellular carcinoma is the slow release of the drug over several weeks to sustain optimal therapeutic concentrations, but also to reduce side effects such as hypocalcaemia.

Drug loaded particles (**S1**) were prepared using the evaporation technique to investigate the release of $VD_3$from its polymeric carrier into an aqueous environment of pH 7.4. An amount of 50 mg of microspheres (equivalent to 8 mg of drug) in the drug–polymer mixture was initially suspended in a 10 ml mixture containing 1 wt% of ethanol in water. The aqueous solution was replaced every 10 days to prevent a saturation effect. The amount of drug in the aqueous system was determined using UV–Vis spectrocopy. The amount of drug release over the first five days was observed to be very minimal and then gradually increased afterwards (Fig. 7). After 40 days, the amount of released VD_3 was found to be only 60 μg, which is equivalent to only 0.75% of the loaded drug. Even though the percentage of drug release was low due to the hydrophobic nature of both the drug and the polymer, the absolute amount of drug released is comparable to current treatments. 0.267 μg of $1,25(OH)_2VD_3$ per kg of body weight is currently administered in a solution of lipidiol. In addition, it should be considered that the drug carrier is designed to release $1,25(OH)_2VD_3$ over several months.

Slow but sustained release of VD_3 is desirable in regional treatment of liver cancer. Therefore, while achieving the target concentrations at the tumor

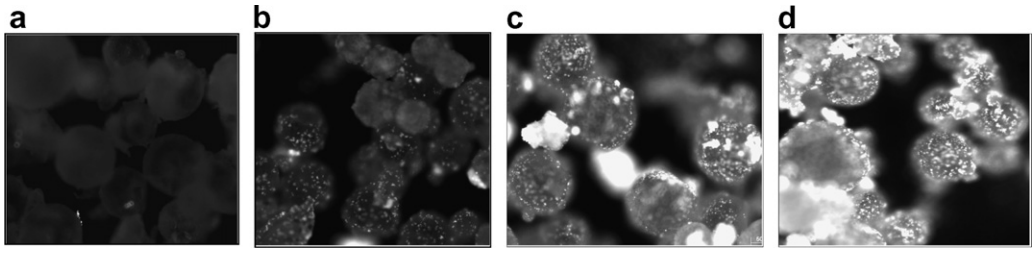


Fig. 6. Fluorescence image of particles loaded with Sudan II: (a) particle before loading; (b) after 20 min; (c) after 40 min and (d) after 60 min.

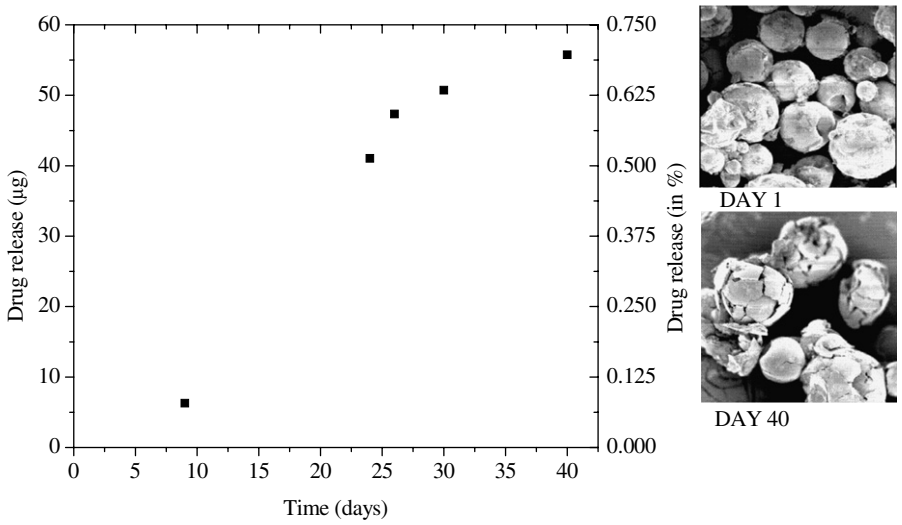


Fig. 7. Drug release over 40 days measured using 50 mg of loaded particles in 10 ml of aqueous solution (pH 7.4) at 37 °C (in orbital shaker at 40 rpm). The amount of VD3 was determined using UV–Vis spectroscopy ($\lambda = 265$ nm). The SEM photos show the microspheres on day 1 and after 40 days in aqueous solution at pH 7.4.

site, the amount of VD_3 entering systemic circulation at any one time may be minimal, hence avoiding the development of hypocalcaemia.

Some unloaded particles were investigated after 40 days in aqueous solution at pH 7.4. The weight loss is below 10% and no apparent degradation has occurred, which would lead to the leaching of water soluble PVA from the sphere. However, the particles were found to show a significant brittleness while newly synthesized particles have a high impact resistance (Fig. 7). These initial signs of degradation were not quantified in detail for this study.

3.5. Cell growth inhibition test

An essential part in the investigation of novel microspheres is their thorough analysis regarding their cytotoxicity. The test was carried out with the extract of the polymer samples. Different microspheres (**S1**, **S2**, **S3**, see experimental part) were incubated in MEM solution for 24 h. This test aims to find any released toxic chemicals. Cell line 929 was utilized to study the cell growth inhibition caused by the presence of these microspheres. Next to the microspheres studied in detail regarding their drug loading and release kinetics (**S1**), microspheres with a higher percentage of crosslinker (30 wt%) (**S2**) and microspheres prepared with a mixture of poly(vinyl alcohol) PVA and poly(vinyl pyrrolidone) PVP as stabilizers were investigated (**S3**). Alterations to the type of stabilizers were considered important to verify the toxicity of stabilizers, which were potentially not fully removed during washing.

In this study, 4% ethanol was used as reference while 5% and 7.5% ethanol solution were used as positive controls. The mean cell number was used to compare with the null sample to find out any toxicity effect. All samples were washed intensively with water and acetone before the assay. All three samples show percentage of inhibition of less than 30% and thus, could be considered as non-toxic to these cells. It can also be seen that different stabilizers do not have an impact on toxicity of the final product as these are removed during the cleaning process (Fig. 8). However, increasing the amount of crosslinker seems to increase the toxicity of the product. This can be explained in terms of excessive double bond on the surface of the particles due to increasing amount of crosslinker. It is known that the synthesis of microspheres using crosslinker can lead to unreacted vinyl groups on the surface, which are potentially toxic. From these results, it can be concluded that poly(vinyl neodecanoate-crosslinked-ethyleneglycol dimethacrylate) microspheres synthesized via suspension polymerization are non-toxic carriers suitable for the preparation of drug carriers aimed at regional use in the treatment of liver cancers. However, further work including testing the efficacy and toxicity of the spheres in appropriate animal models of liver cancer is warranted.

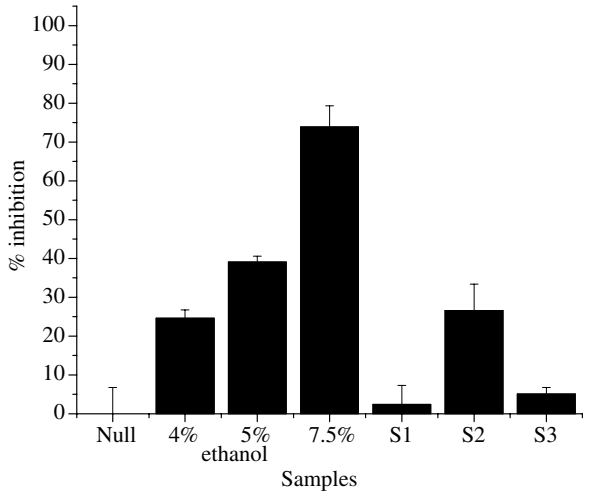


Fig. 8. Cell growth inhibition test using cell line 929 employing microspheres **S1**, **S2** and **S3** and control experiments with MEM and FBS solutions (null) and solutions with varying amounts of ethanol.

3.6. Direct contact cytotoxicity assay and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The assay aimed at finding the effect of the particles on cancer cells – here human colorectal cancer cell line HT-29.

Table 1
Direct contact testing on cell line 929 using microspheres **S1**, latex (positive control) and silastic (negative control)

Sample	Zone grading[a]	Lysis grading[b]
Null	0	0
Positive control	5	4
Negative control	0	0
Microsphere S1	0	0

[a] 0 = No detectable zone, 1 = zone limited to under samples, 2 = zone extends <0.5 cm, 3 = zone between 0.5 and 1 cm, 4 = zone extends >1 cm beyond sample, 5 = zone covers the entire plate.

[b] 0 = No observable cytotoxicity, 1 = less than 20% affected, 2 = less than 40% affected, 3 = less than 60% affected, 4 = less than 80% affected, 5 = greater than 80% affected.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay uses the ability of viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan compound, which can be detected by UV–Vis spectroscopy. Microspheres (**S1**) were incubated with HT-29 cells and the UV–Vis signal was compared to a blank sample. The result of the MTT assay could be compared to the cell growth inhibition test using L929 showing more than 90% of surviving cells. The microspheres could therefore be deemed non-toxic.

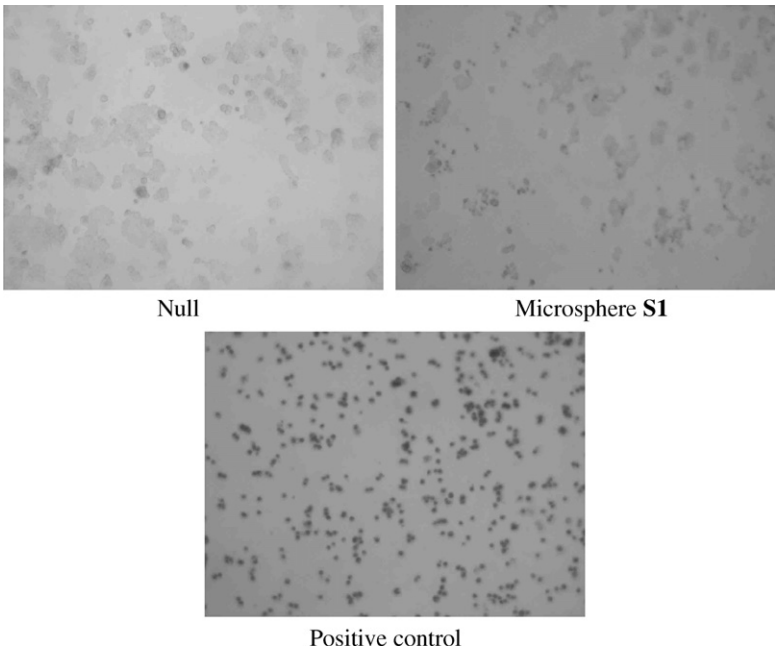


Fig. 9. Microscope images of HT-29 cells after direct contact testing with microspheres (S1) and latex (positive control); the null sample shows HT-29 cells without any added polymer material.

The direct contact cytotoxicity assay is necessary to mimic the clinical situation when spheres are in direct contact with cells. Microspheres (**S1**) as well as latex as positive control and silastic as negative control were used. All three samples were exposed to HT-29 cells. Zone and lysis affects of these samples were compared verifying the non-toxicity of these microspheres as a drug delivery system (Table 1, Fig. 9).

4. Conclusions

Suspension polymerization was employed to successfully synthesize poly(vinyl-neodecanoate) crosslinked with ethyleneglycol dimethacrylate. The resulting microspheres were found to have the desired size of 35 μm, which is regarded as a suitable size for a drug delivery system to be used for transarterial chemoembolisation. However, the optimization of the reaction conditions was essential to avoid agglomeration or collapsing of particles. Especially the temperature was found to have a profound influence on the outcome of the suspension polymerization determining the coalescence and break-up of droplets. The resulting microspheres – obtained after optimization of reaction conditions such as temperature, crosslinker and monomer concentration – were found to be suitable to encapsulate significant amounts of VD3 – a model drug for the expensive 1,25 dihydroxyvitamin D_3. These drug loaded microspheres provide a prolonged release profile with small amounts being released over several weeks. The amount released can be compared to doses currently administered in a single injection. Cytotoxicity tests have shown the spheres to be biocompatible.

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